N-acetyl-β-D-glucosaminidase activity in feral *Carcinus maenas* exposed to cadmium

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23 Abstract

24 Cadmium is a priority hazardous substance, persistent in the aquatic environment, with the capacity to interfere
25 with crustacean moulting. Moulting is a vital process dictating crustacean growth, reproduction and metamorphosis.
26 However, for many organisms, moult disruption is difficult to evaluate in the short term, what limits its inclusion in
27 monitoring programmes. N-acetyl- β -D-glucosaminidase (NAGase) is an enzyme acting in the final steps of the
28 endocrine-regulated moulting cascade, allowing for the cast off of the old exoskeleton, with potential interest as a
29 biomarker of moult disruption. This study investigated responses to waterborne cadmium of NAGase activity of
30 *Carcinus maenas* originating from estuaries with different histories of anthropogenic contamination: a low impacted
31 and a moderately polluted one. Crabs from both sites were individually exposed for seven days to cadmium
32 concentrations ranging from 1.3 to 2000 $\mu\text{g/L}$. At the end of the assays, NAGase activity was assessed in the
33 epidermis and digestive gland. Detoxification, antioxidant, energy production, and oxidative stress biomarkers
34 implicated in cadmium metabolism and tolerance were also assessed to better understand differential NAGase
35 responses: activity of glutathione *S*-transferases (GST), glutathione peroxidase (GPx) glutathione reductase (GR),
36 levels of total glutathiones (TG), lipid peroxidation (LPO), lactate dehydrogenase (LDH), and NADP⁺-dependent
37 isocitrate dehydrogenase (IDH). Animals from the moderately polluted estuary had lower NAGase activity both in
38 the epidermis and digestive gland than in the low impacted site. NAGase activity in the epidermis and digestive
39 gland of *C. maenas* from both estuaries was sensitive to cadmium exposure suggesting its usefulness for inclusion in
40 monitoring programmes. However, in the digestive gland NAGase inhibition was found in crabs from the less
41 impacted site but not in those from the moderately contaminated one. Altered glutathione levels were observed in
42 cadmium-treated crabs from the contaminated site possibly conferring enhanced tolerance to these animals through
43 its chelator action. Investigation of enhanced tolerance should thus be accounted for in monitoring programmes
44 employing NAGase as biomarker to avoid data misinterpretation.

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49 **Keywords:** Shore crab, chitobiase, glutathione, anti-oxidant defences, environmental monitoring.

1. Introduction

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In recent years efforts have been made to promote the sustainable use of water and to reduce pollutant inputs in order to protect and improve the quality of the aquatic environment. The EU Water Framework Directive (WFD) regulates important aspects, related to the prevention of aquatic pollution, wastewater treatment efficiency and impact assessment of regulated and non-regulated substances, among others (Laane et al. 2012; Ruel et al. 2012). The metal cadmium has been identified as a priority hazardous substance by the European Union (Directive 2008/105/EC), representing a risk to human and environmental health. It is commonly found in aquatic systems, both from natural and anthropogenic origins (Jensen and Bro-Rasmussen, 1992), and is persistent in the environment requiring relentless understanding and detection of its detrimental effects.

Assessing the presence and effects of aquatic contaminants requires long-term chemical and biological monitoring. In this context, it is particularly important to improve the range of cost-effective parameters available to evaluate exposure and effects, and complement the data obtained with chemical analysis, providing an early detection of pollution problems (Hagger et al. 2008). Biomarkers have been recognised as strategic tools in the assessment of environmental quality of coastal waters giving a significant contribution to the weight-of-evidence approaches involved in risk assessment and classification of ecosystems' status recognised by the WFD (Picado et al. 2007; Hagger et al. 2008). Biomarker responses reflect the impact of all the stressors to which the organisms may be exposed, providing warning signals about the health status of species. The use of multibiomarker approaches in invertebrates is also of marked importance. Not only do they represent more than 95% of the known animal species but also they supply keystone organisms for ecosystem function (Schulte-Oehlmann et al. 2004). Moreover, several of them, including crustaceans, have been used and are recommended to investigate contaminant effects and evaluate environmental quality (Moreno et al. 2003; Zou 2005; Picado et al. 2007; Mesquita et al. 2011; Rodrigues et al. 2013, 2014).

