Journal Pre-proof

New insights on the impacts of e-waste towards marine bivalves: The case of the rare earth element Dysprosium

Rosa Freitas, Celso Cardoso, Silvana Costa, Tiago Morais, Pedro Moleiro, André F.D. Lima, Márcio Soares, Samuel Figueiredo, Tiago L. Águeda, Pedro Rocha, Gonçalo Amador, Amadeu M.V.M. Soares, Eduarda Pereira

Please cite this article as: Freitas, R., Cardoso, C., Costa, S., Morais, T., Moleiro, P., Lima, André.F.D., Soares, Má., Figueiredo, S., Águeda, T.L., Rocha, P., Amador, Gonç., Soares, A.M.V.M., Pereira, E., New insights on the impacts of e-waste towards marine bivalves: The case of the rare earth element Dysprosium, *Environmental Pollution* (2020), doi: https://doi.org/10.1016/j.envpol.2019.113859.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1 # New insights on the impacts of e-waste towards marine

2 # bivalves: the case of the rare earth element Dysprosium

3

4 Rosa Freitas[a], Celso Cardoso[b], Silvana Costa[a], Tiago Morais[c], Pedro Moleiro[c],

5 André F. D. Lima[c], Márcio Soares[c], Samuel Figueiredo[c], Tiago L. Águeda[c], Pedro

6 Rocha[c], Gonçalo Amador[c], Amadeu M.V.M. Soares[a], Eduarda Pereira[b]

7

8 [a]Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro,

9 Portugal

10 [b]Departamento de Química & LAQV-REQUIMTE, Universidade de Aveiro, 3810-193

11 Aveiro, Portugal

12 [c]Departamento de Química, Universidade de Aveiro, 3810-193 Aveiro, Portugal

13

14

15

16

17

18

19

20

21

22

23

24

25

26 **Corresponding author:** Rosa Freitas

27 Address: Departamento de Biologia, Universidade de Aveiro

28   Campus Universitário de Santiago

29   3810-193 Aveiro, Portugal

30   e-mail address: rosafreitas@ua.pt

31

32

33 **ABSTRACT**

34   With the technological advance and economic development, the multiplicity and wide

35 variety of applications of electrical and electronic equipment have increased, as well as the

36 amount of end-of-life products (waste of electrical and electronic equipment, WEEE).

37 Accompanying their increasing application there is an increasing risk to aquatic ecosystems and

38 inhabiting organisms. Among the most common elements present in WEEE are rare earth

39 elements (REE) such as Dysprosium (Dy). The present study evaluated the metabolic and

40 oxidative stress responses of *mussels Mytilus galloprovincialis* exposed to an increasing range

41 of Dy concentrations, after a 28 days experimental period. The results obtained highlighted that

42 Dy was responsible for mussel's metabolic increase associated with glycogen expenditure,

43 activation of antioxidant and biotransformation defenses and cellular damages, with a clear loss

44 of redox balance. Such effects may greatly impact mussel's physiological functions, including

45 reproduction capacity and growth, with implications to population conservation. Overall the

46 present study pointed out the need for more research on the toxic impacts resulting from these

47 emerging pollutants, specially towards marine and estuarine invertebrate species.

48

49

50 **Keywords:**

51   Toxicity; mussels; e-waste; bioaccumulation; oxidative stress; metabolism.

52

53 **Capsule:** Dysprosium induced metabolic and oxidative stress alterations in *Mytilus*

54 *galloprovincialis*, which may impair mussels physiological mechanisms.

55

56   **1. INTRODUCTION**

57   The growing demand and use of rare earth elements (REEs) by the industries to produce

58   electric and electronic equipment (EEE) led to enormous amount of e-waste generated annually

59   (49.8 Mt in 2018) and consequently to its discharge into the environment. The disposal of e-

60   waste into the environment is mainly due to the lack of government measures to raise public

61   awareness about e-waste recycling and how to recycle it, to the lack of efficient recycling

62   methodologies, by low yields rates obtained and the tedious and costly steps involved in the

63   separation of the elements to further use (Baldé et al., 2017; European Rare Earths

64   Competency Network (ERECON), 2014; Dutta et al., 2016). E-waste is not biodegradable and

65   accumulates in the environment, in the soil, air, water and aquatic organisms (UNEP et al.,

66   2019; ou, 2013). Although it is estimated that e-waste can represent only 2% of solid waste

67   streams, it can represent up to 70% of the hazardous waste that ends up in landfill (UNEP et al.,

68   2019). There is already an increase of REEs concentration in the superficial waters from the

69   mining activities as a result of increased EEEs production demand. Recent studies revealed an

70   increase of REEs concentration from 1 µg L^{-1} (Ouyang et al., 2006) to 3007 µg L^{-1} (He et al.,

71   2010) in Pearl River, China – an extensive river system that across several REEs producing

72   zones.

73   Dysprosium (Dy, Z = 66) is a REE with high economic importance but also with high

74   supply risk (Batinic et al., 2018; Critical Raw Materials - European Commission Report, 2018).

75   For this reason, Dy is identified as a critical element by the EU Commission (Rabe et al., 2017).

76   According to Abrahami et al. (2015), neodymium (Nd), Dy and terbium (Tb) are among the

77   REEs at most supply risk within the next few years. Almost 95% of the total Dy demand is

78   related to its use in magnets (Zapp et al., 2018), due to its high resistance to demagnetization at

79   high temperatures (Kim et al., 2017). Nevertheless, this element is also used in compounds like

80   Dy iodide to be applied in commercial lighting, to produce an intense white light. Moreover, Dy

81   oxide-nickel cermet (composite material made of ceramic and sintered metal) is used in nuclear

82   reactor control rods, to absorb neutrons for a long period without contracting or expanding.

83   Lastly, Dy, when combined with vanadium and other REEs, is used in the production of laser

84   materials (Antić et al., 2016).

85   The concentration of Dy in natural waters varies from a few ng/L in seawater (0.90 ng/L)

86   (Johannesson et al., 1994; Tai et al., 2010), rain water (1.48 – 1.8 ng/L), throughfall (3.62 ng/L),

87   soil solution (19.0 ng/L) and stream water (46.8 ng/L) (Kabata-Pendias and Mukherjee, 2007) to

88   a few µg/L in surface waters (such as Terengganu River Basin, in Malaysia, with 0.0038-1.93

89   µg/L) (Sultan and Shazili, 2009) and groundwaters (13.5 µg/L) (Johannesson et al., 1994).

90   Furthermore, the concentration of Dy in contaminated environments can achieve 172 – 186

91   µg/L (Berkeley Pit lake, a large acidic mining lake in Butte, Montana) (Gammons et al., 2003). A

92   recent review on the spatial concentration of REEs, including Dy, in various water matrices

93   around several continents (including 35 countries) showed that Dy concentrations in

94   groundwater and surface water (both freshwater and seawater) vary from few ng to more than

95   100 µg/L (Adeel et al., 2019). Nevertheless, it has been estimated that the e-waste generated

96   will increase to 52 Mt in 2021 and to 120 Mt in 2050 (Baldé et al., 2017; UNEP et al., 2019),

97   which will lead to an increase in the concentration of REEs, namely Dy, in the aquatic systems.

98   Although it is reported the increasing presence of REEs in marine coastal areas, their

99   toxicological understanding, an in particular for Dy, in such aquatic systems is still almost

100   unknown but of increasing concern. Nevertheless, the emergence of Dy in the aquatic systems

101   has raised attention into the scientific community related to its effects in the living organisms.

102   Oral et al. (2017) investigated the effect of REEs on early life cycle stages in *Paracentrotus*

103   *lividus* sea urchins, exposing the embryos and sperm of these species to trichloride salts of five

104   REEs, including Dy. The results obtained showed that *P. lividus* embryos had a decreased

105   mitotic activity and an increased aberration rate. Sperm exposed to these elements showed

106   decrease in fertilization success along with increase in offspring damage. These authors

107   concluded that REE-associated toxicity affected embryogenesis, fertilization, cytogenetic and

108   redox endpoints. In another study, Anaya et al. (2016) evaluated the effect of Dy oxide

109   nanoparticles (nDy_2O_3) on the bacteria *Escherichia coli*. This nDy_2O_3 has several biomedical

110   applications due to its fluorescence and paramagnetic properties contributing to the location,

111   diagnosis and treatment of diseases. During this study fluorescent dyes (Live/Dead) were used

112   to measure the undisturbed cell membrane (UCM) and respirometric assays allowing the

113   measure of remaining respiration percentage (RRP). After bacteria exposure to nDy_2O_3, the

114   UCM and RRP decreased to 88% and 43%, respectively, evidencing Dy toxicity, with Dy(III) as

115   the main contributor to the overall toxicity. Vukov et al. (2016) compared the toxicological effect

116   of the Dy to the freshwater invertebrates *Daphnia pulex* and *Hyalella azteca*. The results

117   revealed that *H. azteca* is 1.4 times more sensitive than *D. pulex*. In this study, it was also

118   verified the toxicity modifying influence of Ca, Na, Mg, pH and dissolved organic matter (DOM)

119   in the presence of Dy with a more sensitive organism, *H. azteca*. It was concluded that additions

120   of Ca and Na, low pH and DOM provided protection of the organisms against Dy toxicity, while

121   on the contrary the addition of Mg increase the toxicity of Dy.

122   From the literature available it is possible to recognize that no knowledge exists on the

123   toxic effects of Dy towards marine or estuarine bivalves, namely on species with high ecological

124   and economic relevance. Nevertheless, marine coastal systems are frequently final destination

125   of these pollutants putting at risk inhabiting animals and public health in the case of bivalves

126   associated with human consumption. Therefore, the present study aimed to investigate the

127   biochemical alterations induced in the mussel species *Mytilus galloprovincialis*, when exposed

128   to an increasing exposure gradient of Dy, resembling low to highly contaminated areas.

129   Although no studies are known on the impacts of Dy in bivalves, and in particular in mussels,

130   recent studies demonstrated the negative impacts of other REEs (e.g. Neodymium, Lanthanum,

131   Gadolinium) towards *M. galloprovincialis*, including impairments on their metabolic capacity and

132   occurrence of oxidative stress, with alterations on mussels antioxidant capacity and redox

133   balance (Freitas et al., 2020; Henriques et al., 2019; Pinto et al., 2019). For this reason the

134   present study measured biochemical parameters related with alterations on mussel's metabolic

135   capacity (electron transport system activity), energy reserves content (glycogen content, GLY;

136   total protein content, PROT), oxidative stress (activity of antioxidant and biotransformation

137   enzymes), cellular damage (lipid peroxidation and protein carbonyl levels) and redox balance

138   (ratio between reduced glutathione and oxidized glutathione, GSH/GSSG), factors that may

139   compromise the normal physiological functioning of mussels such as filtration and respiration

140   rates, growth and reproductive capacity.

