Propensity to metal accumulation and oxidative stress responses of two infaunal species (*Cerastoderma edule* and *Nephtys hombergii*): are tolerance processes limiting their responsiveness?

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Abstract

The chronic exposure of infaunal organisms to metals in sediments can lead to the development of tolerance mechanisms, thus diminishing their responsiveness. This study aims to evaluate the accumulation profiles of V, Cr, Co, Ni, As, Cd, Pb and Hg and antioxidant system responses of two infaunal organisms (*Cerastoderma edule*, Bivalvia; *Nephtys hombergii*, Polychaeta). This approach will provide clarifications about the ability of each species to signalise metal contamination. Organisms of both species were collected at the Tagus estuary, in two sites with distinct contamination degrees (ALC, slightly contaminated; BAR, highly contaminated). Accordingly, *C. edule* accumulated higher concentrations of As, Pb and Hg at BAR compared to ALC. However, antioxidant responses of *C. edule* were almost unaltered at BAR and no peroxidative damage occurred, suggesting adjustment mechanisms to the metals present. In contrast, *N. hombergii* showed a minor propensity to metal accumulation, only signalising spatial differences for As and Pb and accumulating lower concentrations of metals than *C. edule*. The differences in metal accumulation observed between species might be due to their distinctive foraging behaviour and/or the ability of *N. hombergii* to minimise the metal uptake. Despite that, the accumulation of As and Pb was on the basis of the polychaete antioxidant defences inhibition at BAR, including CAT, SOD, GR and GPx. The integrated biomarker responses index (IBRv2) confirmed that *N. hombergii* was more affected by metal exposure than *C. edule*. In the light of current findings, the information of *C. edule* as a bioindicator should be complemented by that provided by another infaunal species under field studies, since tolerance mechanisms to metals can hinder a correct diagnosis of sediment contamination and the system's health. Overall, the present study contributed to improve the lack of fundamental knowledge of two widespread and common estuarine species, providing insights of the metal accumulation profiles under a scenario of chronic contamination. Finally, this work provided useful information that can be applied in the interpretation of future environmental monitoring studies.

Keywords: Infaunal organisms; Metal accumulation; Antioxidant defences; Integrated Biomarker Response; Tolerance mechanisms; Estuary contamination

1. Introduction

Marine organisms inhabiting highly contaminated environments, such as estuaries, are exposed to multiple stress agents and might develop an ability to cope with those stressors (Amiard-Triquet et al. 2011). In turn, this ability may allow a prevalence of the most well-adjusted specimens (Meyer and Giulio 2003), which could lead to a decrease in the biodiversity due to the extinction of the less adjusted ones (Lotze et al. 2006; Worm et al. 2006; Amiard-Triquet et al. 2011). Both genetic or physiological strategies could be on the basis of the organism adjustment to environmental contaminants (Amiard-Triquet et al. 2011). The first, also named as resistance, often confer a fitness increase at the population level, being inherited by the following generations (Meyer and Giulio 2003). On the other hand, physiological strategies (or tolerance) increase fitness at the individual level, reflecting biochemical, metabolic or even behavioural alterations. Ultimately, these responses enable the organisms to maintain their homeostasis when exposed to contaminants (Meyer and Giulio 2003; Geracitano et al. 2004).

Metals are recognized as long-lasting pollutants in estuarine systems, frequently potentiating the occurrence of biological adjustments in the exposed organisms. Hence, aquatic organisms inhabiting metal-contaminated estuaries may present several physiological responses that can be associated with the toxicokinetics and/or toxicodynamics processes. Regarding the toxicokinetics, the evaluation of metal accumulated levels in organisms represents the temporal integration of uptake, transport, transformation, accumulation, half-life periods and excretion. In turn, toxicodynamics is related to the toxic effects of metals at the organ, cellular, and molecular levels (Nordberg et al. 2014). To perform a realistic and suitable evaluation of the environmental health status of metal contaminated areas based on measurable biological variables (biomarkers), it seems crucial to address both processes mentioned above. Furthermore, the relevance and validity of a given risk assessment strategy also depend on the selection of appropriate sentinel species, thereby raising a

challenging question concerning the adequacy of more resistant and better adjusted species. In fact, at impacted environments, the evaluation of tolerant organisms can limit sub-lethal alterations, diminishing their responsiveness to stressors and, therefore, disabling the discrimination of different contamination degrees.

Since metals present a strong association with the sediment, metals bound to the sediments and in the interstitial water may directly and/or indirectly affect benthic organisms. Additionally, since both the feeding and behaviour of an organism directly affect its contact with sediment contaminants, it is essential to evaluate a range of organisms that have different routes of exposure (King et al. 2004). Therefore, bivalves and polychaetes exhibiting distinctive feeding and living habits are frequently selected to evaluate the biological effects of the sediment metal contamination (Courtney and Clements 2002; Elliott and Quintino 2007; Carvalho et al. 2011; Pereira et al. 2012; Freitas et al. 2012; Maranho et al. 2015).

In what concerns to the strategies adopted by bivalves and polychaetes, and regarding metals toxicokinetics, those directly related to the uptake, detoxification and storage seem of utmost relevance. Besides being modulated by environmental, biological and chemical conditions (Wang 2001; Wang and Rainbow 2005), the metal uptake rates can also be reduced as a response to the metal exposure *per se*, which results in metal-tolerant individuals (Wang and Rainbow 2005). Tolerance may not only be brought about by changes in metal uptake rates but also by changes in rates of excretion and/or storage detoxification (Mason and Jenkins 1995; Wang and Rainbow 2005).

Metal-tolerant bivalves and polychaetes might as well have developed strategies regarding metals' toxicodynamics. There are several studies linking metal exposure to the production of reactive oxygen species (ROS), which, in turn, may result in cellular damage, namely protein oxidation, lipid peroxidation and DNA damage (e.g. Valko et al. 2005; Zhang et al. 2010; Freitas et al. 2012). To counteract these reactive species, bivalves and polychaetes can use a range of low molecular weight enzymatic (e.g. CAT

and SOD) and non-enzymatic (e.g. GSH) players (Regoli 1998; Geracitano et al. 2004; Alves de Almeida et al. 2007; Ahmad et al. 2011, 2012; Freitas et al. 2012). These components interact in a sophisticated network, which main priority is the ROS detoxification and, consequently, the prevention of the cellular injuries referred above (Regoli and Giuliani 2014). Bearing this in mind, a question arises: are the antioxidant system defences somehow able to contribute to the tolerance of the bivalves and polychaetes at metal-contaminated environments? A few studies suggested that the antioxidant system may play an important role in the tolerance skills of the bivalves *Scrobicularia plana* (Ahmad et al. 2012) and *Mytilus galloprovincialis* (Box et al. 2007) under contaminated areas.