Crustacean moulting (ecdysis) is a periodic phase in the crustacean life-cycle, essential for metamorphosis, growth, and reproduction, that involves the cast-off of the old exoskeleton exposing the new soft one formed beneath. Ecdysis is under the control of a complex hormonal

81 cascade. N-acetyl- β -D-glucosaminidase (NAGase), or chitobiase, is the last chitinolytic enzyme
82 of that pathway. It is responsible for the degradation of chitin oligosaccharides into monomers
83 that will be re-absorbed into the new cuticle (Espie and Roff 1995). Several studies have shown
84 that crustacean moulting and/or NAGase activity may be inhibited by exposure to endocrine
85 disrupting compounds such as polychlorinated biphenyls (PCBs), pesticides, 4-octylphenol,
86 diethylphthalate, and several metals (Xie et al. 2004; Zou 2005; Meng and Zou 2009; Zhang et
87 al. 2010). Recently, the levels of epidermal NAGase activity in *Carcinus maenas* were shown to
88 vary in relation to the moult cycle (Mesquita et al. 2011). However, despite the ecological
89 importance of *C. maenas* in estuarine and coastal food webs, and its sensitivity to chemical stress
90 (Mesquita et al. 2011; Rodrigues et al. 2013, 2014; Pereira and Pardal 2014), there is a lack of
91 studies addressing the responses of its NAGase activity to priority contaminants. As a parameter
92 measured at the sub-individual level, of relatively simple and cost-effective determination in
93 tissues of exposed organisms, *C. maenas* NAGase activity could provide an early diagnostic tool
94 for identifying exposure to chemical stress and assess the status of this species in relation to its
95 environment.

96 Earlier works reported adverse effects of cadmium on aquatic wildlife, including the modulation
97 of crustacean ecdysis and NAGase activity. Reddy and Fingerman (1995) exposed the fiddler
98 crab *Uca pugilator* to cadmium and registered alterations in brain and eyestalk neurosecretory
99 tissues responsible for the regulation of ecdysis and other physiological processes. Moreno and
100 colleagues (2003) observed moult arrest in the estuarine crab *Chasmagnathus granulata*
101 triggered by cadmium exposure. Xie et al. (2004) showed that NAGase activity of the prawn
102 *Penaeus vannamei* could be inhibited in vitro by cadmium. Hence, the aim of the present work
103 was to investigate the responses of *C. maenas* NAGase activity to cadmium in relation to its
104 tissue accumulation.

105 Since *C. maenas* from different areas may exhibit differential sensitivity to pollution (Rodrigues
106 et al. 2013, 2014), laboratory experiments were carried out with crabs originating from a low
107 impacted and a moderately contaminated estuary, providing wider characterisation of NAGase
108 interest for biomonitoring. Moreover, increased upregulation of detoxifying enzymes and
109 molecules involved in metal detoxification, often play a role in differential sensitivity of species
110 to contaminants (Knapen et al. 2004, Morgan et al. 2007), possibly with associated metabolic

costs. Therefore, to better understand NAGase responses in crabs from the two estuaries and the consequent implications for biomonitoring, we also included in this study biomarkers involved in the biotransformation process, anti-oxidant defences, oxidative stress and energy production, which are known to be involved in the response to cadmium exposure (Perrin and Watt 1971, Manca et al. 1991, Hatcher et al. 1995, Stohs et al. 2000, Wang and Wang 2009). These were the activities of enzymes glutathione peroxidase (GPx), glutathione S-transferases (GST), glutathione reductase (GR), total glutathione (TG), lactate dehydrogenase (LDH), NADP⁺-dependent isocitrate dehydrogenase (IDH) activities, and lipid peroxidation (LPO) levels.

