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Antioxidant Defenses and Trace Metal Bioaccumulation Capacity of *Cymbula nigra* (Gastropoda: Patellidae)

G. A. Rivera-Ingraham · G. Malanga · S. Puntarulo · A. F. Pérez · A. Ruiz-Tabares · M. Maestre · R. González-Aranda · F. Espinosa · J. C. García-Gómez

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Abstract The present study deals with the effect of trace metals on the endangered limpet *Cymbula nigra*. The Bay of Algeciras (Strait of Gibraltar) was used as the study site. Important industrial activity takes place in the area, including frequent oil spills. However, it is home to important populations of *C. nigra.* The objective of this work was to determine if these animals were being affected at a subcellular level by the pollutants present in their environment and to analyze the trace metal concentrations in the animal's soft tissues. To determine the effects of water quality on the antioxidant activity and concentrations through field experimentation, a total of six sites were selected in Algeciras Bay, three located in the inner areas (environmentally degraded sites with higher levels of pollutants) and three in the outermost areas of the Bay. Stress associated to reactive oxygen species formation was assessed on digestive glands and gills as the enzymatic antioxidant activity of catalase (CAT), superoxide dismutase (SOD), and glutathione S-transferase (GST) and as the concentrations of lipid-soluble (α-tocopherol and β-carotene) and the water-soluble antioxidants (reduced and oxidized glutathione (GSH and GSSG)). Gills and digestive glands of those animals located in the inner areas of Algeciras Bay showed higher CAT activity values than those located in the outer areas. As a general pattern, we observed higher antioxidant activities and concentrations in digestive glands that in gills, suggesting the possibility that pollutants are mainly being incorporated by limpets through the food. As a general rule, larger animals showed greater concentrations of these compounds. Iron, zinc, and manganese, in this order, were present in the tissues at the highest concentrations. Chromium and manganese were found in significantly higher concentrations in those animals collected from the inner areas of the Bay. Through the present study, we provide the first data regarding the antioxidant defense levels and metal accumulation capacity of this species, and we reinforce the idea that this endangered species may be, in fact, relatively tolerant to degraded environments.

G. A. Rivera-Ingraham · A. Ruiz-Tabares · M. Maestre · R. González-Aranda · F. Espinosa · J. C. García-Gómez
Laboratorio de Biología Marina, Departamento de Fisiología y Zoología, Universidad de Sevilla, Avenida Reina Mercedes 6, 41012 Sevilla, Spain

G. A. Rivera-Ingraham (✉)
Functional Ecology, Alfred Wegener Institute for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany
e-mail: grivera@us.es

G. Malanga · S. Puntarulo
Fisicoquímica-IBIMOL Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, Buenos Aires, Argentina

A. F. Pérez
Laboratorio de Ecología de Bentos Marino, Departamento de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

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1 Introduction

Trace metals are naturally present in the marine environment although their concentrations have considerably increased due to technological development. Industrial by-products are often released to water masses, and some metals species may have negative consequences on the ecological balance of the recipient environment (e.g., Farombi et al. 2007) or even cause reduction or elimination of intolerant species and, thereby, produce alterations of the habitat's biodiversity (Peterson 1986). Trace metals may be found dissolved or suspended in the water column and may usually end up concentrating in sediments. Exposure to these compounds often produces alterations in the organisms' fitness (e.g., Hansen et al. 2002; Vosyliene et al. 2003; Harada et al. 2007). One good example of this is how, for certain gastropods species, exposition to metals such as copper can cause sterility through the phenomenon of "imposex" (development of male characters on the female) (e.g., Nias et al. 1993; Miller and Pondick 1984). Furthermore, trace metals can also be uptaken by organisms by direct contact with polluted water through permeable areas of their body and through polluted food (see review by Depledge and Rainbow 1990), and may lead to concentrations several orders of magnitude higher than those of the surrounding water (Casas et al. 2008), involving a growing risk for wildlife. Consequently, measuring chemical and physical variables may not always be adequate procedures for assessing pollution. Aquatic organisms (especially benthic, which cannot escape the negative effects of these compounds) have been frequently used to study and develop models of how pollutants affect the oxidative metabolism of the biological systems (see Di Giulio et al. 1989; Winston and Di Giulio 1991; Livingstone et al. 1994; Cavaletto et al. 2002). Moreover, there has been an increasing interest for using this knowledge, and how organisms may accumulate trace metals, as a biomonitoring tool to determine environmental quality (Stegeman et al. 1992). The organisms selected for this task have usually been aquatic filter feeders such as mussels, clams and oysters (Boening 1999). However, other nonfiltering organisms such as limpets are also considered as good sentinel organisms (Navrot et al. 1974). Limpets are intertidal organisms which forage on rocky substrates feeding from the algal biofilm that develops on these surfaces. Limpets are, moreover, present around the globe, making them good candidates for the assessment of water pollution. So it is that these organisms have also been used as indicators of impacts such as trace metal pollution (e.g., Navrot et al. 1974; Nakhlé et al. 2006).