To the authors' knowledge, there is a significant lack of information regarding the involvement of antioxidant systems modulation on the tolerance mechanisms of marine invertebrates inhabiting metal-contaminated areas, particularly concerning the polychaetes' group. Moreover, there are only a few studies comparing species from different taxonomic groups with distinct ecological functions in the environment (e.g. Pérez et al. 2004; Solé et al. 2009; Freitas et al. 2012). For instance, the common cockle *Cerastoderma edule* (Linnaeus, 1758) and the polychaete worm *Nephtys hombergii* (Savigny in Lamarck, 1818) are two infaunal species with wide geographical distribution (Rodrigues et al. 2006; Silva et al. 2006). The bivalve *C. edule* is an active suspension filter-feeder (Dabouineau and Ponsero 2009) while *N. hombergii* is a scavenger predator that excavates burrows in the sediment on the hunt for food (Budd and Hughes 2005). Thus, both species present a distinct foraging behaviour, which is known to strongly influence metal accumulation patterns (Farag et al. 1998).

Hence, when planning monitoring studies at metal contaminated aquatic environments, it is important to be aware of the accumulation and physiological responses profiles of the candidate species or taxa, to select the most suitable bioindicators. Thus, the main aims of the present study were: (i) to evaluate the metal accumulation and antioxidant system profiles of two organisms that are representative

of their taxa, namely *C. edule* (Bivalvia) and *N. hombergii* (Polychaeta), from a metal-contaminated area and a reference area (Tagus estuary); (ii) to infer about the development of tolerance mechanisms in the organisms under metal exposure, addressing the repercussion on the value of each species as bioindicators of metal contamination. Keeping in view the role of antioxidants modulation in metal tolerance, organisms were analysed for metal bioaccumulation, and a battery of biochemical assays concerning the antioxidant system was performed.

2. Materials and Methods

2.1. Study area characterization

The present study was conducted in the Tagus estuary (Fig. 1), which is located in the most populated area of Portugal (Lisbon metropolitan area) and is one of the largest estuaries in Europe with a broad shallow bay covering an area of about 320 km^2. The Tagus estuary has an extremely high socio-economic value supporting several industries and human population. It has been submitted to intensive anthropogenic pressure, due to effluents from about 2.5 million inhabitants (Chainho et al. 2010), but also by contamination from several chemical, petrochemical, metallurgic, shipbuilding, cement manufacture industries and agriculture fertilizers/pesticides (Duarte et al. 2008). These impacts resulted in a high metal accumulation (e.g. Cr, Ni, Cd, Pb and Hg), among other contaminants, in sediments and organisms, particularly in a confined area - Barreiro (Canário et al. 2005; Neto et al. 2011; Caçador et al. 2012). The Tagus estuary also comprises an important Natural Reserve area with low anthropogenic impacts, which is located in the northern part of the estuary in its south margin, including Alcochete area (Fig. 1).

2.2. Sampling procedures

A field campaign was performed in February 2013. Two sampling areas were selected taking into account the different metal contamination levels previously

described for the sediments (Canário et al. 2005; França et al. 2005; Vale et al. 2008; Neto et al. 2011). The most contaminated area was located at Barreiro city margins (BAR), while Alcochete (ALC) was selected as a reference site since it presents minor contamination levels (Canário et al. 2005; Vale et al. 2008) (Fig. 1). At each sampling area, surface sediments were collected in triplicate with a Van Veen grab (0.05 m^2). From each grab, a sub-sample of sediment for metals determination was taken with a 2.5 – 15 cm corer, placed into plastic bags and transported to the laboratory. At the field, *C. edule* and *N. hombergii* specimens were separated from the sediment by sieving it through a 0.5 mm^2 mesh sieve and transported to the laboratory.

Water physico-chemical parameters (pH, salinity and temperature) were measured *in situ* in both sampling areas with an YSI 650 multi-parameter probe (Yellow Springs, USA).

At the laboratory, organisms were carefully washed to remove the excess of mud and measured. *C. edule* specimens presented a width (mean ± standard deviation) of 1.64 ± 1.01 cm (ALC) and 1.56 ± 0.99 cm (BAR), while *N. hombergii* specimens showed a length (mean ± standard deviation) of 20.6 ± 10.9 mm (ALC) and 29.0 ± 7.8 mm (BAR). Organisms were divided into two sets of samples: one for metal determination and other for oxidative stress evaluation. The organisms allocated to the subset for metals determination were depurated in plastic containers with water from the respective sampling areas and with constant aeration during 48 hours (Boening 1999). After the depuration period, organisms from both species are expected to be clean, minimizing thus the bias caused by adsorbed particles to the tissues. For both sets of samples, *C. edule* (soft tissues) and *N. hombergii* (whole organism), were preserved until further procedures at -20 and -80 ºC for metal determination and oxidative stress evaluation, respectively.

197 **2.3. Analytical procedures**

198 **2.3.1. Determination of metal levels in sediments and biological samples**

199 Prior to the analysis, each sediment sample was oven-dried at 40 ºC to constant
200 weight. Total Hg concentrations were determined in both dried sediment and
201 lyophilized biological samples (*C. edule* and *N. hombergii*) by atomic absorption
202 spectrometry, with no pre-treatment, using a silicon UV diode detector Leco AMA-254,
203 after pyrolysis of each sample in a combustion tube at 750 °C under an oxygen
204 atmosphere and collection on a gold amalgamator (Costley et al. 2000). Total Hg levels
205 were expressed as microgram per gram of dry weight (µg g^{-1}).

206 For determination of V, Cr, Co, Ni, As, Cd and Pb, sediment samples (about 100
207 mg) were completely mineralized with HF (40%) and Aqua Regia (HCl 36%:HNO_3 60%;
208 3:1) in closed Teflon bombs (100 °C for 1 h), evaporated to near dryness
209 (DigiPrepHotBlock – SCP Science), and re-dissolved with 1 mL of doubled-distilled
210 HNO_3 (prepared from 65% pro analysis) and 5 mL of ultra-pure water. Then, the
211 samples were heated for 20 min at 75 °C and diluted to 50 mL with ultra-pure water
212 (Caetano et al. 2007). Freeze dried biological samples were digested with a mixture of
213 HNO_3 (doubled distilled from 65%) and H_2O_2 (suprapure, 30%) at 60 °C for 12 h, at 100
214 °C for 1 h and at 80 °C for 1 h according to the method described in Ferreira et al
215 (1990). Procedural blanks were prepared using the same analytical procedure and
216 reagents but without sample. The metal quantification was made by an ICP-MS in a
217 Thermo Elemental – X Series and the results were expressed as microgram per gram
218 of dry weight (µg g^{-1}).

219 The accuracy of the analytical procedures was verified through the analysis of
220 certified reference materials, 1646a (estuarine sediment), Pacs-2 (marine sediment),
221 Mess-3 (marine sediment), 1566a (oyster tissue), BCR 278 (mussel tissue) and IAEA
222 452 (scallop tissue).