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121 **2. Material and Methods**

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123 **2.1. Crab sampling and maintenance in the laboratory**

124 Crabs were collected at the mouth of the estuaries of the Minho and Lima rivers (Rodrigues et al. 2013) during autumn. The Minho estuary is under low human pressure, with low historical levels of metals and polycyclic aromatic hydrocarbons (PAHs) (Ferreira et al. 2003; Reis et al. 2009). Cadmium concentrations in the sampling site ranged between 6.4 and 8.9 µg/g dw (Guimarães et al., unpublished data). The Lima estuary has several industries located nearby, and a harbour and a shipyard. It shows high population density and receives wastewaters of industrial, soil leaching, livestock and urban origin) (Ferreira et al. 2003). It is contaminated by metals, with cadmium concentrations in the sampling site ranging between 27 and 91.5 µg/g dw (Guimarães et al., unpublished data).

133 Male crabs (41.6 ± 0.97 mm, cephalothorax width; mean $\pm$ SD) in intermoult stage (C4) (Drach 1939; O'Halloran and O'Dor 1988) were captured using baited hand-nets. Water temperature, salinity, dissolved oxygen, and pH were measured, in triplicate, during each sampling, using a multiparametric sea gauge WTW multi 340i with the appropriate probes (pH SenTix 41 and Tetracon 325). The mean values, and corresponding standard deviation (SD), obtained for these parameters were similar for the two sampling sites (Table 1).

139 The animals were immediately transported to the laboratory in thermally insulated boxes and 140 acclimated for at least 14 days to laboratory conditions. During this period the crabs were kept

141 individually in 2 L capacity glass beakers filled with filtered seawater (15 psu), in a temperature
142 ($16 \pm 1^\circ\text{C}$) and photoperiod (14:10 h light:dark) controlled room. The recipients were covered
143 and aeration was provided. Crabs were fed every other day, 1-2 hours prior to medium renewal.

144

145 **2.2. Chemicals**

146 Cadmium chloride (CdCl_2 , Cas no. 10108-64-2) was purchased from Merck (Darmstadt,
147 Germany), and the chemicals for enzymatic analyses were purchased from Sigma–Aldrich
148 Chemical (Steinheim, Germany). The Bradford reagent was purchased from Bio-Rad (Munich,
149 Germany). For cadmium analysis nitric acid (65%, Suprapur®, Merck, Darmstadt, Germany)
150 was used; standard solutions were prepared daily by dilution of a cadmium stock solution (1000
151 mg L^{-1} ; Panreac, Barcelona, Spain) in 0.5% (v/v) aqueous nitric acid solution. Monobasic
152 ammonium phosphate (Merck, Darmstadt, Germany) was used as matrix modifier. The solutions
153 were prepared with ultrapure water (resistivity = $18.2 \text{ M}\Omega\cdot\text{cm}$) obtained from a Simplicity 185
154 water purification system (Millipore, Molsheim, France).

155

156 **2.3. Exposure experiments**

157 Organisms from both populations were exposed to five cadmium concentrations (1.3, 8, 50, 320
158 and $2000 \mu\text{g/L}$). Exposure levels were chosen to include both environmentally relevant
159 concentrations and concentrations reported to cause accumulation in *C. maenas* (Bjerregaard
160 1990). Stock solutions of cadmium were prepared in ultra-pure water and diluted with filtered
161 seawater (15 psu) to prepare the test medium. Control groups prepared only with filtered
162 seawater were included in the assays. Three replicates of each treatment (control and cadmium)
163 were prepared for each population. In each replicate 4 crabs were individually exposed in 2 L
164 glass beakers, under temperature, photoperiod and aeration conditions previously described for
165 the acclimation period. Test solutions were renewed every other day. Water temperature,
166 conductivity, salinity, pH, and dissolved oxygen (DO) were monitored during the assays in the
167 old and the freshly prepared test solutions. No food was provided to the crabs during the assays.
168 Samples of old (48h) and freshly prepared test solutions of the highest exposure concentration
169 were collected for determination of ionic cadmium. At the end of the assays crabs were ice
170 anaesthetised and samples of epidermis (beneath the dorsal carapace), muscle (first pair of

171 locomotor appendices) and digestive gland were collected for biomarker analysis. Each
172 biomarker was determined in nine to twelve crabs per treatment. For each treatment, the
173 remaining epidermis, muscle and digestive gland, together with the rest of the soft tissues of the
174 crabs, hereafter referred to as whole-body soft tissues for simplicity, were collected, pooled, and
175 used for the quantification of cadmium.