There is a wide variety of biomarkers that can be used in order to determine environmental quality, mainly based on different levels of biological organization. At the subcellular level, the oxidant/antioxidant balance is crucial for cellular homeostasis (Livingstone 2001; Valavanidis et al. 2006), but exposure to certain types of pollutants can enhance reactive oxygen species (ROS) formation and create an imbalance between oxidants and antioxidants in favor of the former, producing the so-called oxidative stress (Cadenas and Sies 1985; Sies 1991). A good example of this is the oxidative damage produced by accumulation of transition metals in soft tissues (Viarengo 1989), since many metal species, such as iron (Fe) and copper (Cu), are active catalysts of the formation of ROS, mainly hydroxyl radicals, through both the Fenton-type (Fenton 1894) and the Haber-Weiss reactions (Haber and Weiss 1932) in the presence of H_2O_2 and $O_2^{\bullet -}$. In consequence, biomarkers of oxidative stress, such as antioxidant enzyme activities, are frequently used, as they may be altered when exposure to pollutants occur (e.g., Regoli and Principato 1995) and many studies have previously approached the relationship between the antioxidant activity/contents and exposure to pollution in the aquatic environment (Cajaraville et al. 2000; Ansaldo et al. 2005; Alves de Almeida et al. 2007; Weihe et al. 2010; Fernández et al. 2010), through field (e.g., Niyogi et al. 2001; Lionetto et al. 2003; Alves de Almeida et al. 2007) and laboratory experimentation (Doyotte et al. 1997; Ansaldo et al. 2005).

The aim of the present study was to evaluate, through the analysis of antioxidant activities and concentrations, if *Cymbula nigra* (da Costa, 1771) (Gastropoda: Patellidae) is affected at a subcellular level by the pollutants present in their environment. *C. nigra* is the largest patellid limpet species of the Mediterranean Sea, reaching up to 13.3 cm of shell length (Rivera-Ingraham et al. 2011). The species has been cataloged as "endangered" and "vulnerable" at European and Spanish levels, respectively. Although the species is commonly found in the Strait of Gibraltar, it is quite surprising that very little is known about the biology of the species. Most of studies are related to its reproduction biology (e.g., Renault and Moueza 1971; Frenkiel

1975; Rivera-Ingraham 2010), its phylogenetic status within the Patellidae family (Ridgway et al. 1998; Koufopanou et al. 1999; Sá-Pinto et al. 2005), and population genetics (Espinosa et al. 2011). However, only more recently the species has received more attention, and new works approaching some of its ecological and biological aspects have been published (e.g., Espinosa et al. 2007; Rivera-Ingraham 2010; Rivera-Ingraham et al. 2011). The effects of oxidative stress were analyzed at a cellular level by testing the antioxidant enzymatic activities of catalase (CAT), Glutathione S-transferase (GST), and superoxide dismutase (SOD). SOD catalyzes the dismutation of $O_2^{\bullet -}$ into H_2O_2, while CAT catalyzes the decomposition of the latter into water and oxygen. GST, on the other hand, is involved in the biotransformation of many xenobiotics (Eaton and Bammler 1999) and some endogenous compounds such as the end-products of lipid peroxidation (Leaver and George 1998), and more recently, it has been suggested that it may also play a role in metal homeostasis or detoxification (Yoshinaga et al. 2007). Nonenzymatic lipid-soluble (α-tocopherol and β-carotene) and water-soluble antioxidants (reduced and oxidized glutathione) (GSH and GSSG, respectively) concentrations were also analyzed, being the GSH/GSSG ratio an indication of the redox capacity in the cellular cytosol, since under oxidative stress conditions GSH (reducing power) is decreased and GSSG is increased. Additionally, and because exposition or ingestion of trace metals during grazing can increase oxidative stress, this study attended the arsenic (As), chromium (Cr), copper (Cu), cadmium (Cd), iron (Fe), mercury (Hg), manganese (Mn), nickel (Ni), lead (Pb), vanadium (V), and zinc (Zn) concentrations in limpet soft tissues. The trace metal bioaccumulation capacity of the species was tested, and among the question that were addressed were if trace metal concentrations in the limpets' soft tissues are size-/age-dependent. Finally, and taking into account that trace metal bioavailability is one key factor determining tissue metal concentrations, our results were compared to those obtained by other authors analyzing metal concentration in sediments or water.