141

142

143

144   **2. MATERIALS AND METHODS**

145   2.1 Experimental conditions

146   The Mediterranean mussel *Mytilus galloprovincialis* was selected as biological model for

147   the present study. Among the most widely used mussel species identified as good bioindicator

148   is *Mytilus galloprovincialis*, with several studies demonstrating the capacity of this species to

149   respond to pollutants accumulation with physiological and biochemical alterations (among

150   others, Andrade et al., 2019; Burgos-Aceves and Faggio, 2017; Coppola et al., 2018a;

151   Henriques et al., 2019; Monteiro et al, 2019; Munari et al., 2018; Pirone et al., 2019; Renault,

152   2015).

153   Animals were collected in September 2018, at the Ria de Aveiro lagoon (Portugal).

154   Mussels with similar size (5.7±0.7 cm length; 3.0±0.4 cm width) were selected to avoid

155   differences in biological responses.

156   Bivalves were transported from the field to the laboratory where they were placed in

157   aquaria for depuration and acclimation to laboratory conditions for two weeks. During this

158   period, mussels were maintained under constant aeration in different aquaria with artificial

159   seawater (Tropic Marin® SEA SALT) at temperature, pH and salinity values resembling the

160   sampling site conditions (18.0 ± 1.0 ºC; 8.0 ± 0.1, 30 ± 1, respectively). Seawater was renewed

161   every day during the first seven days and then every three days until the end of the acclimation

162   period.

163   After this period, mussels were distributed in different aquaria (with four aquaria per

164   condition with 3 L of seawater each) and exposed to the following conditions for twenty-eight

165   days: control (CTL, 0 µg L^{-1}), 2.5, 5, 10, 20, 40 µg L^{-1} of Dy (Dy^{3+}). A total of twenty mussels

166   were used per tested concentration (five mussels per aquarium). The selection of the Dy

167   exposure concentrations was based on the levels identified in low to highly contaminated

168   environments (see for review Adeel et al., 2019).

169   To evaluate the stability of Dy in the water medium a parallel experiment was conducted,

170   in the absence of mussels. For this, glass containers with 500 mL of artificial seawater were

171   spiked with 2.5 and 40 µg L^{-1} of Dy (10 containers per concentration) and, during seven days

172   (corresponding to the period between water renewals along the twenty-eight days experimental

173   assay), aliquots of 5 mL were daily collected to assess concentrations of Dy in the water.

174   During the experimental period (twenty-eight days), water was changed every week and

175   the medium conditions re-established, including Dy concentrations and seawater parameters

176   (temperature 17 ± 1.0 °C, pH 8.0± 0.1 and salinity 30 ± 1). During the exposure period water

177   medium in each aquarium was continuously aerated with a photoperiod of 12h light:12h dark.

178   Every week, immediately after water renewal, water samples were collected from each

179   aquarium for Dy quantification, to assess real exposure concentrations. During this period,

180   mussels were fed with Algamac protein plus (150,000 cells/animal) three times per week.

181   Mortality was also daily checked, with 100% of survival recorded during all the experimental

182   period.

183   At the end of the exposure period, mussels were frozen individually with liquid nitrogen

184   and stored at -80ºC, until homogenization of each individual soft tissue under liquid nitrogen.

185   Each homogenized organism was divided into aliquots (each with 0.5 g fresh weigh, FW) for

186   biomarkers analyses and Dy quantification.

187

188   2.2 Dysprosium quantification in water and in mussel tissues

189   To guarantee that nominal and real concentrations were similar, Dy concentrations in

190   water samples, collected every week from each aquaria immediately after water contamination,

191   were quantified using inductively coupled plasma mass spectrometry (ICP-MS), on a Thermo

192   ICP-MS X Series equipped with a Burgener nebulizer after adequate sample dilution and

193   acification to pH <2. Water samples collected daily to evaluate the stability of Dy in seawater (in

194   the absence of mussels), along seven days experimental period, were analysed following the

195   same procedure.

196   Total Dy concentrations in *M. galloprovincialis* whole soft tissues (2 individuals per

197   replicate (8 individuals per condition) were also quantified by ICP-MS, after microwave assisted

198   acid digestion. After freeze-drying, mussel samples with 100–200 mg were digested in a

199   microwave, firstly with 2 mL of HNO_3 (70%) at 170 °C for 15 min, followed by a second i dentical

200   microwave cycle with 0.5 mL of H_2O_2 (30%). After addition of H_2O_2, the mixture was allowed to

201   stand for 15 min so that the microwave reaction was not as violent. The obtained digests were

202   transferred into 25 mL polyethylene vessels and the volume made up with ultrapure water.

203

204

205   2.3 Biochemical markers

206   The whole tissue of mussels was used for biomarkers determination. For each

207 biochemical parameter, 0.5 g of FW tissue per organism was used, with 2 individuals per

208 replicate (8 individuals per condition, the same used for Dy quantification). For each condition,

209 metabolic capacity (electron transport system activity, ETS), energy reserves (glycogen content,

210 GLY; total protein content, PROT), antioxidant and biotransformation defences (activities of

211 superoxide dismutase, SOD; catalase, CAT; glutathione S-transferases, GSTs), cellular

212 damage (lipid peroxidation levels, LPO; protein carbonyl levels, PC) and redox balance (ratio

213 between reduced glutathione and oxidized glutathione, GSH/GSSG) markers were assessed.

214 Each sample was performed at least in duplicate (2 sub-samples from each organism), for

215 operator quality control. All measurements were done using a microplate reader. The extraction

216 for each biomarker was performed with specific buffers: phosphate buffer for SOD, CAT, GSTs,

217 PROT, GLY and PC; magnesium sulphate buffer for ETS; trichloroacetic acid buffer for LPO

218 and KPE buffer for GSH/GSSG. Each sample was sonicated for 15 s at 4 ºC and centrifuged for

219 25 min (or 15 min for GSH/GSSG) at 10,000 g (or 3,000 g for ETS). Supernatants were stored

220 at -80 ºC. Biomarkers quantifications were performed as described previously by Carregosa et

221 al. (2014), Andrade et al. (2019), Coppola et al. (2019a) and Freitas et al. (2018).

222

223   *Antioxidant defences*

224   SOD activity was determined by the Beauchamp and Fridovich (1971) method after

225 adaptations performed by Carregosa et al. (2014). The standard curve was formed using SOD

226 standards (0.25-60 U/mL). Samples' absorbance was read at 560 nm after 20 min of incubation

227 at room temperature. Results were expressed in U per g FW where one unit (U) represents the

228 quantity of the enzyme that catalyzes the conversion of 1 µmol of substrate per min.

229   CAT activity was quantified according to the Johansson and Borg (1988) method and the

230 modifications performed by Carregosa et al. (2014). The standard curve was determined using

231 formaldehyde standards (0–150 µM). Absorbance was measured at 540 nm. The enzymatic

232 activity was expressed in U per g of FW, where U represents the amount of enzyme that caused

233 the formation of 1.0 nmol formaldehyde per min at 25 ℃.

234   GPx activity was quantified following Paglia and Valentine (1967). The absorbance was

235   measured at 340 nm in 10 sec intervals during 5 min and the enzymatic activity was determined

236   using ε = 6.22 mM^{-1}cm^{-1}. The results were expressed as U per g FW, where U represents the

237   amount of enzyme that caused the formation of 1.0 µmol NADPH oxidized per min.

238

239   *Biotransformation defences*

240   GSTs activity was quantified following Habig et al. (1974) protocol with some adaptations

241   performed by Carregosa et al. (2014). GSTs activity was measured spectrophotometrically at

242   340nm (ε = 9.6 mM^{-1} cm^{-1}). The enzymatic activity was expressed in U per g of FW where U is

243   defined as the amount of enzyme that catalysis the formation of 1 µmol of dinitrophenyl

244   thioether per min.

245

246   *Redox balance*

247   GSH and GSSG glutathione contents were measured at 412 nm (Rahman et al., 2007).

248   The ratio GSH/GSSG was determined taking in account the number of thiol equivalents (GSH /

249   2*GSSG).

250

251   *Cellular damage*

252   LPO determination was done following the method described by Ohkawa et al. (1979).

253   LPO levels were measured trough the quantification of malondialdehyde (MDA), a by-product of

254   lipid peroxidation. Absorbance was measured at 535 nm and the extinction coefficient of 156

255   mM^{-1} cm^{-1} was used to calculate LPO levels, expressed in nmol of MDA formed per g of FW.

256   The quantification of carbonyl groups in oxidized proteins (PC) was done following the

257   2,4-dinitrophenylhydrazina (DNPH) alkaline method, described by Mesquita et al. (2014).

258   Absorbance was measured at 450 nm and the extinction coefficient of 22,308 M^{-1} cm^{-1} was

259   used to calculated PC levels, which were expressed in nmol per g of FW.

260

261   *Metabolic capacity and energy reserves*

262   The ETS activity was measured based on King and Packard (1975) and the modifications

263   performed by De Coen and Janssen (1997). Absorbance was measured during 10 min at 490

264 nm with intervals of 25 s and the extinction coefficient of 15,900 $M^{-1}cm^{-1}$ was used to calculate

265 the amount of formazan formed. Results were expressed in nmol per min per g of FW.

266 For GLY quantification the sulphuric acid method was used, as described by Dubois et al.

267 (1956). Glucose standards were used (0–10 mg/ mL) to produce a calibration curve.

268 Absorbance was measured at 492 nm after incubation during 30 min at room temperature.

269 Results were expressed in mg per g FW.

270 The PROT content was determined according to the spectrophotometric Biuret method

271 (Robinson and Hogden, 1940). Bovine serum albumin (BSA) was used as standard calibration

272 curve (0–40 mg/mL). Absorbance was read at 540 nm. The results were expressed in mg per g

273 FW.

274

275 2.4 Integrated biomarker response

276 The integrated biomarker response (IBR) was calculated according to Beliaeff and

277 Burgeot (2002) aiming to evaluate the general mussel's biochemical response among tested

278 concentrations. All biomarkers determined were used in the calculation of the IBR and they

279 were arranged clockwise in the following order: ETS, GLY, PROT, LPO, PC, GSH/GSSG, SOD,

280 CAT, GPx and GSTs. Values were discussed in terms of a general response given by the final

281 IBR value, where higher values correspond to higher mussels' response.

282

283

284 2.5 Statistical analyses

285 Results from biochemical analyses and Dy concentrations in mussel's tissues, obtained

286 from each condition, were submitted to statistical hypothesis testing using permutational

287 analysis of variance (PERMANOVA+add-on in PRIMER v6, Anderson et al., 2008). When

288 significant differences were observed in the main test, pairwise comparisons were performed

289 among conditions. Values of p lower than 0.05 were considered as significantly different and

290 identified in the figures with different lowercase letters and p-values are presented in a Table

291 format.

292 The matrix gathering the Dy concentrations in mussels soft tissues and biochemical

293 results per condition were used to calculate the Euclidean distance similarity matrix, which was .

11

294   simplified through the calculation of the distance among centroids matrix based on the

295   concentration and submitted to ordination analysis (Principal Coordinates, PCO). Pearson

296   correlation vectors of biochemical descriptors (correlation >0.75) were provided as

297   supplementary variables, whcih were superimposed on the top of the PCO graph.