176

177 **2.4. Determination of cadmium in water samples and whole-body soft tissues**

178 The cadmium quantifications were performed at 228.8018 nm with a High Resolution-
179 Continuum Source Atomic Absorption Spectrophotometer (HR-CS-AAS, ContrAA 700,
180 Analytik Jena, Jena, Germany) using electrothermal atomization, after acid mineralization by
181 microwave-assisted digestion (MARS-X, CEM, Mathews, NC, USA) in HP-500 Plus Teflon
182 vessels (CEM, Mathews, NC, USA), according to APHA recommendations (APHA, 1992).
183 Soft tissues were lyophilized, ground and homogenised. The microwave acid digestion of soft
184 tissue samples was performed using 130-mg portions and 10 ml of a 27% nitric acid solution
185 (Reis, 2008). For the digestion of the water samples 7-mL aliquots and 3 ml of a nitric acid
186 solution (65%) were used (Reis, 2008). The optimised microwave digestion temperature was 160
187 °C (20 min) at a maximum pressure of 200 psi. The atomic adsorption parameters were
188 optimised and were based on the guidelines provided by the equipment's manufacturer. The
189 external calibration method was used for cadmium quantification and the analytical procedure
190 was checked using samples spiked with known amounts of cadmium. Satisfactory mean
191 recoveries (90.5 – 114 %) were obtained. All samples were analysed in triplicate.

192

193 **2.5. Biomarkers**

194 All biomarker determinations were performed as described for *C. maenas* by Mesquita et al.
195 (2011) and Rodrigues et al. (2013). A portion of epidermis and digestive gland of each crab was
196 homogenised and centrifuged. The supernatant was recovered for the determination of NAGase
197 activity (Mesquita et al. 2011). Muscle samples were homogenised in the respective buffers,
198 centrifuged, and the supernatants recovered were used for the determination of LDH and IDH
199 activities. Digestive gland was homogenised in K-phosphate buffer. An aliquot of homogenate
200 was reserved for determination of LPO levels. The remaining homogenate was centrifuged and

the post-mitochondrial supernatant recovered was used for the determination of GST, GPx and GR activities, and TG levels as well. Except for LPO, biomarkers were expressed as a function of the concentration of protein in the samples. Protein concentration was determined by the Bradford method (Bradford 1976). Bovine γ -globuline was used as standard. Spectrophotometric readings were performed in a Bio-Tek Power Wave 340 microplate reader or a Jasco 6405 UV/VIS spectrophotometer.

207

208 **2.6. Data analysis**

Results of cadmium concentrations in water are presented as mean $\pm$ SD (standard deviation). All other results are presented as mean $\pm$ SE (standard error of the mean). Significant differences in the biomarker responses in relation to cadmium treatments and the estuary of origin of the crabs were first sought by factorial two-way analysis of variance (ANOVA), to test for significance of the interaction term. When significance of this term was found, one-way ANOVA with planned comparisons was performed for the interaction variable. ANOVA assumptions were tested using the Shapiro–Wilk and the Levene's test. When appropriate, the logarithmic transformation was applied to the data in order to fulfil ANOVA assumptions. Significant differences were accepted for $p < 0.05$ in all tests performed. All statistical analyses were carried out in SPSS IBM v19.0.

219

220

221 **3. Results**

222

223 **3.1. Cadmium in water samples and whole-body soft tissues**

Throughout the experiments water parameters were kept stable in all test treatments. Dissolved oxygen and pH ranged between 9.0-9.2 mg/L and 7.7-7.8, respectively. Salinity varied between 15.0-15.2 and temperature ranged between 16.0-16.3 °C.