2 Materials and Methods

2.1 Study Area

The study was conducted in the rocky shores of Algeciras Bay (Strait of Gibraltar, Southern Spain) (Fig. 1a), which is mainly characterized by a high industrial activity (including petrochemical and thermal power plants, oil refineries, shipyards, and some factories related to paper and steel production) and the intense maritime traffic, especially in the inner zones. A total of six sites (all presenting important *C. nigra* populations) (Rivera-Ingraham 2010) were selected on the coast of Algeciras Bay (Fig. 1b): three of them were located in the inner areas of the Bay (Roquedillo, Guadarranque, and Saladillo), characterized for presenting low water quality (Guerra-García et al. 2010) and important levels of pollutants (e.g., trace metals) (e.g., Morillo and Usero 2008); the other three sites (Europa Point, Outer San García Point, and Inner San García Point) were located in the outermost areas of the Bay, which are subjected to higher levels of hydrodynamics and lower pollutant concentration (Guerra-García et al. 2010).

2.2 Registration of Abiotic Water Parameters

For each six sites, water temperature, pH (WTW Tetra Con 340i with a Sen Tix 41–3 electrode), salinity (WTW Tetra Con 340i), and dissolved oxygen (WTW Oxi 197i) were recorded, as these parameters are considered important to define water quality (e.g., Karr and Dudley 1981).

2.3 Biochemical Analyses

Taking into account that enzymatic activities and antioxidant contents may differ among seasons (Malanga et al. 2007), all individuals were collected during the same month. Furthermore, in order to minimize the intraspecific physiological variability, which can be high among individuals and sizes (Cravo and Bebianno 2005), individuals were chosen based on shell length (41.2± 2.2 mm) and soft wet tissue (2.1±0.5 g). Immediately after collection, both gills and digestive glands were dissected and preserved in dry ice for transportation and finally maintained at −80 °C until analysis.

Homogenates from both digestive glands and gills were prepared in 30 mM potassium phosphate-120 mM KCl buffer at pH7.4. All activity and content results were expressed per milligram of protein performed according to Lowry et al. (1951).

CAT activity was measured as the decomposition of a 0.3 M H_2O_2 solution in a 50 mM potassium phosphate buffer at pH7.0 (Aebi 1984), and results were