298

299   **3. RESULTS**

300   3.1 Dysprosium concentrations in seawater and mussel tissues

301   Results concerning the stability of Dy in seawater medium showed that, in the absence of

302   mussels, concentrations were maintained along seven days' exposure period, with results

303   showing that the mean±STDEV values after exposure to 2.5 and 40 µg/L of Dy were,

304   respectively, 2.5±0.1 and 44±3.2 µg/L of Dy. These results clearly demonstrate the stability of

305   Dy during the seven days' exposure period, the interval used between water renewal along the

306   experimental assay.

307   In what regards to Dy concentrations in seawater from the experimental exposure assay,

308   values obtained in water samples collected immediately after spiking revealed that measured

309   and nominal concentrations were similar, for all the conditions and weeks, validating the Dy

310   spiking process (Table 1).

311   The results obtained from Dy quantification in mussel's tissues showed significant

312   difference among animals exposed to tested conditions, with increasing Dy levels along the

313   increasing exposure concentration (Table 1).

314

315   3.2 Biochemical markers

316   *Antioxidant defences*

317   The activity of SOD was significantly lower at control and at 2.5 µg/L of Dy in comparison

318   to mussels exposed to higher concentrations (Figure 1A, Table 2). The activity of CAT was

319   significantly higher in mussels exposed to 20 and 40 µg/L of Dy in comparison to the remaining

320   conditions (Figure 1B, Table 2). The activity of GPx was significantly higher in mussels exposed

321   to 40 µg/L of Dy in comparison to non-contaminated mussels and the ones exposed to 5.0 and

322   10 µg/L of Dy (Figure 1C, Table 2).

323

324   *Biotransformation defences*

325   The activity of GSTs increased with the increase of Dy exposure concentration, with

326   significantly higher values in mussels exposed to 20 and 40 µg/L of Dy in comparison to animals

327   under control and exposed to the lowest Dy concentration (Figure 2, Table 2).

328

13

329   *Redox balance*

330   The GSH/GSSG values were significantly lower in contaminated mussels compared to

331   control ones, with the lowest values in animals exposed to concentrations 2.5 and 5.0 µg/L of

332   Dy (Figure 3, Table 2).

333

334   *Cellular damage*

335   Levels of LPO were significantly higher in contaminated mussels compared to control

336   ones, with the highest values in mussels exposed to 20 µg/L of Dy. No significant differences

337   were observed among mussels exposed to 2.5, 5.0 and 10 µg/L of Dy (Figure 4A, Table 2). The

338   PC levels increased in mussels exposed to Dy, with significant differences between control and

339   Dy exposed mussels. Although higher PC levels were obtained in animals exposed to 10 µg/L

340   of Dy, no significant differences were observed among contaminated mussels (Figure 4B, Table

341   2).

342

343   *Metabolic capacity and energy reserves*

344   The ETS activity showed no significant differences among conditions, although higher

345   values were observed at the highest Dy exposure concentrations (Figure 5A, Table 2). The GLY

346   content decreased in Dy exposed mussels, with significant differences between the control and

347   mussels exposed to 5, 10, 20 and 40 µg/L of Dy (Figure 5B, Table 2). As for the ETS activity, no

348   significant differences were observed among tested conditions in terms of PROT content,

349   although higher values were noticed at higher Dy concentrations (Figure 5C, Table 2).

350

351   3.3 Integrated Biomarker Response

352   Integrated Biomarker Response (IBR) values showed the highest score (3.4) for

353   mussels exposed to the highest Dy exposure concentration. The lowest IBR values were

354   observed for mussels exposed to the concentrations 2.5 and 5.0 µg/L of Dy (Table 3).

355

356   3.4 Principal Coordinates Analysis

357   The Principal Coordinates Analysis (PCO) representation revealed that PCO1 explained

358   64.2% of the total variation among the data, separating mussels exposed to control and to the

14

359   two lowest Dy exposure concentrations (2.5 and 5 µg/L of Dy) in the positive side from the

360   mussels exposed to higher concentrations (10, 20 and 40 µg/L of Dy) in the negative side.

361   PCO2 explained 16.5% of the total variation, separating control (CTL) and the two highest

362   concentrations in the positive side from the remaining conditions in the negative side. LPO, GLY

363   and GSH/GSSG ratio presented a correlation higher than 0.6 with PCO1 positive side, with Dy,

364   ETS, PROT, GSTs, SOD and PC being the factors that best correlate with PCO1 negative side

365   (r>0.7).

4. DISCUSSION

366

367   The present study evaluated the toxic impacts of Dy in the mussel *M. galloprovincialis*,

368   evaluating the changes induced by this element in mussels oxidative stress status, metabolic

369   capacity and energetic reserves content.

370   It has been reported that when in the presence of pollutants bivalves may increase the

371   production of reactive oxygen species (ROS, the singlet oxygen $^1O_2$, the superoxide anion O_2^-,

372   the hydrogen peroxide (H_2O_2) and the hydroxyl radical (HO)), that are naturally produced during

373   several cellular pathways of aerobic metabolism including oxidative phosphorylation, electron

374   transport chains in mitochondria and microsomes, or during the activation of immune

375   mechanisms (Halliwell and Gutteridge, 2007). Under basal conditions the adverse effects of

376   ROS are prevented by a series of antioxidant defence mechanisms, including Phase I

377   antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione

378   peroxidase (GPx). While SOD is responsible for the removal of O_2^- with formation H_2O_2, CAT

379   and GPx are involved in the reduction of H_2O_2 to H_2O (Regoli and Giulianni, 2014). The present

380   study revealed that *M. galloprovincialis* increased the activity of SOD, CAT and GPx enzymes

381   along with Dy increasing exposure gradient, with the highest activities at the highest exposure

382   concentrations. As previously demonstrated, in the presence of pollutants *M. galloprovincialis*

383   has de capacity to activate antioxidant defence mechanisms, with published data revealing the

384   ability of this species to increase the activity of antioxidant enzymes in the presence of

385   metal(oid)s (Coppola et al., 2018a, 2018b; Freitas et al., 2019b; Monteiro et al, 2019),

386   pharmaceuticals and personal care products (Balbi et al., 2018; Freitas et al., 2019a; Mezzelani

387   et al., 2018; Munari et al., 2018; Pirone et al., 2019), and nanoparticles (Andrade et al., 2019;

388   Barmo et al., 2013; Hull et al., 2013). Most recently is was also shown the capacity of *M.*

389   *galloprovincilais* to increase the activity of antioxidant enzymes when exposed to the REEs

390   Gadolinium (Gd) and Lanthanum (La) (Henriques et a., 2019; Pinto et al., 2019). Thus, the

391   present results highlight that mussels exposed to Dy were able to develop a defence strategy to

392   eliminate the excess of ROS produced, in a tentative to avoid cellular damages and loss of

393   redox balance.

394   Glutathione S-transferases (GSTs) are a superfamily of Phase II detoxification enzymes

395   that detoxify both ROS and toxic xenobiotics, through conjugation to reduced glutathione (GSH)

16

396 with compounds that contain an electrophilic centre through the formation of a thioether bond

397 between the sulfur atom of GSH and a broad range of substrates. Both GSH content and GSTs

398 enzyme activities are under tight homeostatic control, with higher GSTs activity often associated

399 with low GSH content, indicating loss of cellular redox balance. Under stressful conditions,

400 GSTs activity are induced to achieve efficient protection. In this way, since xenobiotics are a

401 primary source of oxidative stress, GSTs also play an important albeit indirect role in antioxidant

402 defense, by eliminating toxic substances and preventing subsequent deleterious effects. The

403 present study demonstrated the capacity of GSTs to detoxify Dy, with higher activity at higher

404 exposure concentrations. Also previous studies already demonstrated the increased activity of

405 GSTs in mussels exposed to different pollutants, including metal(oid)s (Coppola et al., 2018a,

406 2018b; Freitas et al., 2019b; Peric and Buric, 2019), drugs (Gonzalez-Rey and Bebianno, 2013;

407 Martin-Diaz et al., 2009) and nanoparticles (Canesi et al., 2010; Ciacci et al., 2012; Huang et

408 al., 2018) and most recently Pinto et al. (2019) and Henriques et al. (2019b) also demonstrated

409 the capacity of this group of enzymes to detoxify other REEs, namely La and Gd, respectively.

410 Thus, previous studies and the presents findings highlight the efficiency of this group of

411 enzymes to detoxify mussels from REE.

412   Besides antioxidant and biotransformation enzymes, low molecular weight scavengers

413 are also able to neutralize ROS by direct reaction with them. The most abundant cytosolic

414 scavenger is GSH. In particular, GSH can be oxidized to GSSG (oxidized glutathione) by GPx.

415 For this, H_2O_2 acts also as substrate for GPx, using GSH as electron donor to catalyse the

416 reduction of H_2O_2 to H_2O. Thus, when under a stressful condition GSSG content is enhanced

417 above the reducing capacity of glutathione reductase (GRed) and the ratio GSH/GSSG is

418 altered, decreasing along the increasing stress level. For this reason, the ratio GSH/GSSG has

419 been frequently used as an indicator of cellular redox status after exposure to pollutants. The

420 results obtained in the present study also demonstrated that mussels exposed to Dy strongly

421 decreased the ratio GSH/GSSG, a clear sign of cells loss redox homeostasis when in the

422 presence of this REE. Similarly, Coppola et al. (2017) showed a decrease of GSH/GSSG values

423 along the exposure to metals. Other authors showed a similar pattern in bivalves exposed to

424 pharmaceuticals and personal care products (Almeida et al. 2014, 2015; Freitas et al., 2019a).

425 Recently, studies published by Pinto et al. (2019) and Henriques et al. (2019b) also highlighted

426 the use of GSH/GSSG ratio as an indicator of redox balance in bivalves exposed to REEs (La

427 and Gd, respectively), with significantly lower values in contaminated animals in comparison to

428 control ones.

429   When enzymatic and non-enzymatic antioxidant defences are not sufficient to eliminate

430 the excess of ROS these free radicals can easily react with organism's membrane lipids,

431 inducing an alteration of membrane permeability, as well as with proteins, causing oxidative

432 modifications which might result in catalytically less active enzymes or proteins more

433 susceptible to proteolytic degradation. Such events are normally assessed by measuring lipid

434 peroxidation (LPO) and protein carbonylation (PC) in animals. Lipid peroxidation is the oxidative

435 degradation of lipids in cell membranes, resulting in cell damage, being commonly measured by

436 the content of malondialdehyde (MDA), one of the most abundant aldehydes generated during

437 lipid oxidation and also probably the most commonly used as an oxidative stress marker. In the

438 present study *M. galloprovincialis* showed increased LPO levels in Dy contaminated specimens.