In freshly prepared medium of the highest test treatment (2000 $\mu\text{g/L}$), measured dissolved cadmium was between 95% and 104% of the nominal concentration, corresponding to $1905 \pm 50.2 \mu\text{g/L}$ (mean $\pm$ SD) and $2076 \pm 27.6 \mu\text{g/L}$, respectively. In the old exposure medium measured dissolved cadmium was 4% to 15% lower than in the freshly prepared one. At the end

231 of the 7-day experiments a significant concentration-dependent accumulation of cadmium in the
232 whole-body soft tissues was observed, which peaked at $89.6 \pm 14.7 \mu\text{g/g dw}$ (mean $\pm$ SD) in
233 crabs from the Minho and at $99.9 \pm 17.5 \mu\text{g/g dw}$ in crabs from the Lima estuary exposed to
234 2000 $\mu\text{g/L}$ (Fig. 1).

235

236 **3.2. Biomarkers**

237 *3.2.1. Moulting*

238 Crabs collected from the Lima estuary showed significantly lower epidermal NAGase activity
239 (*ca.* 65% on average) than those collected from the Minho estuary (Table 2, Fig. 2). However,
240 the response pattern of crabs from both estuaries was similar. High exposure concentrations
241 (2000 $\mu\text{g/L}$) significantly decreased NAGase activity (by 36% in Minho crabs and 45% in Lima
242 crabs, $p < 0.05$).

243 NAGase activity in the digestive gland was also significantly lower (about 40% on average) in
244 crabs from the Lima than in those from the Minho estuary (Table 2, Fig. 2). Cadmium also
245 caused a decrease of NAGase activity in Minho crabs, which was significantly lower than
246 controls at 2000 $\mu\text{g/L}$ (-34%, $p < 0.05$). However, no significant differences among experimental
247 groups were found for Lima crabs.

248

249 *3.2.2. Biotransformation, anti-oxidant defences and oxidative stress*

250 GST activity in the digestive gland was strikingly higher (*ca.* 7.5 folds, $p < 0.05$) in Minho crabs
251 than in Lima crabs (Table 2, Fig. 3). Moreover, a different response pattern to cadmium exposure
252 was found for the two study sites. In Minho crabs, induction of GST increased with the cadmium
253 concentration, so that at 2000 $\mu\text{g/L}$ the enzyme activity was 45% higher than that of the control
254 group ($p < 0.05$). In Lima crabs, no significant differences among experimental groups could be
255 depicted.

256 Opposite results were obtained for TG levels. The levels of glutathione were significantly higher
257 in Lima than in Minho crabs (about 8 folds, $p < 0.05$) (Table 2, Fig. 3). In Lima crabs, TG levels
258 increased with cadmium concentrations and were 50% higher in the 2000 $\mu\text{g/L}$ group, compared
259 to the respective control group ($p < 0.05$). No significant differences among experimental groups
260 were found for Minho crabs.

261 Lima crabs additionally showed significantly lower GPx activity (*ca.* 55% on average) than
262 Minho crabs (Table 2, Fig. 3). However, no differences among treatment groups were found in
263 either site. Also, for GR activity and LPO levels there were no differences between estuaries or
264 treatment groups (Table 2, Figs. 3 and 4).

265

266 3.2.3. Energy production

267 Crabs from the Minho and the Lima estuaries exhibited similar levels of IDH activity and
268 patterns of response to cadmium exposure. IDH activity increased with the cadmium
269 concentration, in crabs from both sites, with significant differences from the respective control
270 group found at >50 µg/L cadmium (Table 2, Fig. 5). Such results suggest an increase in the
271 aerobic pathway of energy production to cope with the exposure. Compared to the control groups,
272 at 2000 µg/L cadmium IDH was increased by 92% ($p<0.05$) and 56% ($p<0.05$) in the Minho and
273 the Lima crabs, respectively.

274 Concerning LDH, differences observed were only related to the origin of the crabs. Crabs from
275 the Lima exhibited higher LDH activity (*ca.* 44%, $p<0.05$) than those from the Minho estuary.