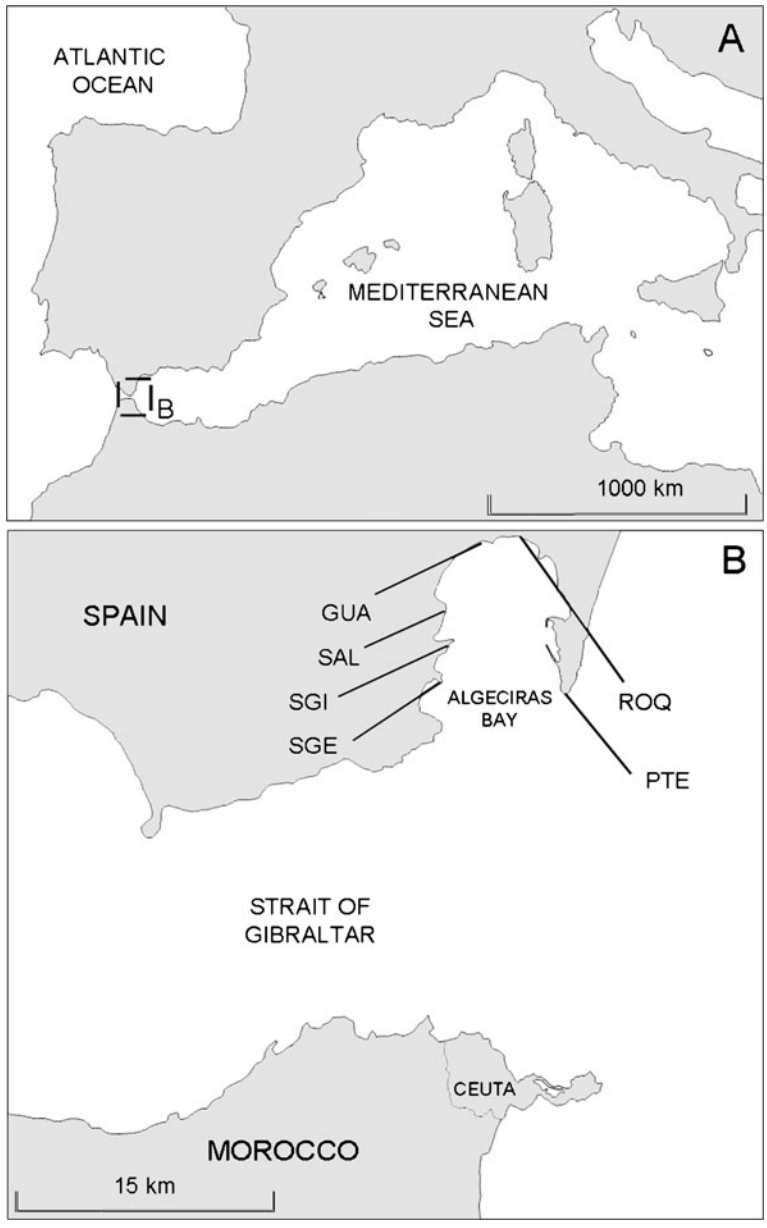

Fig. 1 Location of the study area. **a** Western Europe. The *black box* indicates the location of the Strait of Gibraltar. **b** Strait of Gibraltar. *Black lines* indicate the location of each of the sites considered in the study. In Outer Algeciras Bay: *SGE* Outer San García Point, *SGI* Inner San García Point, *PTE* Europa Point. In Inner Algeciras Bay: *SAL* Saladillo Marina, *GUA* Guadarranque, *ROQ* Roquedillo

expressed as picomole per milligram of protein. GST was assessed using 1 chlorine-2,4-dinitrobenzene 1 mM in ethanol (Habig et al. 1974) while total SOD activity was registered by cytochrome detection system using the protocol originally described by McCord and Fridovich (1969) and later modified by Flohe and Otting (1984). For the two later analyses, results were expressed as USOD or UGST per milligram of protein. All measurements were carried out spectrophotometrically at room temperature (20–25 °C).

The content of α-tocopherol and β-carotene was quantified using reverse-phase HPLC at an oxidation potential of 0.6 V (Desai 1984) in the same homogenates in which the enzymatic assays were carried out. Samples were extracted with methanol: hexane (1:4), and were centrifuged at 6,000×*g* for 10 min. The hexane phase was then removed and evaporated to dryness using N_2. Samples were then dissolved in methanol:ethanol (1:1) and injected for isocratic HPLC analysis (Desai 1984). Standards of α-tocopherol and β-carotene were provided by

Sigma©. Results were expressed as picomole per milligram of protein.

In order to determine the content of water-soluble antioxidants, sample homogenates were prepared in $HClO_4$ 1 M and 2 mM EDTA, and centrifuged at 29,000×*g* for 20 min at 4 °C. The content of GSH and GSSG were quantified by isocratic HPLC analysis with a 20 mM sodium phosphate buffer, pH2.7 as the mobile phase (Rodríguez-Ariza et al. 1994) and through a standard curve with a linear relationship between 5–200 ng GSH or GSSG. Results were expressed as microgram per milligram of wet weight.

2.4 Trace Metal Concentration in Soft Tissues of *C. nigra*

For each site, six additional individuals were collected (two per size class, which ranged in 1-cm intervals from 4 to 7 cm of maximum shell length), regardless their sex or reproductive stage. Animals were measured to the nearest millimeter using a caliper. The shells were then removed, and the complete animals were weighted and then preserved at −20 °C.

Analyses were carried out following the indications described by Guerra-García et al. (2010): samples were finely ground after drying them at 30 °C until constant weight. The resulting powders were accurately weighed in a dry, pre-cleaned Teflon digestion vessel. To each vessel, 2 ml of HNO_3, 1 ml of HCl, and 3 ml of H_2O_2 were added. The vessels were then sealed and placed in the microwave chamber (Anton Paar, Multiwave 3000) at 240 °C for 20 min with a maximum pressure of 40 bar. After digestion, the solution was brought to 25 ml volume with deionised waters. Analytical determinations were performed by using inductively coupled plasma-optical emission spectrophotometer (ICP-OES Horiba Jobin-Yvon, Ultima 2). Patterns used for ICP measurements included Merck ICP multi-element calibration standard solutions IV (HC612727) and XII (OC461429). The following trace metals were analyzed: As, Cr, Cu, Cd, Fe, Hg, Mn, Ni, Pb, V, and Zn. All values were expressed as microgram of trace metal per gram of limpet soft wet tissue.

2.5 Statistical Analyses

One-way ANOVA analyses of variance were carried out when data satisfied the assumptions of normality and homoscedasticity. When data did not meet these requirements, Kruskal–Wallis tests were carried out. Linear and nonlinear regression analyses were conducted to find the best relationship among factors. A total of 11 models were tested: linear, logarithmic, inverse, quadratic, cubic, power, compound, *S*, logistic, growth, and exponential. Only when the linear model did not significantly explain our results, nonlinear models were taken into account, and among those showing significant relationships, the model presenting the better adjustment (R) was selected as optimum. The level of significance was set at 5 %. All statistical analyses (parametric and nonparametric analyses) were carried out with SPSS 14.0.