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225 **2.3.2. Biochemical analyses in *C. edule* and *N. hombergii***

226 Biological samples (*C. edule* soft tissues and *N. hombergii* whole tissues) were
227 homogenized using a Potter-Elvehjem homogenizer, in chilled phosphate buffer (0.1 M,
228 pH 7.4) in a 1:10 ratio [tissue mass (mg):buffer volume (mL)]. Eight individuals of *C.*
229 *edule* were homogenized for each site. In the particular case of *N. hombergii*,
230 organisms were homogenized as pools (2 individuals *per* pool, in a total of 5 pools),
231 due to the small sample mass. The resulted homogenate was then divided into two
232 aliquots for lipid peroxidation (LPO) measurement and post-mitochondrial supernatant
233 (PMS) preparation. The PMS preparation was obtained by centrifugation in a
234 refrigerated centrifuge (Eppendorf 5415R) at 13,400 g for 20 min at 4 °C. Aliquots of
235 PMS were then divided into microtubes and stored at -80 °C until further analyses.

236 Catalase (CAT) activity was assayed in PMS (at 25 ºC) by Claiborne (1985)
237 method, with slight modifications. Briefly, the assay mixture consisted of 0.195 mL
238 phosphate buffer (0.05 M, pH 7.0) with hydrogen peroxide (H_2O_2; 0.010 M) and 0.005
239 mL of PMS in a final volume of 0.2 mL. Change in absorbance was measured in
240 appropriated UV-transparent microplates (UV-Star® flat-bottom microplates, Greiner
241 Bio-One GmbH, Germany), recorded in a SpectraMax 190 microplate reader at 240 nm
242 and CAT activity was calculated in terms of μmol H_2O_2 consumed min^{-1} mg $protein^{-1}$
243 using a molar extinction coefficient of 43.5 M^{-1} cm^{-1}.

244 Superoxide dismutase (SOD) was assayed in PMS (at 25 ºC) with a Ransod kit
245 (Randox Laboratories Ltd., UK). The method employs xanthine and xanthine oxidase to
246 generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-
247 phenyltetrazolium chloride (INT), forming a red formazan dye determined at 505 nm in
248 a SpectraMax 190 microplate reader. Then, SOD activity is measured by the degree of
249 inhibition of this reaction, considering that one SOD unit causes a 50% inhibition of the
250 INT reduction rate, under the conditions of the assay. Results were expressed as SOD
251 units mg $protein^{-1}$.

252 Glutathione peroxidase (GPx) activity was determined in PMS (at 25 °C) according
253 to the method described by Mohandas et al. (1984) as modified by Athar and Iqbal
254 (1998). The assay mixture consisted of 0.09 mL phosphate buffer (0.05 M, pH 7.0),
255 0.03 mL ethylenediaminetetraacetic acid (EDTA; 0.010 M), 0.03 mL sodium azide
256 (0.010 M), 0.03 mL glutathione reductase (GR; 2.4 U mL^{-1}), 0.03 mL reduced
257 glutathione (GSH; 0.010 M), 0.03 mL nicotinamide adenine dinucleotide phosphate-
258 oxidase (NADPH; 0.0015 M), 0.03 mL H_2O_2 (0.0025 M) and 0.03 mL of PMS in a total
259 volume of 0.3 mL. Oxidation of NADPH to $NADP^+$ was recorded at 340 nm in a
260 SpectraMax 190 microplate reader and GPx activity was calculated in terms of nmol
261 NADPH oxidized min^{-1} mg $protein^{-1}$ using a molar extinction coefficient of 6.22×10^3 M^{-1}
262 cm^{-1}.

263 Glutathione reductase (GR) activity was assayed in PMS (at 25 °C) by the method
264 of Cribb et al. (1989) with some modifications. Briefly, the assay mixture contained
265 0.050 mL of PMS fraction and 0.250 mL of reaction medium consisted of phosphate
266 buffer (0.05 M, pH 7.0), NADPH (0.0002 M), glutathione disulfide (GSSG; 0.001 M) and
267 diethylenetriaminepentaacetic acid (DTPA; 0.0005 M). The enzyme activity was
268 determined by measuring the oxidation of NADPH at 340 nm in a SpectraMax 190
269 microplate reader and calculated as nmol NADPH oxidized min^{-1} mg $protein^{-1}$ using a
270 molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}.

271 Glutathione-S-transferase (GST) activity was determined in PMS (at 25 °C) using
272 1-chloro-2,4-dinitrobenzene (CDNB) as substrate according to the method of Habig et
273 al. (1974). The assay mixture consisted in 0.2 mL of phosphate buffer (0.2 M, pH 7.9),
274 CDNB (0.060 M) and GSH (0.010 M). The reaction was initiated by the addition of 0.1
275 mL of PMS and the increase in absorbance was recorded at 340 nm in a SpectraMax
276 190 microplate reader. GST activity was calculated as nmol CDNB conjugate formed
277 min^{-1} mg $protein^{-1}$ using a molar extinction coefficient of 9.6×10^3 M^{-1} cm^{-1}.

278 Total glutathione (GSHt) content in PMS was precipitated with trichloroacetic acid
279 (TCA; 12%) for 1 h (4 ºC) and then centrifuged at 12,000 g for 5 min at 4 °C. GSHt

content was determined in the resulting supernatant (deproteinated PMS) (at 25 °C) adopting the enzymatic recycling method using GR excess, whereby the sulfhydryl group of GSH reacts with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB; Ellman's reagent) producing a yellow colored 5-thio-2-nitrobenzoic acid (TNB) (Tietze 1969; Baker et al. 1990). The rate of TNB production is directly proportional to this recycling reaction, which is in turn directly proportional to the GSH concentration in the sample. The assay mixture consisted in 0.2 mL sodium phosphate buffer (0.143 M, pH 8), EDTA (0.0063 M), DTNB (0.001 M) and NADPH (0.00034 M), added to 0.04 mL of deproteinated PMS. The reaction is initiated with 0.04 mL of GR (8.5 U mL^{-1}). Formation of TNB was measured in a SpectraMax 190 microplate reader at 415 nm. It should be noted that GSSG is converted to GSH by GR in this system, which consequently measures total GSH. The results were expressed as nmol TNB formed min^{-1} mg $protein^{-1}$ using a molar extinction coefficient of 14.1×10^{3} M^{-1} cm^{-1}.

As estimation of LPO, TBARS quantification was carried out in the previously prepared homogenate according to the procedure of Ohkawa (1979) and Bird and Draper (1984) and adapted by Wilhelm Filho et al. (2001a; 2001b). Briefly, 0.005 mL of butylatedhydroxytoluene (BHT; 4% in methanol) and 0.045 mL of phosphate buffer (0.05 M, pH 7.4) were added to 0.05 mL of homogenate and mixed well to prevent oxidation. To this aliquot, 1 mL of TCA (12%), 0.9 mL of Tris-HCl (0.060 M, pH 7.4 and 0.0001 M DTPA) and 1 mL of thiobarbituric acid (TBA; 0.73%) were added and well mixed. This mixture was heated for 1 h in a water bath set at 100 ºC and then cooled to room temperature, transferred into 2-mL microtubes and centrifuged at 15,700 g for 5 min. The absorbance of each sample was measured at 535 nm in a SpectraMax 190 microplate reader. The rate of LPO was expressed in nmol of thiobarbituric acid reactive substances (TBARS) formed mg $protein^{-1}$ using a molar extinction coefficient of 1.56×10^{5} M^{-1} cm^{-1}.