276

277

278 4. Discussion

279

280 In the present study a concentration-dependent accumulation of cadmium in *C. maenas* tissues
281 was found, which was linearly proportional to the cadmium level in the exposure media.
282 Cadmium concentrations measured in whole body soft tissues of exposed organisms were in
283 accordance with previously reported accumulation for *C. maenas* exposed to similar levels for an
284 equivalent period (Nørum et al. 2005 and references therein). The residual levels detected in
285 control animals (~0.5 µg/L) from both sites were within the range of background concentrations
286 previously found for *C. maenas* males collected in uncontaminated areas (Bjerregaard et al.
287 2005). The data are also suggestive of similar cadmium uptake in crabs from the two studied
288 sites. On this regard, it has been reported that uptake rates of cadmium did not show relevant
289 significant differences between *C. maenas* from metal-rich and control estuaries (Rainbow et al.
290 1999). According to these authors, the chronic exposure to raised trace metal availabilities was
291 not sufficient to select for a reduction in dissolved trace metal uptake rate (Rainbow et al. 1999).

292 While moulting is a process that may be affected by environmental contaminants, its disruption
293 is difficult to evaluate in an expeditious manner. The importance of this process for growth,
294 reproduction and metamorphosis of aquatic crustaceans sets the need for cost-effective
295 environmental biomarkers of moulting disruption to be assessed in species recommended for use
296 in pollution monitoring, such as *C. maenas* (Picado et al. 2007). As an enzyme acting in the final
297 step of the endocrine cascade regulating moulting, the potential of NAGase activity as biomarker
298 has been shown for some crustacean species (Zou 2005). However, available information on
299 differential sensitivity of *C. maenas* NAGase activity to priority contaminants is scarce. The
300 present study shows the enzyme activity may be inhibited by cadmium exposure, indicating its
301 potential for inclusion in monitoring programmes. Further, the previous history of exposure to
302 contamination enhanced tolerance to cadmium, eliciting differential responses of NAGase
303 enzyme, probably through the involvement of glutathione.

304 The NAGase activity levels recorded here are in the same range of those previously obtained for
305 *C. maenas* epidermis and digestive gland (Mesquita et al. 2011). They were, however, clearly
306 higher than those observed for the fiddler crab (Zou and Fingerman 1999). In addition to
307 differences inherent to the species, a gender effect may also account for these dissimilarities, as
308 Zou and Fingerman (1999) determined the enzyme activity in females, whereas males have been
309 used here. In the epidermis of *C. maenas*, NAGase activity was significantly inhibited by 2000
310 $\mu\text{g/L}$ cadmium, independently of the crabs' site of origin, suggesting that upon prolonged
311 exposure crabs may persist longer on intermoult stage or suffer moult arrest. The inhibition may
312 result from cadmium interference with early steps of the endocrine moulting cascade and/or from
313 direct effect on the enzyme. Previous studies with eyestalk-ablated adult crabs of the species *C.*
314 *granulata* suggest that cadmium inhibits moulting by inhibiting the secretion of ecdysone
315 (Moreno et al. 2003). Conversely, *in vitro* studies performed with purified NAGase extracts from
316 the prawn *Litopenaeus vannamei* have shown that cadmium was able to inhibit this enzyme to
317 some extent (Xie et al. 2004). Future research should address the mechanism by which inhibition
318 may occur. NAGase activity in the digestive gland appeared to be more sensitive to cadmium
319 than that of the epidermis, although only in Minho crabs. In these organisms, inhibition of the
320 enzyme activity was observed in all cadmium treatments (1.3-2000 $\mu\text{g/L}$). The differences found
321 between the epidermis and digestive gland of Minho crabs may be related to the differential