3 Results

3.1 Abiotic Water Parameters

The average values recorded are shown in Table 1. No significant differences were found among sites located in inner and outer Algeciras Bay regarding water temperature, dissolved oxygen or pH. However, a one-way ANOVA analysis evidenced that the outer Algeciras sites (36.67±0.03‰) show significantly higher water salinity values than inner (36.43±0.03‰) Algeciras sites (F=24.50; p<0.01) (Table 1).

Table 1 One-way ANOVA results for the influence of the site location on the environmental parameters registered

Source of variation	Mean±SE	*P*
Water temperature (°C)		n.s.
Outer Bay	20.00±0.50	
Inner Bay	21.53±0.52	
Dissolved oxygen (mg/l)		n.s.
Outer Bay	6.28±0.67	
Inner Bay	7.56±0.43	
Water salinity (‰)		*
Outer Bay	36.67±0.03	
Inner Bay	36.43±0.03	
pH		n.s.
Outer Bay	8.15±0.10	
Inner Bay	8.26±0.06	

n.s. non significant

*p<0.01

3.2 Biochemical Analyses

Only CAT activity was significantly different among sites (see Table 2). For both gills and digestive glands, the activity values recorded were significantly higher in those animals collected from the inner areas of Algeciras Bay. Regarding water soluble antioxidants, although differences were observed in the content of GSH and GSSG between sites, these did not reflect in the GSH/GSSG ratio (Table 3).

The overall antioxidant activity/content values was significantly higher in digestive glands than in gills for GST (K=24.23; p<0.001), α-tocopherol (K=23.87; p<0.001), β-carotene (K=25.53; p<0.001), and GSH/GSSG ratio (K=12.75; p< 0.001). No significant differences were recorded for either CAT (K=1.66; p=0.20) or SOD (F=0.51; p= 0.48) activities between organs.

3.3 Metal Concentration in Soft Tissues

In general terms, trace metal concentrations in *C. nigra* soft tissues presented high variability. The Fe values were at least one order of magnitude greater than the rest of metal species considered during the study (see Table 4). For the specific case of those animals collected from the inner areas of Algeciras Bay, metal concentrations decreased following the sequence: Fe≫Zn≫As> Mn>Cu>Cr≈Ni≈V>Pb>Cd≈Hg. For the case of the outer areas, concentrations decreased according to the sequence: Fe≫Zn≫As>Cu>V≈Ni≈Mn>Pb≈Cr> Cd≈Hg. No significant differences were recorded between the two areas. However, animals collected from the inner areas of Algeciras Bay showed significantly higher concentrations of Cr and Mn in their tissues than those collected from the outer sites. On the other hand, V concentrations followed the opposite pattern and presented significantly increased concentrations in those animals located in the outer sites of Algeciras Bay (Table 4).

The results obtained from the animals collected in inner areas of Algeciras Bay were additionally used to test if there was any correlation between the sizes/weights of the animals and trace metal bioaccumulation. Although as a general rule, trace metal concentrations increase with the animal's shell length (see Table 4), no significant relationship was found. The same was true when considering the animals' soft weight tissue except for the case of V, for which a significantly correlation (best explained by a linear model) was found (R=0.559; p=0.016) (Fig. 2).

4 Discussion

The inner zone of Algeciras Bay constitutes one of the most polluted areas in Spain. This is mainly due to the high industrial activity that is carried out nearby, which includes, as aforementioned, oil refining and steel and paper production. Moreover, the fact that the Bay holds one of the largest and busiest

Table 2 One-way ANOVA results for the influence of the site's location on the mean values of the parameters taken into consideration

		Digestive glands				Gills			
		Inner Algeciras Bay	Outer Algeciras Bay	F	p	Inner Algeciras Bay	Outer Algeciras Bay	F	p
Enzymatic antioxidante activities	Catalase (pmol·mg prot^{-1})	12.54±1.14	6.10±1.17	12.28	*	19.67±2.13	5.49±1.84	25.59	*
	GST(U·mg prot^{-1})	19.66±3.64	13.64±1.33	2.65	n.s.	2.41±1.02	1.94±0.84	0.11	n.s.
	SOD (U·mg prot^{-1})	0.01±0.00	0.01±0.00	0.02	n.s.	0.01±0.00	0.02±0.01	1.75	n.s.
Nonenzymatic antioxidante concentrations	α-tocopherol (pmol·mg prot^{-1})	1.44±0.53	0.75±0.09	1.69	n.s.	0.17±0.04	0.10±0.02	1.80	n.s.
	β-carotene (pmol·mg prot^{-1})	2.35±0.49	3.55±1.13	0.86	n.s.	0.08±0.03	0.13±0.03	1.35	n.s.