Total protein contents were determined according to the Biuret method (Gornall et al. 1949), using bovine serum albumin (E. Merck-Darmstadt, Germany) as a standard.

308 In the particular case of *N. hombergii*, it was not possible to measure GST activity
309 as well as GSHt content, since those parameters presented values under the detection
310 limit of the methodology.
311
312 **2.4. Data analysis**
313 Statistica 8.0 software was used for the data analysis. Data were first tested for
314 normality and homogeneity of variances and transformed by ln (x) whenever normality
315 or homogeneity was not met. Differences were considered significant at $p < 0.05$. A
316 one-way analysis of variance (ANOVA) was applied to compare study areas (ALC
317 versus BAR) for metal concentrations (in sediment and in biological tissues) and
318 oxidative stress biomarkers in individuals.
319 To compare the total metal content in sediments from the two sampling areas, the
320 Metal Pollution Index (MPI) was obtained according to the following equation (AMA
321 1992; Usero et al. 1996):
322 $$MPI = (Cf_1 \times Cf_2 x \ldots\ldots \times Cf_n)^{1/n}$$
323 Where, Cf_1 is the concentration of the first metal, Cf_2 is the concentration of the
324 second metal and Cf_n is the concentration of the n^{th} metal.
325 To compare metal accumulation patterns between both species, two ratios (Ratio 1
326 and Ratio 2) were calculated according to the following formulas:
327 $$Ratio\ 1 = [Metal]_{BAR}/[Metal]_{ALC}$$
328 Ratio 1 was calculated both for sediments and the indicator species. [Metal] is the
329 mean concentration of a metal determined at each site in the sediment, in *C. edule* and
330 in *N. hombergii*. Values above 1 indicate that the sediment/organisms from BAR
331 presented/accumulated higher levels of that metal than sediment/individuals from ALC.
332 Values below 1 indicate that the sediment/organisms from ALC presented/accumulated
333 higher metal levels than sediment/individuals from BAR.
334
335 $$Ratio\ 2 = [Metal]_{C.\ edule}/[Metal]_{N.\ hombergii}$$

Where, [Metal] is the mean concentration of a metal determined in each of the indicator species. Values above 1 indicate that *C. edule* accumulated higher levels of a specific metal than *N. hombergii*, while values below 1 show a higher metal accumulation at *N. hombergii* than *C. edule*. Ratio 2 was calculated for both sites separately.

Biomarkers described in section 2.3.2 (CAT, SOD, GPx, GR, GST, GSHt and LPO) were combined into a stress index termed “integrated biomarker response version 2” (IBRv2) described by Sanchez et al. (2013). The IBRv2 is an improvement of the IBR calculation, previously described by Beliaeff and Burgeot (2002), since it allows the simultaneous integration of both up- and down-regulated biomarkers. This index was calculated for both species (*C. edule* and *N. hombergii*). Briefly, the mean of the individual biomarkers data (X_i) was compared to the mean reference value (X_0; mean value of each biomarker at the reference site - ALC) previously estimated for each biomarker; then, a log transformation was applied to reduce variance $Y_i = \log (X_i / X_0)$; in a next step, the general mean (μ) and the standard deviation (σ) of Y_i were computed as described by Beliaeff and Burgeot (2002), and Y_i is standardized $Z_i = (Y_i - \mu) / \sigma$. To create a basal line to represent the biomarker variation, the mean of standardized biomarker response (Z_i) and the mean of reference biomarker data (Z_0) were used to define a biomarker deviation index $A = Z_i - Z_0$. To obtain IBRv2, the absolute values of A parameters calculated for each biomarker were summed $IBRv2 = \sum |A|$. Finally, A parameters were depicted in a star plot to represent the deviation of each investigated biomarker in relation to reference values. The area up to 0 reflects biomarker induction, and the area down to 0 indicates a biomarker inhibition.

3. Results

3.1. Water and sediment characteristics

Water physico-chemical parameters, measured in ALC and BAR sites (Fig. 1) showed similar values between the two locals, namely pH of 8.9 ± 0.3 (ALC) and 8.4 ±

364 0.2 (BAR), salinity of 25.9 ± 0.1 (ALC) and 24.4 ± 0.1 (BAR) and temperature of 12.9 ±
365 0.1 ºC (at both ALC and BAR).

366 In general, BAR presented higher values of V, As, Cd, Pb and Hg than ALC site,
367 resulting in a greater metal pollution index (MPI) in the first site (Table 1). Moreover,
368 both ALC and BAR sites presented values of Ni, As, Pb and Hg above the effects
369 range low (ERL), where values below this threshold are unlikely to exert toxicity,
370 whereas only BAR site showed values of Hg above the effects range median (ERM),
371 where values above this threshold are likely to exert toxicity.

372

373 **3.2. Metal levels in *C. edule* and *N. hombergii***

374 Individuals of *C. edule* from BAR accumulated significantly higher levels of As, Pb
375 and Hg than those collected in ALC (Table 2). *N. hombergii* from BAR showed
376 significantly higher levels of As and Pb compared to the polychaetes collected in ALC
377 (Table 2).

378 The quotient between metal levels in organisms and sediment from BAR and ALC
379 was calculated for both species and sediment (Fig. 2A). In general, the sediment ratio
380 was higher than 1, confirming the higher metal contamination at BAR. Regarding *C.*
381 *edule*, ratios were higher than 1 for V, Co, As, Pb and Hg, while ratios lower than 1
382 were observed for Cr, Ni and Cd, which was, in general, in agreement with the
383 sediment ratios. Ratios of *N. hombergii* were greater than 1 for Cr, As and Pb.
384 Otherwise, ratios lower than 1 were found for V, Co, Ni, Cd and Hg in *N. hombergii*.

385 The metal accumulation profiles of both species in each local showed that, in
386 general, *C. edule* accumulates higher levels of metals than *N. hombergii* (Fig. 2B).
387 Those differences can range from two-fold (e.g. Cd or Hg) to eleven-fold for Ni in *C.*
388 *edule* relatively to *N. hombergii*. Co and As are the exceptions to this pattern,
389 presenting higher accumulation levels in *N. hombergii* than in *C. edule* and similar
390 levels between the organisms, respectively.

391

3.3. Biochemical analyses in *C. edule* and *N. hombergii*

C. edule showed significant differences in oxidative stress responses between sites only for the GPx activity, which was greater in BAR than ALC site (Table 3).

N. hombergii from BAR showed significant lower activities of CAT, SOD, GR and GPx, than those collected in ALC (Table 3).

The oxidative damage, measured as LPO levels, showed no differences between sites for both species (Table 3).