distribution and physiological relevance of organ-specific NAGase isoforms (Mesquita et al. 2011; Peters et al. 1999). On the other hand, the absence of NAGase inhibition noted in the digestive gland of Lima crabs suggests that these animals coped better with cadmium exposure. Interestingly, upon cadmium exposure Minho crabs exhibited increased GST activity (at >50 µg Cd/L), with no alterations of TG levels. Lima crabs showed no changes in GST but significant increases in TG levels (at 1.3 and 50-2000 µg/L). Glutathione is a key component of the redox cycle, and potent free radical scavenger that also acts as cofactor of detoxification and antioxidant enzymes (e.g., GST, GR and GPx). The molecule deals with metal stress mainly by two mechanisms: by relieving oxidative stress effects caused by metals through the formation of oxidised glutathione; and by forming complexes with metals (Hatcher et al. 1995). Other authors, working with cell lines, have reported that elevation of cellular glutathione level is involved in cadmium resistance, rather than decreased cadmium uptake or enhanced cellular metallothionein synthesis (Hatcher et al. 1995). They have also observed that, despite the elevation of cellular glutathione level, the activities of glutathione redox cycle enzymes GPx and GR, and anti-oxidative enzymes catalase (CAT) and superoxide dismutase (SOD) remained unchanged (Hatcher et al. 1995). Hence, these authors suggested that enhanced expression of γ-glutamylcysteine synthetase, the rate-limiting enzyme for glutathione biosynthesis, was responsible for elevated cellular glutathione levels. In the present work, for both Minho and Lima crabs, no changes relative to the respective controls were found in GPx or GR (the enzyme involved in glutathione recycling). Enhanced production of glutathione in Lima crabs appears, thus, to be an effective mechanism in preventing oxidative damage and cadmium NAGase inhibition in the digestive gland. In contrast, GST induction in Minho crabs, appear to protect against lipid peroxidation, but were not able to prevent NAGase inhibition in the hepatopancreas upon cadmium exposure. GSTs are multifunctional proteins, which in addition to their intrinsic transferase activity, also have glutathione peroxidase activity toward ROS (Casalino et al. 2007). The activation of GST enzymes triggered by cadmium exposure was also observed in several marine worms (Won et al. 2011 and references herein). Moreover, according to previous studies GST may have a protective role against Cd toxicity, in terms of physiological alterations related to metabolism and cell protection (Wang et al. 2009, Won et al. 2011). Overall, the results from the present work suggest that animals from Minho and Lima estuaries use different detoxifying

mechanisms to deal with cadmium exposure, and support the role of glutathione as chelating agent, conferring enhanced tolerance to this metal. GST induction may represent an initial response to cadmium, as seen in Minho crabs, which in animals under chronic exposure to contamination, as Lima crabs, may give way to glutathione chelation to deal with the chemical challenge and achieve a new physiological steady state, allowing homeostasis maintenance (Mouneyrac et al., 2011). Both mechanisms appear to impose increased energetic burden in the aerobic pathway of energy production as indicated by the significant induction, relatively to controls, of IDH activity found in both Minho and Lima crabs. Mitochondrial IDH catalyses decarboxilation of isocitrate into α -ketoglutarate with concurrent production of NADPH (Jo et al. 2001). It is also known to be induced by the presence of reactive oxygen species (ROS), providing NADPH for the regeneration of GSH in the mitochondria, which in turn protects against oxidative damage and cell death (Jo et al. 2001).

§

5. Conclusions

Overall, *C. maenas* NAGase activity was sensitive to cadmium exposure suggesting its usefulness for incorporation in monitoring programmes. Glutathione appears to confer enhanced tolerance to cadmium, preventing NAGase inhibition, in animals with a previous history of exposure to moderate contamination. Investigation of the enhanced tolerance should thus be accounted for in monitoring programmes employing NAGase as biomarker, to avoid data misinterpretation. Tolerance to metal pollution has been considered as a trait that may facilitate adaptation to contaminated estuaries (Dafforn et al. 2009), an important aspect given that *Carcinus maenas* is also a well known widespread non-indigenous species that has colonised metal polluted areas (Cohen et al. 1995; Klassen and Locke 2007).

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Figure captions

Fig. 1. Cadmium accumulation (mean $\pm$ SE) in whole body soft tissues of *C. maenas* from the Minho and the Lima estuaries after a 7-day exposure.