Values are expressed as mean±SE. U(GST): One unit GST is defined as the enzyme amount which catalyzes the formation of 1 μmol of GS-DNB per min at 30 °C. U(SOD): One unit SOD is defined as the enzyme amount that inhibits the rate of reaction by 50 %.

n.s. non significant

*p<0.001

Table 3 One-way ANOVA results for the influence of the site's location on the mean values of the different glutathione species taken into consideration

	Digestive glands				Gills			
	Inner Algeciras Bay	Outer Algeciras Bay	*F*	*p*	Inner Algeciras Bay	Outer Algeciras Bay	*F*	*p*
GSH (μg/mg wet weight)	0.006±0.001	0.01±0.002	4.54	n.s.	0.007±0.001	0.025±0.008	5.75	*
GSSG (μg/mg wet weight)	0.006±0.001	0.020±0.004	11.93	**	0.003±0.001	0.013±0.005	3.54	n.s.
GSH/GSSG	2.74±1.16	2.45±0.58	0.05	n.s.	0.61±0.21	0.53±0.16	0.10	n.s.

Values are expressed as mean±SE

n.s. non significant

*$p<0.05$; **$p<0.01$

commercial ports of Spain also contributes to the high levels of pollution that have been registered in the area. The Bay, and specially the innermost area, presents the highest concentrations of pollutants (e.g., Carballo et al. 1996; Conradi et al. 1997; Guerra-García et al. 2006), including trace metals (e.g., Morillo and Usero 2008) and is chronically affected by oil spills (Morales-Caselles et al. 2007). This makes the area an interesting place to conduct toxicological analyses and studies which is proven by the high number of articles published in the last decade related to the subject (e.g., Carballo et al. 1996; Conradi and López-González 1999; Guerra-García et al. 2006; Morillo and Usero 2008; Guerra-García et al. 2010)

Based on limpet abundance, previous studies have indicated that *C. nigra* may be relatively more tolerant to certain human impacts such as the presence of sewage outfalls than other limpet species present in the study site (Espinosa et al. 2007). This may be supported by the fact that *C. nigra* shows important populations in the inner areas of Algeciras Bay (Rivera-Ingraham 2010), despite the pollution levels registered in the area. However, it is widely accepted that toxicity events will manifest themselves at a subcellular level before they are evident at other levels of organization (Cajaraville et al. 2000). Catalase activities, which are widely considered as an important and sensitive biomarker of stress (Regoli, Gorbi et al. 2002; Regoli, Nigro et al. 2002) were up to 3.5-fold higher in those animals collected from the inner areas than in the outer areas of the Bay, supporting the hypothesis that *C. nigra* individuals located in Algeciras Bay (and especially those in the inner areas) may be subject to some degree of stress. Among the factors that could be inducing this oxidative stress, pollutants (such as trace metals) should be considered. In fact, *C. nigra* individuals from the inner areas also showed the highest concentrations of Cr and Mn per unit of wet tissue, and these could be responsible for the enhanced catalase activities recorded. Other authors have also reported that invertebrate species like mussels show an increase in catalase activity when exposed to metals (Vlahogianni et al. 2007). For the specific case of limpets, some species like *Patella vulgata* also show increased CAT activity when they are subject to low-quality environments (Douhri and Sayah 2009) and have been reported to be especially sensitive to copper exposure (Brown et al. 2004).

It is also interesting to comment the different activity rates/antioxidant content among the gills and digestive glands used in the study. As a general pattern, the latter showed higher values than gills. This allows us to think that digestive glands may be subject to higher exposure to pollution. Even though gills (compared to other tissues) are highly sensitive to genotoxic damage (Manna and Sadhukhan 1986; Hayashi et al. 1998), it is known that marine organisms, and specially mollusks, can uptake trace metals from food (e.g., Phillips 1977; Depledge and Rainbow 1990) and may end up increasing the oxidative stress in digestive glands. Any cyanobacterium and diatom species (which constitute the microalgal biofilm on rocks and are the limpets' food resource) (see Della Santina and Naylor 1993) have the capacity to bioaccumulate certain pollutants (Dwivedi et al. 2006). Through this accumulation processes, these compounds can be transmitted through the food chain (Vasconcelos 1995; Vasconcelos et al. 2001; Lance et al. 2010) and affect consumers at a subcellular level, which would explain our results. This can also be considered as an important conclusion, as it should be taken into account