439 Similarly, several other studies used LPO as a marker of the oxidative stress generated by the

440 exposure of bivalves to different pollutants, including in mussels, exposed to classical pollutants

441 as metal(oid)s (among others, Vlahogianni and Valavanidis, 2006; Coppola et al., 2018a), and

442 emerging pollutants, such as pharmaceuticals and personal care products (Gonzalez-Rey and

443 Bebianno, 2011, 2014; Quinn et al., 2011; Teixeira et al., 2017), nanoparticles (Gornati et al.,

444 2016; Gomes et al., 2011, 2014) and the REEs La and Gd (Henriques et al., 2019b; Pinto et al.,

445 2019). Malondialdehyde (MDA) levels also increased in the sea urchin *Paracentrotus lividus*

446 larvae exposed to Dy (Oral et al., 2017). Protein carbonylation is also one biomarker of

447 oxidative stress, resulting from the oxidation of proteins by ROS and corresponding to the

448 presence of carbonyl groups, such as aldehyde and ketone, in specific amino acid side chains

449 such as lysine, proline, arginine and threonine. The present findings also highlighted the

450 capacity of Dy to induce oxidation of proteins, with higher PC levels in contaminated mussels.

451 Although less used as oxidative stress biomarker in bivalves, and especially in clams and

452 mussels (Andreade et al., 2019; Matozzo et al., 2016; Merad et al., 2016; Parolini et al., 2016),

453 few studies revealed increased PC levels in *M. galloprovincialis* exposed to the drugs triclosan

454 and diclofenac (Freitas et al., 2019a), cadmium (Dailianis et al., 2009), the pesticides

455 chlorpyrifos and penoxsulam (Patetsini et al., 2012).

456   Apart from alterations induced by Dy in oxidative stress related biomarkers, the impacts

457   induced in *M. galloprovincialis* by this REE can be also assessed by evaluating mussel

458   metabolism. For this, the electron transport system (ETS) activity is commonly used as a

459   measure of the potential respiration that could be supported by the enzymatic machinery activity

460   (Cammen et al., 1990). The results obtained in the present study indicate that mitochondrial

461   respiration tended to increase at higher Dy concentrations, probably to fuel up mussels defence

462   mechanisms, with higher ETS activity at higher exposure concentrations (especially at 20 and

463   40 µg/L of Dy). Accompanying this metabolic enhance the obtained results further demonstrated

464   an increase of the total protein (PROT) content at higher exposure concentrations, which may

465   result from higher production of enzymes, namely antioxidant enzymes, to fight against the

466   stress induced by Dy. As a consequence of increased metabolic capacity and activation of

467   defence mechanisms, bivalves tended to decrease their glycogen (GLY) concentration,

468   evidencing that under Dy exposure conditions mussels may use this energy reserve. In aquatic

469   species, including bivalves, ETS activity has been used also to assess the oxygen demand to

470   evaluate environmental changes (Sokolova et al., 2012), including the ones related with

471   seasonal alterations (Fanslow et al., 2001), pH decrease (De Marchi et al., 2017; Simcic and

472   Brancelj, 2006), and the presence of pollutants (De Marchi et al., 2018; Bielen et al., 2016;

473   Gagné et al., 2016; Duquesne et al., 2004; Hamza-Chaffai et al., 2003). A similar response was

474   already demonstrated by other authors when exposing mussels to different pollutants, with

475   increasing ETS activity and decreasing GLY content along the increasing exposure gradient of

476   different pollutants (Coppola et al., 2017; Freitas et al., 2019a; Monteiro et al., 2019a, 2019b;

477   Pinto et al., 2019).

478

479   Overall the present study clearly demonstrated a dose-dependent response, with

480   mussels showing higher biological impacts at to higher exposure concentrations. Nevertheless,

481   at intermediate exposure concentrations, namely at 5 µg/L of Dy, it seems that mussels were

482   able to avoid injuries by efficiently activating their defence mechanisms (including increase of

483   antioxidant enzymes activities), while at the lowest concentration these strategies were not

484   activated due to low stress levels. At higher concentrations, although enzymes activities

485   increased mussels these defence mechanisms were not enough to avoid injuries, leading to

486 higher impacts. Such effects were corroborated by IBR values, based on measured biochemical

487 markers, with the highest values at the highest exposure concentration and the lowest value at

488 5.0 µg/L of Dy. Such results are in accordance with previous studies that already highlighted

489 IBR as a useful tool for quantitative assessment of stress levels in mussels exposed to different

490 pollutants (Pinto et al., 2019; Yuan et al., 2017; Beliaeff and Burgeot, 2002). The lowest IBR

491 values obtained at 5 µg/L of Dy may corroborate the hypothesis of an hormesis response,

492 indicating a certain adaptive response of organisms to a moderate stress. Furthermore, PCO

493 analysis applied to Dy concentration levels and biochemical responses, demonstrated a clear

494 distinction between: i) mussels exposed to control and the two lowest Dy concentrations (PCO

495 axis 1, positive side), associated to lower cellular damage and the maintenance of redox

496 balance; ii) and mussels exposed to the three higher Dy concentrations (PCO axis 1, negative

497 side), close associated with higher metabolic capacity, higher antioxidant and biotransformation

498 defences, and higher protein content.

499

500

501 **5. CONCLUSIONS**

502 The present study clearly demonstrated the impacts of Dy in *M. galloprovincialis*, with

503 increasing antioxidant defences, cellular damages and oxidative stress in Dy contaminated

504 mussels. The results obtained further demonstrated that mussels exposed to Dy increased their

505 metabolic capacity, with expenditure of GLY, indicating higher metabolic requirements under Dy

506 contamination. With the increasing use of REE and the associated risks due to the increasing e-

507 wastes resulting from electronic and electric devices, aquatic environments are increasingly

508 endangered by the presence of these hazardous elements. Therefore, the present findings

509 highlight the potential toxic impacts of REEs in marine animals, with oxidative stress and

510 metabolic changes that might compromise mussel's physiological functions, such as respiration

511 and filtration rates, growth, and reproduction. Considering that even at the lowest tested

512 concentrations (2.5 and 5.0 µg/L of Dy) significant biochemical impairments were observed, the

513 present study highlights the risk of toxic effects under actual concentration levels, identifying Dy

514 as an hazardous element towards mussel's populations, and potential other bivalve species.

515

516   **Acknowledgments**

527

528

REFERENCES

529

530   Abrahami, S. T., Xiao, Y., Yang, Y., 2015. Rare-earth elements recovery from post-
531   consumer hard-disc drives. *Mineral Processing and Extractive Metallurgy*, *124*(2), 106-115.

532   Adeel, M., Lee, J. Y., Zain, M., Rizwan, M., Nawab, A., Ahmad, M. A., Shafiq, M., Yi, H.,
533   Jilani, G., Javed, R., Horton, R., Rui, Y., Tsang, D.C.W., Xing, B., 2019. Cryptic footprints of rare
534   earth elements on natural resources and living organisms. *Environment international*, *127*, 785-
535   800.

536   Almeida, Â., Calisto, V., Esteves, V. I., Schneider, R. J., Soares, A. M., Figueira, E.,
537   Freitas, R., 2014. Presence of the pharmaceutical drug carbamazepine in coastal systems:
538   effects on bivalves. *Aquatic Toxicology*, *156*, 74-87.

539   Almeida, Â., Freitas, R., Calisto, V., Esteves, V. I., Schneider, R. J., Soares, A. M.,
540   Figueira, E., 2015. Chronic toxicity of the antiepileptic carbamazepine on the clam *Ruditapes*
541   *philippinarum. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*,
542   *172*, 26-35.

543   Anaya, N. M., Solomon, F., Oyanedel-Craver, V., 2016. Effects of dysprosium oxide
544   nanoparticles on *Escherichia coli. Environmental Science: Nano*, *3*(1), 67-73.

545   Anderson, M.J., Gorley, R.N., Clarke, K.R., 2008. PERMANOVA for PRIMER: Guide to
546   software and statistical methods. University of Auckland and PRIMER-E, Plymouth.

547   Andrade, M., De Marchi, L., Pretti, C., Chiellini, F., Morelli, A., Figueira E., Rocha R.J.M.,
548   Soares, A.M.V.M., Freitas, R., 2019. The impacts of warming on the toxicity of carbon
549   nanotubes in mussels. *Marine Environmental Research* 145, 11-21.

550   Antić, Ž.,* Dramićanin,M.D., Prashanthi, K., Jovanović, D., Kuzman, S., Thundat, T.,
551   2016. Pulsed Laser Deposited Dysprosium-Doped Gadolinium–Vanadate Thin Films for
552   Noncontact, Self-Referencing Luminescence Thermometry. Advanced Materials. DOI:
553   10.1002/adma.201601176.

554   Balbi, T., Montagna, M., Fabbri, R., Carbone, C., Franzellitti, S., Fabbri, E. and Canesi,
555   L., 2018. Diclofenac affects early embryo development in the marine bivalve *Mytilus*
556   *galloprovincialis. Science Total Environment* 642, 601-609.

557   Baldé CP, Forti V, Gray V, Kuehr R, Stegmann P. The Global E-Waste Monitor - 2017.
558   Bonn/Geneva/Vienna: 2017)

559   Barmo, C., Ciacci, C., Canonico, B., Fabbri, R., Cortese, K., Balbi, T., Marcomini, A.,

560   Pojana, G., Gallo G., Canesi, L., 2013. In vivo effects of n-TiO2 on digestive gland and immune

561   function of the marine bivalve *Mytilus galloprovincialis*. *Aquatic Toxicology* 132, 9-18.

562   Batinic, B., Vaccari, M., Savvilotidou, V., Kousaiti, A., Gidarakos, E., Marinkovic, T., Fiore,

563   S., 2018. Applied WEEE pre-treatment methods: Opportunities to maximizing the recovery of

564   critical metals. *Global Nest Journal*, *20*(4), 706-711.

565   Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assays and an

566   assay applicable to acrylamide gels. *Analytical Biochemistry* 44, 276–287.

567   Beliaeff B., Burgeot T., 2002. Integrated biomarker response: a useful tool for ecological

568   risk assessment. *Environmental Toxicology Chemistry*, 21, 1316–1322.

569   Bielen, A., Bošnjak, I., Sepčić, K., Jaklič, M., Cvitanić, M., Lušić, J., Lajtner, J., Simčič, T.,

570   Hudina, S., 2016. Differences in tolerance to anthropogenic stress between invasive and native

571   bivalves. *Science Total Environment* 543, 449-459.

572   Burgos-Aceves, M. A., Faggio, C., 2017. An approach to the study of the immunity

573   functions of bivalve haemocytes: physiology and molecular aspects. *Fish Shellfish Immunology*

574   67, 513-517.

575   Cammen, L. M., Corwin, S., Christensen, J. P., 1990. Electron transport system (ETS)

576   activity as a measure of benthic macrofaunal metabolism. *Marine Ecology Progress Series*,

577   *65*(1), 171-182.

578   Canesi, L., Fabbri, R., Gallo, G., Vallotto, D., Marcomini, A., Pojana, G., 2010.

579   Biomarkers in *Mytilus galloprovincialis* exposed to suspensions of selected nanoparticles (Nano

580   carbon black, C60 fullerene, Nano-TiO2, Nano-SiO2). *Aquatic Toxicology* 100(2), 168-177.