In general, *C. edule* presented higher levels of the antioxidant enzymatic activities (with the exception of GPx) than *N. hombergii*, for the same sites.

The IBRv2 results showed that *N. hombergii* (Fig. 3B) was the most sensitive bioindicator species when compared with *C. edule* (Fig. 3A), since the first presented a higher IBRv2 value (IBRv2 = 6.43 and IBRv2 = 3.62, respectively). Through the observation of the IBRv2 star plots, it is possible to observe that for *N. hombergii* (Fig. 3B), CAT, SOD, GR and GPx activities were the most discriminating factors between sites, since BAR individuals (black line) demonstrated lower activities of those enzymes than the reference (grey dashed line). Besides, a slightly increasing tendency in LPO levels is also evident in individuals from BAR. In *C. edule* (Fig. 3A), CAT, GR, GSHt and especially GPx were the most important factors, concerning spatial discrimination, since BAR individuals (black line) demonstrated higher activities of those than the reference (grey dashed line).

4. Discussion

4.1. Metal accumulation kinetics not always reflect sediment contamination

The evaluation of the physico-chemical characteristics of sediments from estuarine sites confirmed the distinction between the two zones, particularly regarding metal contamination, namely ALC and BAR areas. In general, the first presented lower sediment metal concentrations than the latter, resulting in a minor metal pollution index (MPI), identifying BAR as a metal-contaminated area. In fact, Hg levels in sediments of

BAR were above its ERM thus, toxicity related with this element is likely to occur (Long et al. 1995). Moreover, levels of Ni, As and Pb in sediments of BAR were above their respective ERL, suggesting a probable risk to organisms. Despite sediments of ALC presented levels of Ni, As, Cd, Pb and Hg above their ERL, a higher toxicity risk is expected to be occurring at BAR, since metal levels in sediments were higher than in ALC. Our results are in agreement with several previous studies that reported similar levels of Ni, As, Cd, Pb and Hg in the sediments from the same estuarine areas (Vale et al. 2008; Canário et al. 2010; Neto et al. 2011).

The present study revealed different metal accumulation kinetics for *C. edule* and *N. hombergii*. *C. edule* accumulation patterns better mirrored the sediment contamination by metals when compared with *N. hombergii*. The polychaete accumulated significantly lower concentrations of metal, regardless the site. These results may, in part, be explained by the distinct foraging behaviour presented by both species. *N. hombergii* is a predator and, consequently, more likely to uptake metals adsorbed in its preys (Robinson et al. 2003) than in the water or sediment particles, as expected to occur in suspension filter-feeders as *C. edule* (Bergayou et al. 2009).

A very distinct pattern regarding the accumulation of Hg was observed for both species. Only *C. edule* showed a significantly higher accumulation of Hg at the highest contaminated site (BAR). The inability of *N. hombergii* to discriminate sites regarding Hg accumulation is a remarkable result, especially considering the elevated persistence and bioaccumulation propensity of Hg, as well as the higher levels observed in the sediments from BAR. These dissimilar responses of *C. edule* and *N. hombergii* to the sediment metal contamination may also indicate a tolerant behaviour by the polychaetes living in the BAR area. *N. hombergii* might be able to perform a selection of the particles regarding their metal contamination (based on the taste) and then to avoid them (Mason and Jenkins 1995; Rainbow et al. 2004). Another possibility is the regulation of metals' intake as observed by King et al. (2004) for the polychaetes *Australonereis ehlersi* and *Aglaophamus australiensis* that were able to minimise the

448 uptake of Zn from the sediment and water compartments, although the opposite
449 behaviour occurred with Cu. These mechanisms allowed the organisms to tolerate high
450 concentrations of metals in the environment (Berthet et al. 2003; King et al. 2004).
451 *C. edule* populations were able to survive and inhabit at BAR, despite the elevated
452 metal bioaccumulation, which also suggests some tolerance mechanisms acquired by
453 the bivalve. This might be due to the ability of the compartmentalization of metals as
454 metal-sensitive fractions (i.e. organelles and heat-sensitive proteins) and biological
455 detoxification of metals (i.e. metallothioneins and metal-rich granules) (Wallace et al.
456 2003). Such compartmentalization of the metal ions will reduce their bioavailability to
457 body tissues and consequent toxicity to the organisms. This process has been
458 previously described as a metal tolerance mechanism in bivalves (Wallace et al. 2003;
459 Vijver et al. 2004; Freitas et al. 2012).
460 Despite the lack of studies comparing patterns of metal accumulation in both *C.*
461 *edule* and *N. hombergii,* they are known to be influenced by several geo- and physico-
462 chemical parameters as well as biological factors (Sanchiz et al. 2001; King et al. 2004;
463 Baudrimont et al. 2005). The biology of the selected species, including different feeding
464 behaviours, respiration, mobility and living habits, are important variables to take into
465 account concerning metal uptake and accumulation (King et al. 2004). Nevertheless, it
466 is interesting to notice that the species, which whole body is in a direct contact with the
467 sediment (*N. hombergii*), presents significantly lower metal levels than the bivalve (*C.*
468 *edule*), protected by the shells, suggesting a certain impermeability of the integument
469 that can hinder the uptake of metals.
470
471 **4.2. Relationship between oxidative stress profiles and metal**
472 **bioaccumulation levels in *C. edule* and *N. hombergii***
473 The balance between the pro-oxidant challenge promoted by ROS and the
474 organism's ability to detoxify the reactive intermediates, namely through the antioxidant
475 defence system, is crucial to the evolution of severe cellular damage, either in DNA,

proteins or lipids (Muniz et al. 2008). Regarding the toxicity mechanisms of metals, one of the most important is the enhancement of the intracellular ROS levels, through Fenton-like reactions (Ercal et al. 2001; Frenzilli et al. 2001). Besides, some metals, namely Cd, Pb and Hg, due to their electron-sharing affinities, can form covalent attachments with the enzymes from the antioxidant system, inactivating them (Ercal et al. 2001).

The evaluation of oxidative stress profiles in *N. hombergii* suggested that metals, namely As and Pb, could be acting by inhibiting the antioxidant system enzymes. Such inhibition occurred at the highest contaminated site (BAR) in comparison to reference site (ALC) and, thus, antioxidant enzymes levels were able to perform site discrimination. Since *N. hombergii* from BAR accumulated significantly higher concentrations of As and Pb than individuals from ALC, those metals might be the trigger of the CAT, SOD, GR and GPx inhibitions. In particular, CAT, SOD and GPx were described as Pb targets, by the formation of complexes with their substrates or even enzymatic synthesis inhibition (see Ercal et al. 2001). The majority of previous studies reported that after the exposure to metals, antioxidant defences of polychaetes are enhanced (Geracitano et al. 2004; Pérez et al. 2004; Díaz-Jaramillo et al. 2013; Gomes et al. 2013). As observed in the present study, Sandrini et al. (2008) also found a relationship between the exposure of the polychaete *Laeonereis acuta* to Cd and decrease of CAT activity. In another study, Nusetti et al. (2001) found that after seven days of exposure to sublethal Cu concentrations, GR activity in the polychaete *Eurythoe complanata* decreased. Despite the antioxidant defences inhibition, the polychaete *N. hombergii* was able to prevent the induction of LPO, probably either by other enzymatic and non-enzymatic antioxidant players not measured in the present study or different detoxifying mechanisms. Regardless the absence of LPO, different possible cellular injuries as DNA oxidative damage or protein oxidation should not be disregarded.