Table 4 One-way ANOVA / Kruskal–Wallis results for the influence of the site's location on the overall heavy metal concentrations in *C. nigra* soft tissues

	Inner Algeciras Bay				Outer Algeciras Bay				
	Mean±SE				Mean±SE				
	Overall	4–5 cm	5–6 cm	6–7 cm	Overall	4–5 cm	5–6 cm	6–7 cm	Overall *p*
Cr	2.42±0.28	1.88±0.37	2.61±0.56	2.77±0.49	1.08±0.13	0.89±0.16	0.99±0.27	0.92±0.11	***[a]
Cu	3.14±0.20	3.08±0.41	3.23±0.37	3.07±0.34	4.57±0.75	3.79±0.69	4.16±1.39	4.04±0.72	n.s.[a]
Fe	542.72±59.89	563.35±83.99	576.32±140.18	488.48±94.79	439.42±95.74	471.79±140.40	493.54±153.21	400.92±111.84	n.s.[b]
Ni	2.36±0.40	2.07±0.66	3.07±0.81	1.94±0.64	2.17±0.64	1.40±0.57	4.05±2.07	1.59±0.54	n.s.[b]
Pb	1.38±0.14	1.14±0.11	1.28±0.22	1.71±0.31	1.88±0.31	1.68±0.34	1.79±0.42	2.18±0.81	n.s.[a]
Zn	90.68±11.54	69.79±14.98	100.86±21.00	101.41±23.69	72.55±8.97	68.39±10.10	60.99±4.51	87.44±16.58	n.s.[b]
Cd	0.68±0.16	0.76±0.40	0.86±0.27	0.41±0.11	0.46±0.19	0.44±0.23	0.16±0.04	0.85±0.35	n.s.[a]
As	9.49±0.52	9.12±0.91	10.13±0.48	9.22±1.24	9.62±0.61	8.26±0.70	8.03±0.86	9.87±1.16	n.s.[b]
V	2.07±0.07	1.93±0.09	2.06±0.14	2.23±0.13	2.49±0.16	2.37±0.24	2.33±0.23	2.17±0.23	**[a]
Mn	5.51±0.93	6.08±2.42	5.02±1. 26	5.45±1.13	2.13±0.21	2.42±0.48	1.65±0.10	2.30±0.39	***[a]
Hg	0.11±0.01	0.11±0.01	0.13±0.01	0.11±0.01	0.13±0.02	0.12±0.03	0.10±0.02	0.11±0.02	n.s.[a]

Summary data of heavy metal concentrations (microgram per gram, wet mass) in *C. nigra* limpets for each of the size classes considered are also included. Data are expressed as mean ±SE

[a] Kruskal–Wallis test

[b] One-way ANOVA test

n.s. non significant

*$p<0.001$; **$p<0.05$; ***$p<0.01$

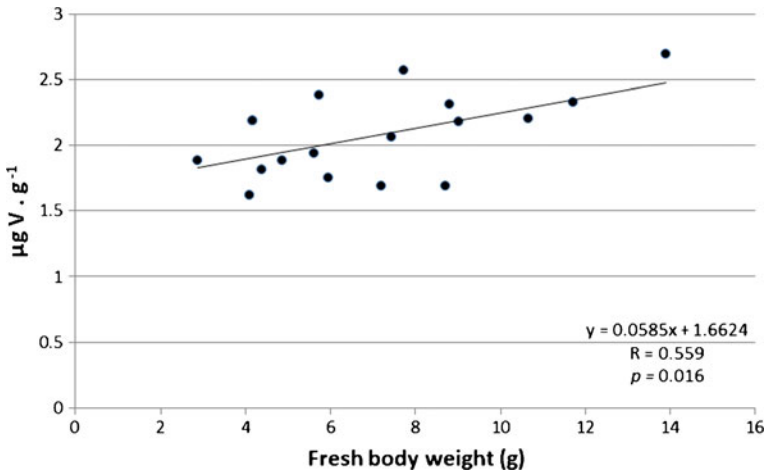

Fig. 2 Relationship between fresh body weight and V concentration in *C. nigra* individuals collected in the inner areas of Algeciras Bay

that limpet are commonly consumed in some countries such as Portugal, Italy or even Spain. In fact, in the past years, *C. nigra* collection rate in Algeciras Bay has significantly increased, presumably to be used in part for human consumption (Rivera-Ingraham 2010). In consequence, special attention should be paid to this fact and how uncontrolled collection and consumption of polluted organisms may constitute a risk for human health.