581   Carregosa, V., Velez, C., Soares, A.M.V.M., Figueira, E., Freitas, R., 2014. Physiological

582   and biochemical responses of three Veneridae clams exposed to salinity changes. *Comparative*

583   *Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 177–178, 1–9.

584   Ciacci, C., Canonico, B., Bilaniĉovă, D., Fabbri, R., Cortese, K., Gallo, G., Marcomini, A.,

585   Pojana, G., Canesi, L., 2012. Immunomodulation by different types of N-oxides in the

586   hemocytes of the marine bivalve *Mytilus galloprovincialis*. *PLoS One* 7(5), e36937.

587   Coppola, F., Almeida, Â., Henriques, B., Soares, A.M.V.M., Figueira, E., Pereira, E.,

588   Freitas, R., 2018a. Biochemical responses and accumulation patterns of *Mytilus*

589 *galloprovincialis* exposed to thermal stress and Arsenic contamination. *Ecotoxicology and*

590 *Environmental Safety* 147, 954–962.

591 Coppola, F., Almeida, Â., Henriques, B., Soares, A.M.V.M., Figueira, E., Pereira, E.,

592 Freitas, R., 2017. Biochemical impacts of Hg in *Mytilus galloprovincialis* under present and

593 predicted warming scenarios. *Science Total Environment* 601–602, 1129–1138.

594 Coppola, F., Henriques, B., Soares, A.M., Figueira, E., Pereira, E., Freitas, R., 2018b.

595 Influence of temperature rise on the recovery capacity of *Mytilus galloprovincialis* exposed to

596 mercury pollution. *Ecological Indicators* 93, 1060-1069.

597 Dailianis, S., Patetsini, E., Kaloyianni, M., 2009. The role of signalling molecules on actin

598 glutathionylation and protein carbonylation induced by cadmium in haemocytes of mussel

599 *Mytilus galloprovincialis* (Lmk). *Journal of Experimental Biology*, *212*(22), 3612-3620.

600 De Coen, W.M., Janssen, C.R., 1997. The use of biomarkers in Daphnia magna toxicity

601 testing. IV. Cellular energy allocation: a new methodology to assess the energy budget of

602 toxicant-stressed Daphnia populations. *Journal of Aquatic Ecosystem Stress and Recovery* 6,

603 43–55.

604 De Marchi, L., Neto, V., Pretti, C., Figueira, E., Chiellini, F., Morelli, A., Soares,

605 A.M.V.M., Freitas, R., 2018. Toxic effects of multi-walled carbon nanotubes on bivalves:

606 Comparison between functionalized and nonfunctionalized nanoparticles. *Science Total*

607 *Environment* 622–623, 1532–1542.

608 De Marchi, L., Neto, V., Pretti, C., Figueira, E., Chiellini, F., Morelli, A., Soares, A.M.V.M.,

609 Freitas, R., 2017. The impacts of seawater acidification on *Ruditapes philippinarum* sensitivity to

610 carbon nanoparticles. *Environmental Science: Nano* 4(8), 1692-1704.

611 Dia et al., 2000

612 Duquesne, S., Liess, M. and Bird, D.J., 2004. Sub-lethal effects of metal exposure:

613 physiological and behavioural responses of the estuarine bivalve *Macoma balthica*. *Marine*

614 *Envrionmental Research* 58(2-5), 245-250.

615 Dutta, T., Kim, K. H., Uchimiya, M., Kwon, E. E., Jeon, B. H., Deep, A., Yun, S. T., 2016.

616 Global demand for rare earth resources and strategies for green mining. *Environmental*

617 *Research*, *150*, 182-190.

618   Fanslow, D. L., Nalepa, T. F., & Johengen, T. H., 2001. Seasonal changes in the

619   respiratory electron transport system (ETS) and respiration of the zebra mussel, *Dreissena*

620   *polymorpha* in Saginaw Bay, Lake Huron. *Hydrobiologia, 448*(1-3), 61-70.

621   Freitas, R., Coppola, F., De Marchi, L., Codella, V., Pretti, C., Chiellini, F., Morelli, A.,

622   Polese, G., Soares, A.M.V.M., Figueira, E., 2018. The influence of Arsenic on the toxicity of

623   carbon nanoparticles in bivalves. *Journal of Hazardous Materials* 358, 484–493.

624   Freitas R., Coppola F., Costa S., Pretti C., Intorre L., Meucci V., Soares A.M.V.M., Sole

625   M., 2019a. The influence of temperature on the effects induced by Triclosan and Diclofenac in

626   mussels. *Science of Total Environment* 663, 992-999.

627   Freitas, R., Leite, C., Pinto, J., Costa, M., Monteiro, R., Henriques, B., Di Martino, F.,

628   Coppola, F., Soares, A.M.V.M., Solé, M., Pereira, E., 2019b. The influence of temperature and

629   salinity on the impacts of Lead in *Mytilus galloprovincialis*. *Science of Total Environment* 601,

630   1129-1138.

631   Freitas, R., Costa, S., Cardoso, C., Morais, T., Moleiro, P., Matias, A.C., Pereira, A.F.,

632   Machado, J., Correia, B., Pinheiro D., Rodrigues, A., Colónia J., Soares, A.M.V.M., Pereira, E.,

633   2020. Toxicological effects of the rare earth element Neodymium in *Mytilus galloprovincialis.*

634   *Chemosphere.* 244, 125457

635   Gagné, F., Auclair, J., Trépanier, S., Turcotte, P., Pilote, M., Gagnon, C., 2016. The

636   impact of zinc oxide nanoparticles in freshwater mussels exposed to municipal effluents.

637   *Invertebrate Survival Journal* 13(1), 281-290.

638   Gammons, C.H., Wood, S.A., Jonas, J.P., Madison, J.P., 2003. Geochemistry of the rare-

639   earth elements and uranium in the acidic Berkeley Pit lake, Butte, Montana. *Chemical Geology*

640   198, 269–288.

641   Gomes, T., Pereira, C.G., Cardoso, C., Sousa, V.S., Teixeira, M.R., Pinheiro, J.P.,

642   Bebianno, M.J., 2014. Effects of silver nanoparticles exposure in the mussel *Mytilus*

643   *galloprovincialis*. *Marine Environmental Research* 101, 208-214.

644   Gomes, T., Pinheiro, J.P., Cancio, I., Pereira, C.G., Cardoso, C., Bebianno, M.J., 2011.

645   Effects of copper nanoparticles exposure in the mussel *Mytilus galloprovincialis*. *Environmental*

646   *Science and Technology* 45(21), 9356-9362.

647   Gomes, T., Pinheiro, J.P., Cancio, I., Pereira, C.G., Cardoso, C., Bebianno, M.J., 2011.

648   Effects of copper nanoparticles exposure in the mussel *Mytilus galloprovincialis*. *Environmental*

649   *Science and Technology* 45(21), 9356-9362.

650   Gonzalez-Rey, M., Bebianno, M.J., 2011. Non-steroidal anti-inflammatory drug (NSAID)

651   ibuprofen distresses antioxidant defense system in mussel *Mytilus galloprovincialis* gills. *Aquatic*

652   *Toxicology* 105(3-4), 264-269.

653   Gonzalez-Rey, M., Bebianno, M. J., 2013. Does selective serotonin reuptake inhibitor

654   (SSRI) fluoxetine affects mussel *Mytilus galloprovincialis*? *Environmental Pollution* 173, 200-

655   209.

656   Gonzalez-Rey, M., Bebianno, M.J., 2014. Effects of non-steroidal anti-inflammatory drug

657   (NSAID) diclofenac exposure in mussel *Mytilus galloprovincialis*. *Aquatic Toxicology* 148, 221-

658   230.

659   Gornati, R., Longo, A., Rossi, F., Maisano, M., Sabatino, G., Mauceri, A., Bernardini, G.,

660   Fasulo, S., 2016. Effects of titanium dioxide nanoparticle exposure in *Mytilus galloprovincialis*

661   gills and digestive gland. *Nanotoxicology*, 10(6), 807-817.

662   Gornati, R., Longo, A., Rossi, F., Maisano, M., Sabatino, G., Mauceri, A., Bernardini, G.,

663   Fasulo, S., 2016. Effects of titanium dioxide nanoparticle exposure in *Mytilus galloprovincialis*

664   gills and digestive gland. *Nanotoxicology*, 10(6), 807-817.

665   Grant, K., 2013Health consequences of exposure to e-waste: a systematic review. *The*

666   *Lancet Global Health* 1, 350–361.

667   Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases. The first

668   enzymatic step in mercapturic acid formation. *The Journal of Biological Chemistry* 249, 7130–

669   7139.

670   Halliwell, B., & Gutteridge, J. M. C., 2007. Antioxidant defences: endogenous and diet

671   derived. *Free radicals in biology and medicine*, *4*, 79-186.

672   Hamza-Chaffai, A., Pellerin, J. and Amiard, J.C., 2003. Health assessment of a marine

673   bivalve *Ruditapes decussatus* from the Gulf of Gabès (Tunisia). *Environment International*

674   28(7), pp.609-617.

675   He J., Lü C., Xue H., Liang Y., Bai S., Sun Y., Shen, L.L., Mi, N., Fan, Q.-Y., 2010.

676   Species and distribution of rare earth elements in the Baotou section of the Yellow River in

677   China. *Environmental Geochemistry and Health* 32, 45–58.

678   Henriques, B., Coppola, F., Monteiro, R., Pinto, J., Viana, T., Pretti, C., Soares, A.,

679   Freitas, R., Pereira, E., 2019. Toxicological assessment of anthropogenic Gadolinium in

680   seawater: Biochemical effects in mussels *Mytilus galloprovincialis. Science of the Total*

681   *Environment* 664, 626-634.

682   Huang, X., Liu, Z., Xie, Z., Dupont, S., Huang, W., Wu, F., Kong, H., Liu, L., Sui, Y., Lin,

683   D., Lu, W., 2018. Oxidative stress induced by titanium dioxide nanoparticles increases under

684   seawater acidification in the thick shell mussel *Mytilus coruscus. Marine Environmental*

685   *Research* 137, 49-59.

686   Hull, M. S., Vikesland, P. J., Schultz, I. R., 2013. Uptake and retention of metallic

687   nanoparticles in the Mediterranean mussel (*Mytilus galloprovincialis*). *Aquatic Toxicology* 140,

688   89-97.

689   Johannesson, K. H., Lyons, W. B., Fee, J. H., Gaudette, H. E., McArthur, J. M., 1994.

690   Geochemical processes affecting the acidic groundwaters of Lake Gilmore, Yilgarn Block,

691   Western Australia: a preliminary study using neodymium, samarium, and dysprosium. *Journal of*

692   *Hydrology*, *154*(1-4), 271-289.

693   Johansson, L. H., Borg, L. H., 1988. A spectrophotometric method for determination of

694   catalase activity in small tissue samples. *Analytical biochemistry*, *174*(1), 331-336.