As previously stated, *C. edule* was the species that better reflected the sediment metal contamination. This species showed higher metal accumulation levels but seems to be more adjusted than *N. hombergii* regarding the modulation of the antioxidant defences. This tolerance is evident since *C. edule* antioxidant defence parameters were almost unaltered between the two assessed sites, ALC and BAR. However, minor increases in CAT and GR activities and significantly higher GPx were found at BAR. Such responses might indicate a proactive action by the cockle antioxidant system to prevent the induction of damage, as LPO, which not detected. Similar results were observed by Freitas et al. (2012), who found that none of the *C. edule* biochemical parameters studied, namely CAT, SOD and GPx, reflected the contamination gradient under analysis. Moreover, Zhang et al. (2010) did not find significant alterations concerning the antioxidant defences (CAT, SOD and GST) of the bivalve *Chlamys farreri* after an exposure to Cu. Contrastingly, a few studies revealed different patterns, namely an enhancement of CAT and SOD activities in *M. galloprovincialis* in polluted areas (Box et al. 2007) and the increase of CAT and LPO in *C. edule* under metal exposure (Bergayou et al. 2009).

In a recent work, Regoli and Giuliani (2014) stated that constantly higher levels of antioxidants activity in exposed organisms than in reference ones reflect their need to maintain a more elevated protection toward the environmental pro-oxidant challenge. Additionally, a comparable antioxidant efficiency between polluted and reference populations would indicate the occurrence of tolerant or counteractive mechanisms (Regoli and Giuliani 2014), which seems to be in line with *C. edule* responses. On the other hand, depressed defences indicate the inability of organisms to counteract the toxicity of ROS (Regoli and Giuliani 2014) and the occurrence of oxidative damage can be expected. Regardless the absence of LPO in *N. hombergii* from BAR, the polychaete antioxidant defences were depressed, thus the oxidative damage at different cellular structures, namely DNA and proteins, are plausible to be occurring.

4.3. Insights into environmental health assessment using *C. edule* and *N. hombergii*: a pros and cons perspective

When planning environmental health assessment studies using bioindicator species, it is important to have a comprehensive knowledge of the ecological and biochemical behaviour of the candidate sentinel species. In particular, the metal accumulation patterns and the oxidative stress profiles comprise two relevant topics, if we intend to investigate tolerance mechanisms in the selected organisms. In the present study, both species showed distinctive patterns. Even if both organisms were able to develop mechanisms that allow their survival in the contaminated area of the estuary (BAR), considering their high abundance in both areas (Piló et al. 2015), they seem to present different degrees of adaptation/acclimatisation. *C. edule* clearly reflected the metal contamination in the sediments from both areas of study. Thus, if one study aims to assess a direct relationship between the metals in the sediment and the bioaccumulation levels on the organism, the common cockle *C. edule* seems to be a good candidate. On the other hand, if the aim of the study is to evaluate early biochemical effects in the exposed organisms, the polychaete *N. hombergii* seems more appropriate. In particular, if we consider the antioxidant system responses, reflected at lower enzymatic activities, this species seems more responsive.

The clear interspecific variability in their responses can result from their different habits, physiology and life styles. The cockle is a filter-feeder while the polychaete lives burrowed in the sediment thus, they will better inform about water- and sediment-bound chemicals, respectively (Pérez et al. 2004). Freitas et al. (2012) also found differences between the bivalve *C. edule* and the polychaete *Diopatra neapolitana*, in what concerns to the metal accumulation and compartmentalization as well as oxidative stress parameters. In that study, the authors stated that the bivalve handled the metal chelation and precipitation in a more successful way while the polychaete was the best bioindicator to assess the oxidative stress status, which is in agreement with the current study.

559 Nevertheless, considering the numerous factors modulating metals
560 bioaccumulation and antioxidant defences and taking into account the diversity of
561 bivalves and polychaetes' habits, biology, life styles, the extrapolation of the results of
562 *C. edule* and *N. hombergii* to their respective taxonomic groups (i.e. Bivalvia and
563 Polychaeta) cannot be performed. Ultimately, when possible, both bioindicators should
564 be adopted and integrated to allow for a more comprehensive approach, particularly if
565 the study aims to assess the general environmental and ecotoxicological health status
566 of a system and their communities, as the macrobenthos at the estuaries.
567
568 **5. Conclusions**
569 Overall, the current findings highlighted distinctive patterns of the two potential
570 bioindicator species from Bivalvia and Polychaeta taxonomic groups regarding metal
571 accumulation and antioxidant system responses, possibly related with their distinct
572 foraging behaviour. Under a metal contamination scenario, *C. edule* better reflected the
573 sediment metal contamination patterns, accumulating higher concentrations of As, Pb
574 and Hg. Differently, *N. hombergii* showed a proficient behaviour in the reduction of
575 metal uptake, resulting in metal-tolerant polychaetes. *N. hombergii* antioxidant system
576 responses were more informative than *C. edule*'s. Nevertheless, the polychaete
577 defences were inhibited in the most contaminated site, probably due to the higher
578 accumulation of As and Pb.
579 *C. edule* demonstrated to be the most efficient species showing a similar trend to
580 metal contamination in sediments. This tolerance might be diminishing its
581 responsiveness to metal exposure, limiting the value of *C. edule* as a bioindicator
582 species. Thus, it is of utmost importance to complement the information of *C. edule*
583 with that of another infaunal species, namely *N. hombergii*, to better evaluate the
584 sediment quality and the system health.
585 Furthermore, the present study contributed to improve the lack of fundamental
586 knowledge concerning *C. edule* and *N. hombergii* metal accumulation patterns and

antioxidant system responses under environmentally metal exposure. Hence, this work provided useful information that can be adopted, supporting the interpretation of future environmental monitoring studies data.

Conflict of interest

The authors declare that they have no conflict of interest.