Because of their ecological and economic importance, molluscs have been very frequently used for bioaccumulation studies, even more than others such as polychaeta and crustaceans (Feldstein et al. 2003). Up to date, there are no available data regarding trace metal concentration in the soft tissue of *Cymbula* species, but other Patellid limpets have also been frequently analyzed under different water quality conditions. In general terms, marine gastropods, and *Patella* species in particular, accumulate in their soft tissues preferentially Fe≫Zn≫Mn (Cravo and Bebianno 2005). Taking into account that the same pattern was observed for *C. nigra* in the present study, we can suggest that this accumulation pattern is maintained across genera. Fe was the predominant metal in *C. nigra* soft tissues and was at least one order of magnitude greater than the rest of metals analyzed during the present study. Fe is an essential metal which is required by organisms at low concentrations. It is known that Fe, due to its low solubility in oxygenated water, does not enter freely in marine organisms (Depledge and Rainbow 1990). However, it has been reported to be abundant in marine invertebrates (Depledge et al. 1994), and other authors studying patellid limpets have attributed this to the fact that Fe is an important constituent of the limpet radula (Cravo and Bebianno 2005). On the other hand, Zn (yet another essential metal) also showed one of the highest concentration values, but unlike Fe, organisms can in many cases easily accumulate Zn by passive uptake (Bryan 1968). Fe, Zn, and Mn are additionally essential metals incorporated in the soft tissue in metabolically important biomolecules namely proteins (including enzymes), metalloenzymes, and respiratory pigments (Bryan et al. 1985; Catsiki et al. 1994; Depledge et al. 1994; Rainbow 1997; Langston et al. 1998). Moreover, it is interesting to note the important differences recorded in trace metal concentrations even among individuals collected from the same site, which has been previously reported in marine invertebrates (Depledge and Rainbow 1990) and limpets in particular (Cravo and Bebianno 2005). Since the influence of environmental factors can be ruled out due to the experiment's design, the physiological state of the individuals under study arises as the most plausible explanation for the abovementioned differences. Nutritional state and reproductive condition have been observed to be the physiological factors that could in greater measure contribute to these interindividual differences (see review by Depledge and Rainbow 1990). However, since these parameters have not been taken into consideration during the study, we cannot contrast this hypothesis. This idea is in any case supported by the fact that the two available reproduction studies that have been carried out for the species coincide in the fact that, on the contrary of what happens with other patellid species such as *Patella ferruginea* or *Patella ulyssiponensis*, an important proportion of *C. nigra* individuals can monthly be found in different reproductive states (Frenkiel 1975; Rivera-Ingraham 2010), which would increase the probability of having analyzed individuals in different reproductive

stages and thus, contributing to the high metal variability observed in the study.

As aforementioned, in the inner areas of Algeciras Bay, individuals showed significantly increased Cr and Mn concentrations, and previous authors have also pointed out the high Mn concentration values in the same area (Morillo and Usero 2008). However, it should also be taken into account the possibility that other biological factors may be influencing our results like: sex (Orren et al. 1980; Boening 1999), weight (Krantzberg 1989), biochemical composition (Frazier et al. 1985), or size of organisms (Boyden and Zeldis 1979; Boening 1999). Even though we did not record significant differences, our results agree with these reports; as we observed, as a general rule, higher trace metal concentration accumulation in larger individuals. This was, however, only statistically supported for V. It is also known that geochemical factors such as metal bioavailability, water temperature, pH, dissolved oxygen, and water salinity (Phillips 1976; Lares and Orians 1997) among others, can influence trace metal uptake. For example, lower water salinities (such as those found in the inner locations of Algeciras Bay, probably due to the fresh water supplied by several rivers as the "Guadarranque" and the residual effluents of coastal cities and smaller towns) (e.g., Sánchez-Moyano 1996) can increase trace metal uptake in marine invertebrates (e.g., Hutcheson 1974; Denton and Burdon-Jones 1981).

The present study can be considered as a first approximation to the study of the effects of pollutants and physicochemical parameters on the antioxidant metabolism of *C. nigra*. Future studies should include the analysis of the oxidative damage (measurements of protein carbonyls, DNA damage or malondialdehyde) to directly assess the impact of environmental quality on these organisms. Studies should furthermore be carried out in controlled conditions in order to confirm the results of the present study. The determination of the accumulated concentration of trace metals in *C. nigra* soft tissues in relation to the available concentrations of the same compounds in the environment should be deeply studied in order to determine the possibility of locally using this species as a pollution indicator. Finally, the determination of the ranges of tolerance of exposure to certain pollutants would be undoubtedly useful for the future management of the species.

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