695   Kabata-Pendias, A., & Mukherjee, A. B., 2007. *Trace elements from soil to human.*

696   Springer Science & Business Media.

697   Kim, C. J., Yoon, H. S., Chung, K. W., 2017. Precipitation Properties of Neodymium and

698   Dysprosium Double Salt in Sulfuric Acid Leaching Solution from Permanent Magnet Scrap. In

699   *2017-Sustainable Industrial Processing Summit* (Vol. 1, pp. 259-260). Flogen Star Outreach.

700   King, F.D., Packard, T.T., 1975. Respiration and the respiratory electron transport in

701   marine zooplankton. *Limnology and Oceanography* 2849–2854.

702   Martin-Diaz, L., Franzellitti, S., Buratti, S., Valbonesi, P., Capuzzo, A., Fabbri, E., 2009.

703   Effects of environmental concentrations of the antiepilectic drug carbamazepine on biomarkers

27

704 and cAMP-mediated cell signaling in the mussel *Mytilus galloprovincialis*. *Aquatic Toxicology*

705 94(3), 177-185.

706   Matozzo, V., Battistara, M., Marisa, I., Bertin, V., Orsetti, A., 2016. Assessing the effects

707 of amoxicillin on antioxidant enzyme activities, lipid peroxidation and protein carbonyl content in

708 the clam *Ruditapes philippinarum* and the mussel *Mytilus galloprovincialis*. *Bulletin of*

709 *environmental contamination and toxicology*, *97*(4), 521-527.

710   Merad, I., Bairi, Y., Sifi, K., Soltani, N., 2016. Protein carbonyls as biomarkers of oxidative

711 stress induced by cadmium in *Donax trunculus*: gonad contents during exposure and recovery.

712 *Fresenius Environmental Bulletin*, *25*, 5889-5895.

713   Mesquita, C. S., Oliveira, R., Bento, F., Geraldo, D., Rodrigues, J. V., Marcos, J. C.,

714 2014. Simplified 2, 4-dinitrophenylhydrazine spectrophotometric assay for quantification of

715 carbonyls in oxidized proteins. *Analytical biochemistry* 458, 69-71.

716   Mezzelani, M., Gorbi, S., Fattorini, D., d'Errico, G., Consolandi, G., Milan, M., Bargelloni,

717 L., Regoli, F., 2018. Long-term exposure of *Mytilus galloprovincialis* to diclofenac, Ibuprofen and

718 Ketoprofen: Insights into bioavailability, biomarkers and transcriptomic changes. *Chemosphere*

719 198, 238-248.

720   Monteiro, R., Costa, S., Coppola, F., Freitas R., Vale, C., Pereira E., 2019. Evidences of

721 metabolic alterations and cellular damages in mussels after short pulses of Ti contamination.

722 *Science of the Total Environment* 650, 987-995.

723   Munari, M., Matozzo, V., Gagné, F., Chemello, G., Riedl, V., Finos, L., Pastore, P.,

724 Badocco, D., Marin, M.G., 2018. Does exposure to reduced pH and diclofenac induce oxidative

725 stress in marine bivalves? A comparative study with the mussel *Mytilus galloprovincialis* and the

726 clam *Ruditapes philippinarum*. *Environmental Pollution* 240, 925-937.

727   Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by

728 thiobarbituric acid reaction. *Analytical Biochemistry* 95, 351–358.

729   Oral, R., Pagano, G., Siciliano, A., Gravina, M., Palumbo, A., Castellano, I., Trifuoggi, M.,

730 2017. Heavy rare earth elements affect early life stages in *Paracentrotus lividus* and *Arbacia*

731 *lixula* sea urchins. *Environmental research*, *154*, 240-246.

732   Ouyang, T.P., Zhu, Z.Y., Kuang, Y.Q., Huang, N.S., Tan, J.J., Guo, G.Z., Gu, L.S., Sun,

733   B., 2006. Dissolved trace elements in river water: spatial distribution and the influencing factor,

734   a study for the Pearl River Delta Economic Zone, China. *Environmental Geology* 49, 733–742.

735   Paglia D.E., Valentine W.N., 1967. Studies on quantitative and qualitative

736   characterization of erythrocyte glutathione peroxidase. *Journal of Laboratory and Clinical*

737   *Medicine.* 70, 158-169.

738   Parolini, M., Magni, S., Castiglioni, S., Binelli, A., 2016. Amphetamine exposure

739   imbalanced antioxidant activity in the bivalve *Dreissena polymorpha* causing oxidative and

740   genetic damage. *Chemosphere*, *144*, 207-213.

741   Patetsini, E., Dimitriadis, B. K., Kaloyianni, M., 2012. Exposure of mussels Mytilus

742   galloprovinciallis to environmental pesticides. Study of LMS, ROS, DNA damage, protein

743   carbonylation and antioxidant capacity for their use as biomarkers. *Comparative Biochemistry*

744   *and Physiology, Part A*, (163), S26.

745   Perić, L., Burić, P., 2019. The effect of copper and chlorpyrifos co-exposure on

746   biomarkers in the marine mussel Mytilus galloprovincialis. *Chemosphere*, *225*, 126-134.

747   Pinto, J., Costa, M., Leite, C., Borges, C., Coppola, F., Henriques, B., Monteiro, R.,

748   Russo, T., Di Cosmo, A., Soares, A.M.V.M., Polese, G., Pereira, M., Freitas, R., 2019.

749   Ecotoxicological effects of lanthanum in Mytilus galloprovincialis: Biochemical and

750   histopathological impacts. *Aquatic Toxicology*, *211*, 181-192.

751   Pirone G., Coppola F., Pretti C., Soares A.M.V.M., Sole M., Freitas R., 2019. The effect of

752   temperature on Triclosan and Lead exposed mussels. *Comparative Biochemistry and*

753   *Physiology Part B* 232, 42-50.

754   Quinn, B., Schmidt, W., O'Rourke, K., Hernan, R., 2011. Effects of the pharmaceuticals

755   gemfibrozil and diclofenac on biomarker expression in the zebra mussel (*Dreissena*

756   *polymorpha*) and their comparison with standardised toxicity tests. *Chemosphere* 84(5), 657-

757   663.

758   Rabe, W., Kostka, G., Stegen, K. S., 2017. China's supply of critical raw materials:

759   Risks for Europe's solar and wind industries? *Energy Policy*, *101*, 692-699.

760 Rahman, I., Kode, A., Biswas, S.K., 2007. Assay for quantitative determination of

761 glutathione and glutathione disulfide levels using enzymatic recycling method. *Nature Protocols*

762 1, 3159–3165.

763 Regoli, F., Giuliani, M.E., 2014. Oxidative pathways of chemical toxicity and oxidative

764 stress biomarkers in marine organisms. *Marine Environmental Research* 93, 106–117.

765 Robinson, H.W., Hogden, C.G., 1940. The biuret reaction in the determination of serum

766 proteins. *The Journal of Biological Chemistry* 135, 707–725.

767 Simčič, T., Brancelj, A., 2006. Effects of pH on electron transport system (ETS) activity

768 and oxygen consumption in *Gammarus fossarum*, *Asellus aquaticus* and *Niphargus*

769 *sphagnicolus. Freshwater Biology, 51*(4), 686-694.

770 Sokolova, I.M., Frederich, M., Bagwe, R., Lannig, G., Sukhotin, A.A., 2012. Energy

771 homeostasis as an integrative tool for assessing limits of environmental stress tolerance in

772 aquatic invertebrates. *Marine Environmental Research* 79, 1-15.

773 Sultan, K., Shazili, N. A., 2009. Distribution and geochemical baselines of major, minor

774 and trace elements in tropical topsoils of the Terengganu River basin, Malaysia. *Journal of*

775 *geochemical exploration, 103*(2-3), 57-68.

776 Tai, P., Zhao, Q., Su, D., Li, P., Stagnitti, F., 2010. Biological toxicity of lanthanide

777 elements on algae. *Chemosphere, 80*(9), 1031-1035.

778 Teixeira, M., Almeida, Â., Calisto, V., Esteves, V.I., Schneider, R.J., Wrona, F.J., Soares,

779 A.M., Figueira, E., Freitas, R., 2017. Toxic effects of the antihistamine cetirizine in mussel

780 *Mytilus galloprovincialis. Water Research* 114, 316-326.

781 UNEP, PACE, ITU, ILO, UNIDO, UNU, et al. A New Circular Vision for Electronics Time

782 for a Global Reboot. 2019.

783 Valavanidis, A., Vlahogianni, T., Dassenakis, M., Scoullos, M., 2006. Molecular

784 biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants.

785 *Ecotoxicology and environmental safety, 64*(2), 178-189.

786 Vukov, O., Smith, D. S., McGeer, J. C., 2016. Acute dysprosium toxicity to Daphnia pulex

787 and Hyalella azteca and development of the biotic ligand approach. *Aquatic Toxicology, 170*,

788 142-151.

789   Yuan M., Wang Y., Zhou B., Jian X., Dong W., Tang X., 2017. An integrated biomarker

790   response index for the mussel *Mytilus edulis* based on laboratory exposure to anthracene and

791   field transplantation experiments. C*hinese Journal of Oceanology and Limnology* 35, 1165-

792   1178.

793   Zapp, P., Marx, J., Schreiber, A., Friedrich, B., Voßenkaul, D., 2018. Comparison of

794   dysprosium production from different resources by life cycle assessment. *Resources,*

795   *Conservation and Recycling*, *130*, 248-259.

796

797

FIGURE CAPTIONS

Figure 1. A: Superoxide dismutase activity (SOD); B: Catalase activity (CAT); and C: Glutathione peroxidase activity (GPx), in *Mytilus galloprovincialis* exposed to different Dysprosium concentrations (CTL-0, 2.5, 5.0, 10, 20 and 40 µg/L). Values are mean + standard deviation. Significant differences among concentrations are represented with different letters.

Figure 2. Glutathione S-transferases activity (GSTs), in *Mytilus galloprovincialis* exposed to different Dysprosium concentrations (CTL-0, 2.5, 5.0, 10, 20 and 40 µg/L). Values are mean + standard deviation. Significant differences among concentrations are represented with different letters.

Figure 3. Ratio between reduced (GSH) and oxidized (GSSG) glutathione (GSH/GSSG), in *Mytilus galloprovincialis* exposed to different Dysprosium concentrations (CTL-0, 2.5, 5.0, 10, 20 and 40 µg/L). Values are mean + standard deviation. Significant differences among concentrations are represented with different letters.

Figure 4. Lipid peroxidation levels (LPO); and B: Protein Carbonylation (PC), in *Mytilus galloprovincialis* exposed to different Dysprosium concentrations (CTL-0, 2.5, 5.0, 10, 20 and 40 µg/L). Values are mean + standard deviation. Significant differences among concentrations are represented with different letters.

Figure 5. A: Electron transport system activity (ETS), B: Glycogen content (GLY) and C: Total Protein content (PROT), in *Mytilus galloprovincialis* exposed to different Dysprosium concentrations (CTL-0, 2.5, 5.0, 10, 20 and 40 µg/L). Values are mean + standard deviation. Significant differences among concentrations are represented with different letters.