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857

858 **Figure captions**

859 **Fig. 1** Map of the Tagus estuary (Portugal), with the locations of the sediments
860 sampling and organisms collection, namely Alcochete – ALC (Reference area) and
861 Barreiro – BAR (Contaminated area)

862

863 **Fig. 2** Comparison of metal accumulation patterns between *C. edule* and *N. hombergii*.
864 **Ratio 1 (A):** ratio of metal accumulation levels between sites for sediments and the
865 indicator species; values above 1 – sediment/organisms from BAR
866 presented/accumulated higher levels of that metal than sediment/individuals from ALC
867 and values below 1 – sediment/organisms from ALC presented/accumulated higher
868 metal levels than sediment/individuals from BAR. **Ratio 2 (B):** ratio of metal
869 accumulation levels between species for each site; values above 1 – *C. edule*
870 accumulated higher levels of metal than *N. hombergii* and values below 1 – *N.*
871 *hombergii* accumulated higher levels of metal than *C. edule*. The metal As did not show
872 accumulation differences between species, for both sites, neither did Co at ALC

873

874 **Fig. 3** Integrated biomarker response (IBRv2) values of *C. edule* (A) and *N. hombergii*
875 (B) collected at the Tagus estuary and associated star plots. Black line corresponds to
876 IBRv2 index of individuals from contaminated site (BAR) and is represented in relation
877 to the reference site (ALC; 0; grey dashed line). Values above 0 reflect induction of the
878 biomarker; while below 0 indicate reduction of the biomarker

879

880 **Fig. 1**

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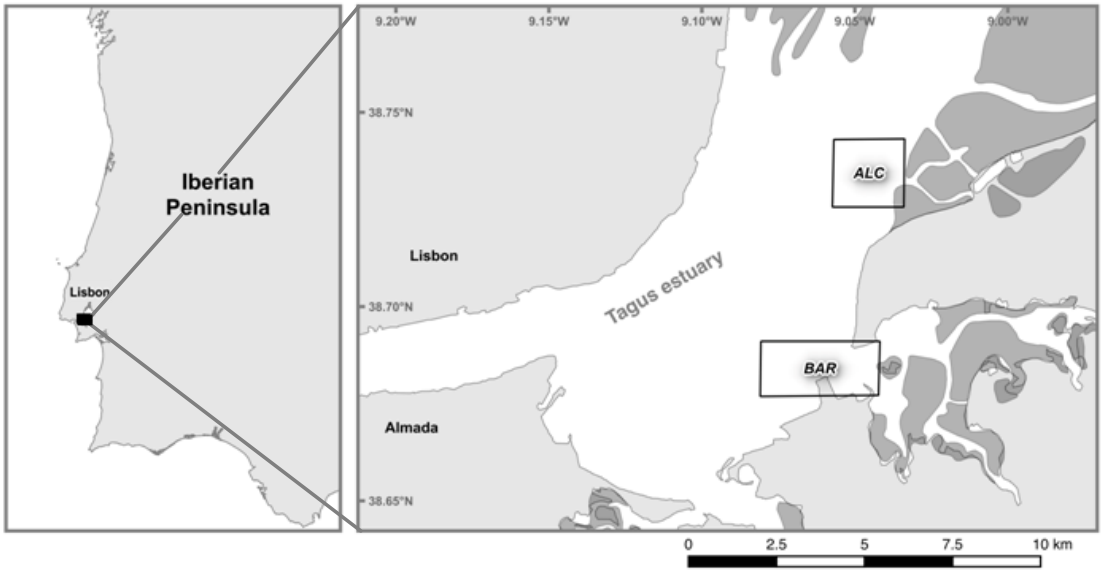

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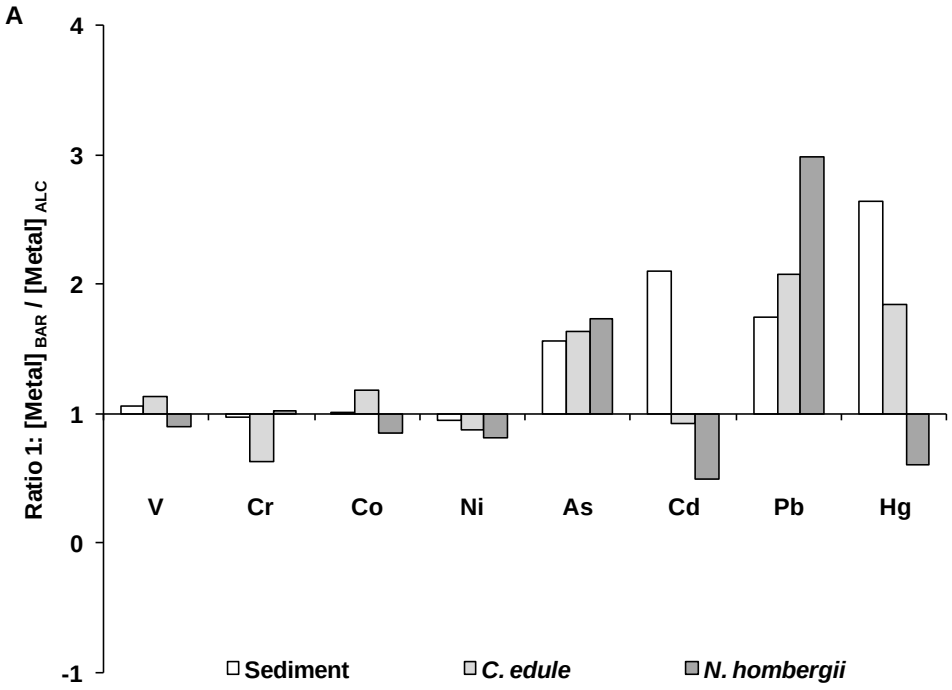
884 **Fig. 2**



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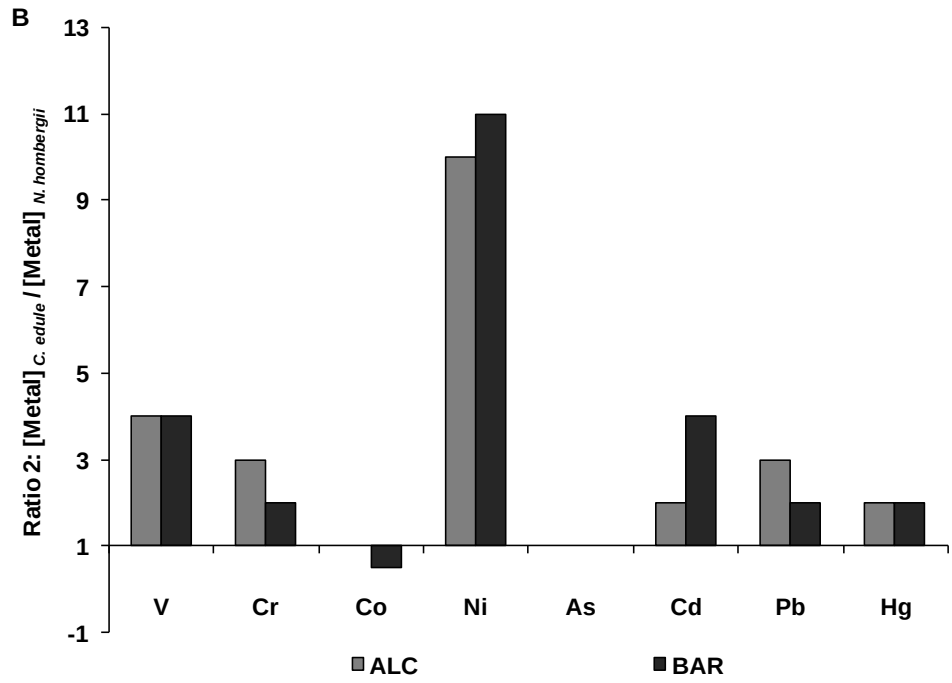