Fig. 2. N-acetyl- β -D-glucosaminidase (NAGase) activity (mean $\pm$ SE) in the epidermis and digestive gland of *C. maenas*, collected from the Minho and the Lima estuaries, after 7-day exposure to cadmium. *Indicates significant differences in relation to the control at $p < 0.05$. Different letters indicate significant differences between sampling sites within each treatment level at $p < 0.05$.

Fig. 3. Levels of total glutathiones (TG) and activity of the enzymes glutathione *S*-transferases (GST), glutathione reductase (GR) and glutathione peroxidase (GPx) in the digestive gland of *C. maenas*, from the Minho and the Lima estuaries, after 7-day exposure to cadmium. *Indicates significant differences in relation to the control at $p < 0.05$. Different letters indicate significant differences between sampling sites within each treatment level at $p < 0.05$.

Fig. 4. Lipid peroxidation (LPO) levels (mean $\pm$ SE) in the digestive gland of *C. maenas*, collected from the Minho and the Lima estuaries, after 7-day exposure to cadmium.

Fig. 5. Activity of the enzymes NADP⁺-dependent isocitrate dehydrogenase (IDH) and lactate dehydrogenase (LDH) in muscle of *C. maenas*, from the Minho and the Lima estuaries, after 7-day exposure to cadmium. *Indicates significant differences in relation to the control at $p < 0.05$. Different letters indicate significant differences between sampling sites within each treatment level at $p < 0.05$.

581 **Table captions**

582

583 Table 1. Physico-chemical parameters (mean $\pm$ SD) measured (in triplicate) in the local water
584 during each sampling campaign. T, temperature ($^{\circ}$ C); Sal, salinity (psu); DO, dissolved oxygen
585 (mg/L).

586

587 Table 2. Results of the full-factorial two-way ANOVAs carried out to assess the effects of cadmium and
588 the sampling site on moulting and detoxification biomarkers of *C. maenas*.

589

Table 1

	Minho	Lima
T	14.1 ± 0.13	14.1 ± 0.16
pH	7.9 ± 0.2	7.7 ± 0.3
Sal	8.2 ± 2.4	17.6 ± 3.6
DO	9.5 ± 1.9	9 ± 2.9

Table 2

Parameter	Source of variation	<i>df</i>	<i>F</i>	<i>P</i>
<i>Moulting</i>				
NAGase	Estuary	1, 106	68.04	<0.001
Epidermis	Cadmium	5, 106	2.90	0.017
	Estuary x Cadmium	5, 106	0.47	0.799
NAGase	Estuary	1, 122	136.88	<0.001
Digestive gland	Cadmium	5, 122	2.93	0.016
	Estuary x Cadmium	5, 122	3.08	0.012
<i>Biotransformation and anti-oxidant defences</i>				
GST	Estuary	1, 111	96.96	<0.001
	Cadmium	5, 111	2.25	0.049
	Estuary x Cadmium	5, 111	2.38	0.043
GR	Estuary	1, 114	0.38	0.542
	Cadmium	5, 114	2.20	0.059
	Estuary x Cadmium	5, 114	1.32	0.259
GPx	Estuary	1, 115	61.20	<0.001
	Cadmium	5, 115	1.22	0.306
	Estuary x Cadmium	5, 115	0.48	0.791
TG	Estuary	1, 116	345.80	<0.001
	Cadmium	5, 116	5.37	<0.001
	Estuary x Cadmium	5, 116	4.02	0.002
<i>Oxidative damage</i>				
LPO	Estuary	1, 116	3.73	0.056
	Cadmium	5, 116	1.29	0.274
	Estuary x Cadmium	5, 116	0.51	0.768
<i>Energy production</i>				
IDH	Estuary	1, 122	0.75	0.388
	Cadmium	5, 122	2.87	0.018
	Estuary x Cadmium	5, 122	0.23	0.950
LDH	Estuary	1, 122	6.53	0.012
	Cadmium	5, 122	0.39	0.854
	Estuary x Cadmium	5, 122	0.48	0.787

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5