Figure 6. Centroids ordination diagram (PCO) based on Dy concentrations and biochemical parameters, measured in *Mytilus galloprovincialis* exposed to different Dy concentrations (CTL: control, C1: 2.5; C2: 5; C3: 10; C4: 20; C5: 40 µg L^{-1} of Dy). Pearson correlation vectors

are superimposed as supplementary variables, namely biochemical data (r > 0.75): Dy, ETS,

GLY, PROT, SOD, GSTs, LPO, PC, GSH/GSSG.

Table 1- Dysprosium (Dy) concentrations in water (µg/L), collected immediately after spiking at the 1st, 2nd, 3rd and 4th weeks of exposure (mean values for the four weeks ± STDEV) and in mussel's tissues (µg/g dry weight) collected at the end of the experimental period (4th week) (mean values of 8 individuals per condition ± STDEV), from each condition (CTL-0, 2.5, 5.0, 10, 20, 40 µg/L of Dy). Different letters denote statistical significance among tested concentrations. Limit of quantification (LOQ) for water samples 10 ng/L; LOQ for tissue samples 0.00125 µg/g.

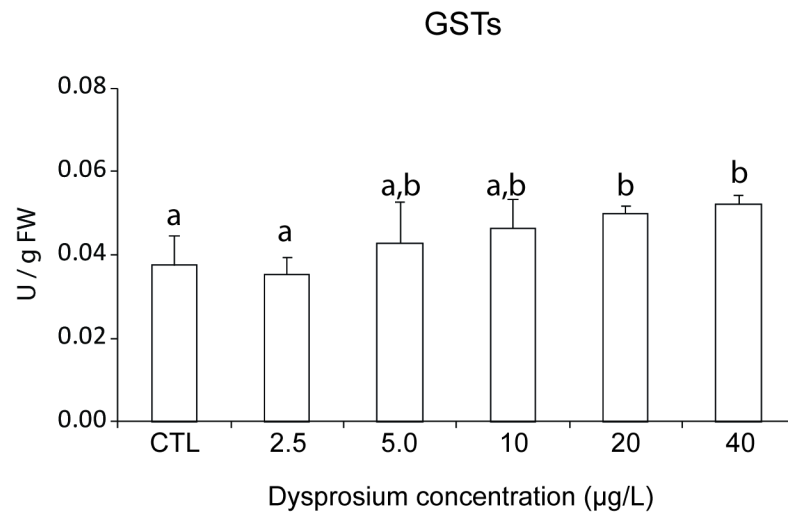| Dy concentrations	Seawater medium	Mussels tissues
	Weekly, after spiking	End of the 4th week
CTL	<LOQ	0.013±0.002[a]
2.5	2.6±0.18	0.036[b]
5.0	5.3±0.27	0.080±0.004[c]
10	10±1.8	0.147±0.001[d]
20	22±4.6	0.226±0.002[e]
40	44±7.0	0.484±0.009[f]

Table 2- p-values obtained through pairwise comparisons performed on biochemical results using PERMANOVA routine from the software PRIMER (PERMANOVA+add-on in PRIMER v6. Anderson et al., 2008).
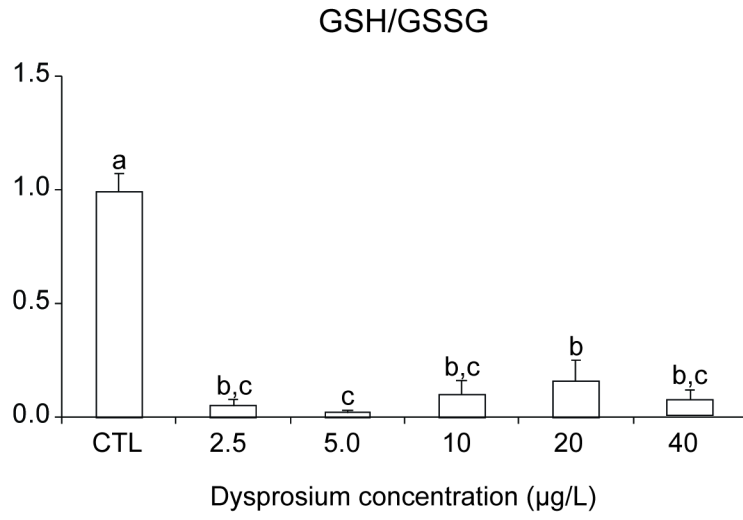
	SOD	CAT	GPx	GSTs	GSH/GSSG	LPO	PC	ETS	GLY	PROT
Main test	0.0496	0.0023	0.0581	0.0005	0.0001	0.0002	0.0023	0.3306	0.0082	0.2168
	Pairwise									
CTL vs 2.5	0.3789	0.9562	0.0814	0.235	**0.0001**	**0.0026**	**0.0187**		0.0834	
CTL vs 5.0	**0.0345**	0.9299	0.4525	0.2132	**0.0002**	**0.0035**	**0.0488**		**0.0285**	
CTL vs 10	**0.0252**	0.3879	0.2624	0.0902	**0.0002**	**0.023**	**0.008**		**0.0209**	
CTL vs 20	**0.0013**	**0.0063**	0.0994	**0.0022**	**0.0001**	**0.0077**	**0.0338**		**0.0369**	
CTL vs 40	**0.0079**	**0.019**	**0.0198**	**0.0006**	**0.0002**	**0.0013**	**0.0102**		**0.0016**	
2.5 vs 5.0	**0.0286**	0.8765	0.0699	0.9127	0.2654	0.4141	0.4931		0.9972	
2.5 vs 10	**0.0426**	0.4258	0.2498	0.1462	0.1502	0.5984	0.0883		0.6492	
2.5 vs 20	**0.0004**	**0.0025**	0.2712	**0.0201**	0.0895	**0.0023**	0.2534		0.1588	
2.5 vs 40	**0.0269**	**0.0228**	0.4814	**0.0119**	0.1129	**0.0158**	0.6932		0.4415	
5.0 vs 10	0.4207	0.3843	0.6752	0.1857	0.2893	0.4532	0.0962		0.6397	
5.0 vs 20	0.198	**0.0016**	0.4728	0.1271	**0.0467**	**0.0005**	0.1428		0.138	
5.0 vs 40	0.2083	**0.0155**	**0.0497**	0.1143	0.1172	**0.027**	0.2207		0.4174	
10 vs 20	0.8913	**0.0359**	0.8013	0.2559	0.1355	**0.0386**	0.0743		0.097	
10 vs 40	0.7579	**0.0008**	**0.0224**	0.3963	0.083	**0.001**	0.0639		0.9353	
20 vs 40	0.7438	0.3842	0.2256	0.4877	0.3451	**0.0317**	0.3408		0.0912	

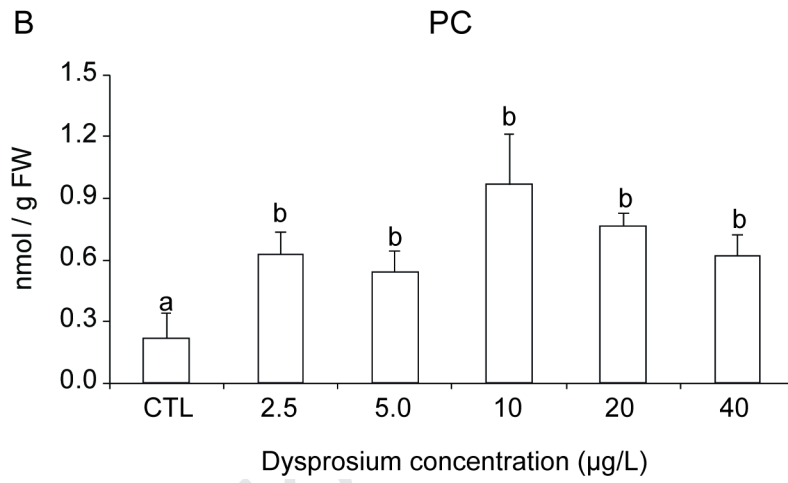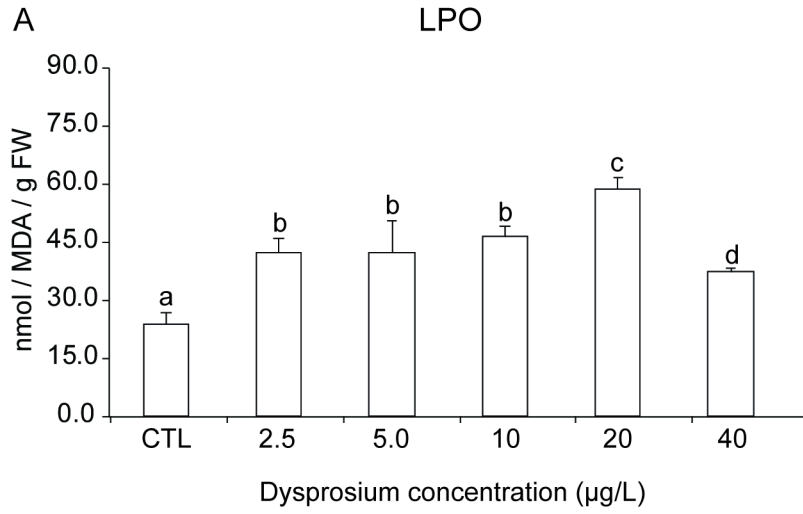**Table 3-** Integrated Biomarker Response (IBR) obtained for each condition (CTL-0, 2.5, 5.0, 10, 20, 40 µg/L of Dy).
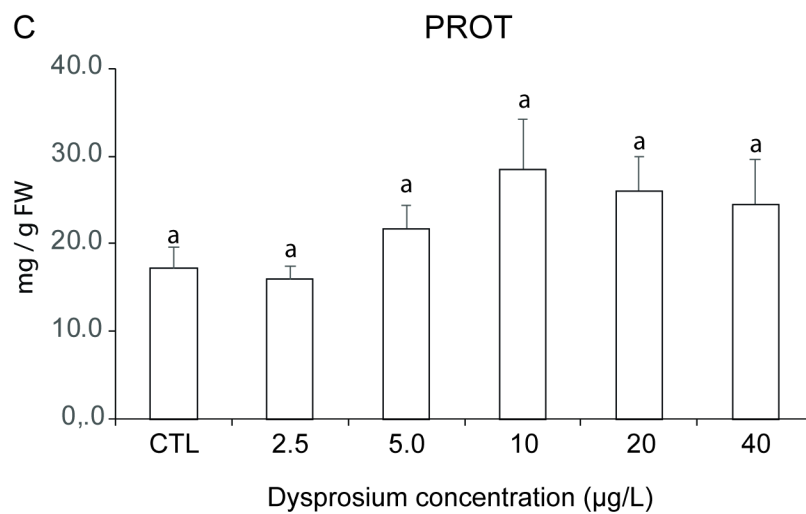
Dy exposure conditions µg/L	IBR values
CTL	-
2.5	1.1
5.0	0.22
10	2.1
20	1.9
40	3.4