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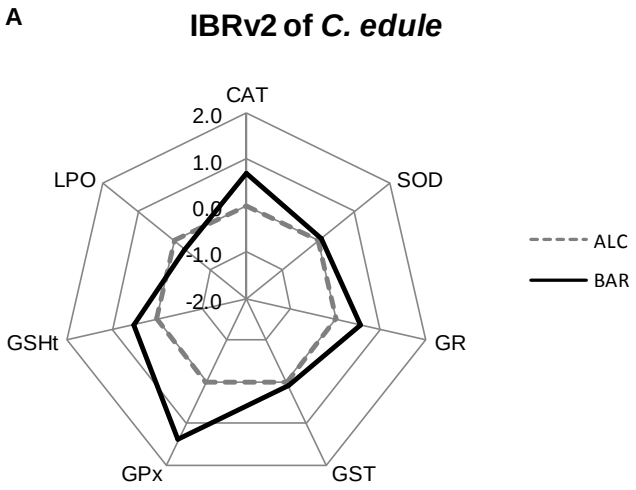
889 **Fig. 3**



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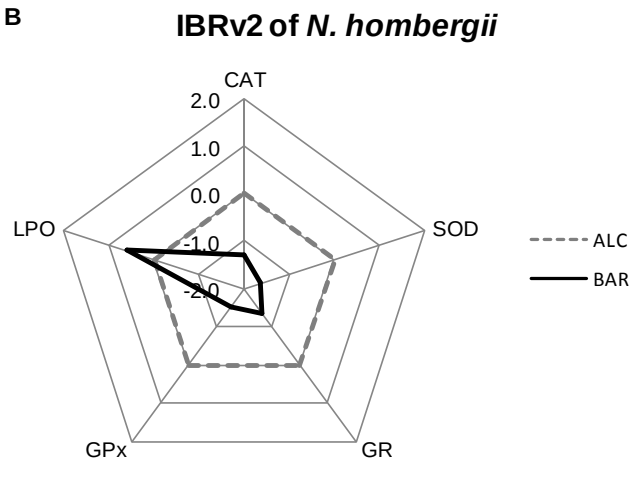

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Table 1 Metal concentrations (µg g^{-1}) in surface sediments collected in Alcochete (ALC) and Barreiro (BAR) at the Tagus estuary. ERL (Effects Range Low), where values below this threshold are unlikely to exert toxicity, and ERM (Effects Range Median), where values above this threshold are likely to exert toxicity, are represented for Cr, Ni, As, Cd, Pb and Hg, as well as MPI (Metal Pollution Index) of each site; (a) values above ERL and (b) values above ERM (Long et al. 1995)

			Metals (µg g^{-1})								MPI
			V	Cr	Co	Ni	As	Cd	Pb	Hg	
Site	ALC	Range	66.3 - 87.0	61.9 - 70.8	9.7 - 12.2	26.3 - 31.8	14.0 - 20.8	0.21 - 0.29	48.4 - 59.3	0.35 - 0.45	11 ± 1
		Mean	76.6	66.2	10.9	27.7[a]	17.3[a]	0.25	54.3[a]	0.40[a]	
	BAR	Range	58.8 - 95.8	46.5 - 75.9	6.9 - 14.4	21.4 - 31.8	12.6 - 59.2	0.17 - 1.24	26.0 - 225.7	0.17 - 3.71	14 ± 6
		Mean	81.0	64.1	11.1	29.2[a]	26.9[a]	0.54	94.7[a]	1.07[ab]	
	ERL	-	-	81.0	-	20.9	8.2	1.2	46.7	0.15	-
	ERM	-	-	370.0	-	51.6	70.0	9.6	218.0	0.71	-

Table 2 Metal concentrations (µg g^{-1}) in *C. edule* and *N. hombergii* (mean ± standard error) collected in ALC (Alcochete) and BAR (Barreiro). Statistically significant differences ($p < 0.05$) are: (a) in relation to ALC

		Metals (µg g^{-1})							
	Site	**V**	**Cr**	**Co**	**Ni**	**As**	**Cd**	**Pb**	**Hg**
C. edule	**ALC**	6.6 ± 0.77	3.2 ± 1.0	1.7 ± 0.28	47 ± 5.0	20 ± 0.75	0.73 ± 0.10	4.7 ± 1.2	0.31 ± 0.010
	BAR	7.5 ± 0.75	2.0 ± 0.31	2.0 ± 0.12	41 ± 4.2	33 ± 2.8[a]	0.67 ± 0.12	9.9 ± 1.3[a]	0.58 ± 0.11[a]
N. hombergii	**ALC**	1.9 ± 0.12	1.1 ± 0.16	3.1 ± 0.32	4.5 ± 0.97	18 ± 2.6	0.30 ± 0.060	1.6 ± 0.38	0.56 ± 0.18
	BAR	1.7 ± 0.11	1.1 ± 0.20	2.6 ± 0.42	3.7 ± 0.26	31 ± 2.9[a]	0.15 ± 0.010	4.7 ± 1.4[a]	0.34 ± 0.060

Table 3 Oxidative stress parameters in *C. edule* and *N. hombergii* (mean ± standard error) collected in ALC (Alcochete) and BAR (Barreiro), including catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione-S-transferase (GST) and glutathione peroxidase (GPx) activities, as well as total glutathione content (GSHt) and lipid peroxidation (LPO). Statistically significant differences ($p < 0.05$) are: (a) in relation to ALC. It was not possible to measure GST activity as well as GSHt content at *N. hombergii* individuals, since those presented values under the detection limit of the methodology

		Oxidative Stress Parameters						
	Site	**CAT** (μmol min^{-1} mg $prot^{-1}$)	**SOD** (U mg $prot^{-1}$)	**GR**	**GST**	**GPx**	**GSHt**	**LPO** (nmol TBARS mg $prot^{-1}$)
				(nmol min^{-1} mg $prot^{-1}$)				
C. edule	**ALC**	31 ± 3.4	36 ± 4.2	2.5 ± 0.30	2.2 ± 0.27	1.2 ± 0.16	0.20 ± 0.050	0.33 ± 0.030
	BAR	40 ± 4.2	36 ± 3.3	3.0 ± 0.31	2.2 ± 0.26	2.7 ± 0.46[a]	0.29 ± 0.050	0.32 ± 0.050
N. hombergii	**ALC**	13 ± 1.5	9.3 ± 1.0	1.6 ± 0.34	-	7.2 ± 1.9	-	0.090 ± 0.020
	BAR	5.4 ± 1.7[a]	3.5 ± 0.59[a]	0.80 ± 0.13[a]	-	2.4 ± 0.32[a]	-	0.11 ± 0.020