A

SOD

B

CAT

C

GPx

GSTs

GSH/GSSG

A LPO

B PC

A

ETS

B

GLY

C

PROT

CTL

C5

ETS

LPO

C4 Dy
GSTs

GLY
GSH/GSSG

SOD PROT

-4

4

PCO1
64.2% of total variation

C3

C2

PC

C1

2

-2

PCO2
16.5% of total variation
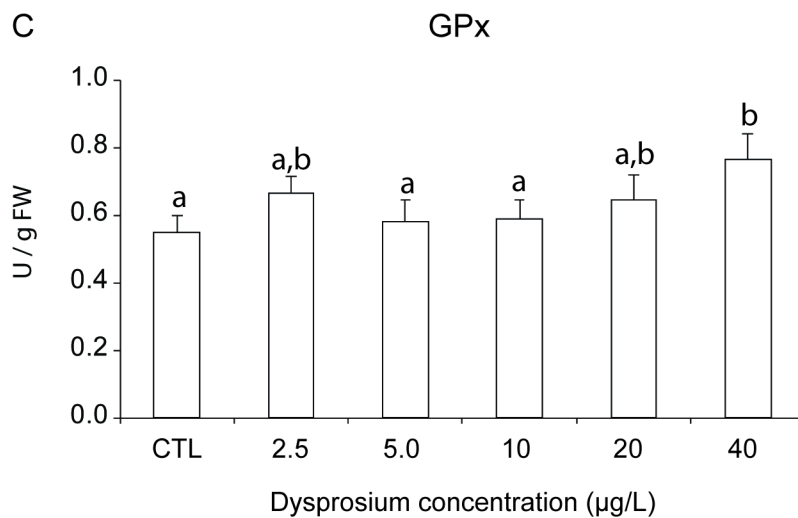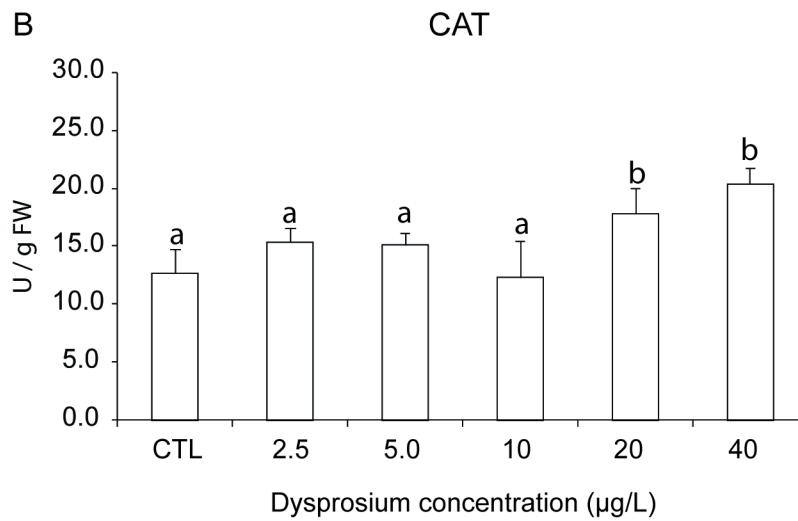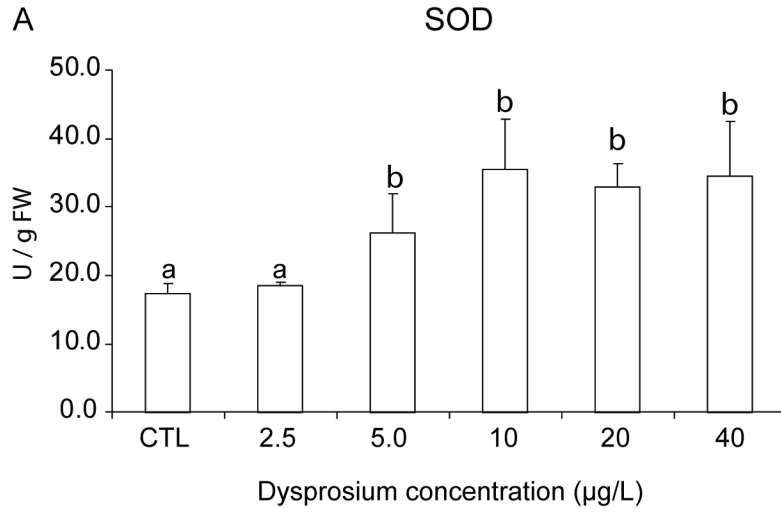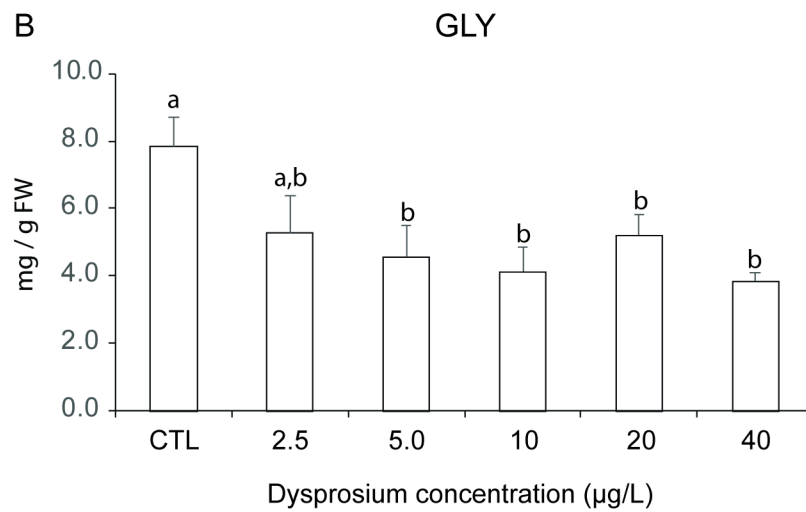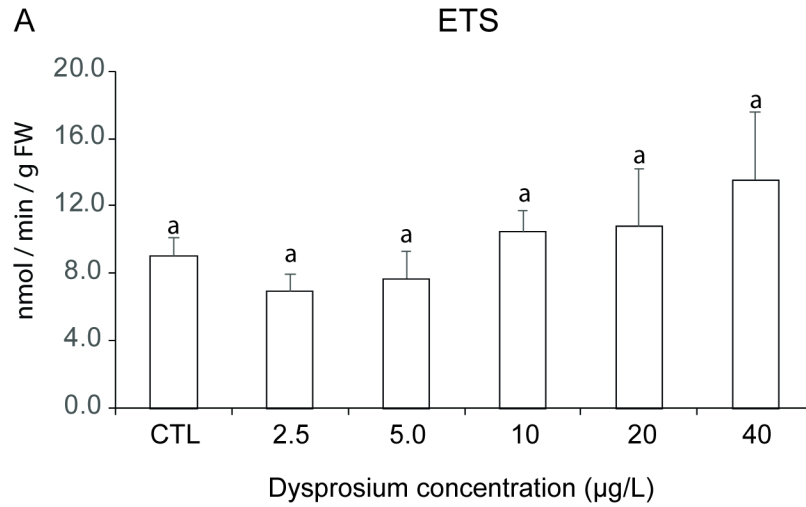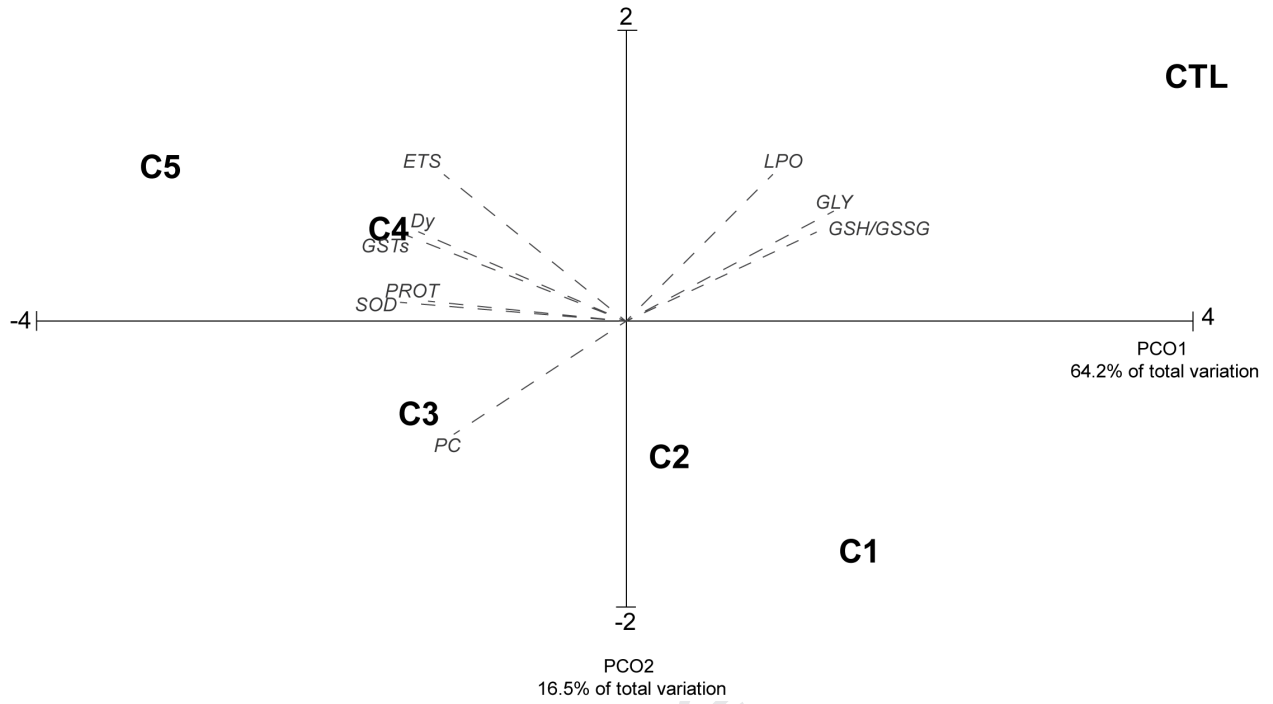
- *Mytilus galloprovincialis* bioaccumulated Dysprosium

- Mussels exposed to Dy decreased their metabolic capacity

- Contaminated mussels increased antioxidant and biotransformation defences

- Lipid peroxidation occurred in contaminated mussels

- Oxidative stress was observed in mussels exposed to Dy

Rosa Freitas and Eduarda Pereira are supervisors of the students that co-authored this ms (Silvana Costa, Celso Cardoso, Tiago Morais, Pedro Moleiro, André F. D. Lima, Márcio Soares, Samuel Figueiredo, Tiago L. Águeda, Pedro Rocha, Gonçalo Amador). Students did the exposure assay (for 28 days under controlled conditions), performed all methods and analyses for Nd quantification and biomarkers determination.

Rosa Freitas and Eduarda Pereira gave the idea of this study to the students that accepted this challenge and performed all the analyses during their last year of their bachelor degree. Eduarda Pereira is the responsible for the laboratory where Nd quantification was done. Rosa Freitas and Amadeu Soares are the responsible persons for the labs where biomarkers were determined. Eduarda Pereira, Rosa Freitas and Amadeu Soares funded this study.

Conflict of Interest

The Authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affi liations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.