

1 **Environmental hazard assessment of a marine mine tailings deposit site and**  
2 **potential implications for deep-sea mining**

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23 **Abstract**

24 Portmán Bay is a heavily contaminated area resulting from decades of metal mine tailings  
25 disposal, and is considered a suitable shallow-water analogue to investigate the potential  
26 ecotoxicological impact of deep-sea mining. Resuspension plumes were artificially  
27 created by removing the top layer of the mine tailings deposit by bottom trawling. Mussels  
28 were deployed at three sites: i) off the mine tailings deposit area; ii) on the mine tailings  
29 deposit beyond the influence from the resuspension plumes; iii) under the influence of  
30 the artificially generated resuspension plumes. Surface sediment samples were collected  
31 at the same sites for metal analysis and ecotoxicity assessment. Metal concentrations and  
32 a battery of biomarkers (oxidative stress, metal exposure, biotransformation and oxidative  
33 damage) were measured in different mussel tissues. The environmental hazard posed by  
34 the resuspension plumes was investigated by a quantitative weight of evidence (WOE)  
35 model that integrated all the data. The resuspension of sediments loaded with metal mine  
36 tails demonstrated that chemical contaminants were released by trawling subsequently  
37 inducing ecotoxicological impact in mussels' health. Considering as sediment quality  
38 guidelines (SQGs) those indicated in Spanish action level B for the disposal of dredged  
39 material at sea, the WOE model indicates that the hazard is slight off the mine tailings  
40 deposit, moderate on the mine tailings deposit without the influence from the  
41 resuspension plumes, and major under the influence of the resuspension plumes. Portmán  
42 Bay mine tailings deposit is a by-product of sulphide mining, and despite differences in  
43 environmental setting, it can reflect the potential ecotoxic effects to marine fauna from  
44 the impact of resuspension of plumes created by deep-sea mining of polymetallic  
45 sulphides. A similar approach as in this study could be applied in other areas affected by  
46 sediment resuspension and for testing future deep-sea mining sites in order to assess the  
47 associated environmental hazards.

48

49 ***Capsule***

50 Sediment resuspension plumes on a sulphide mine tailings deposit cause major  
51 environmental hazard. Similar hazard may be expected in plumes from deep-sea mining.

52

53 **Keywords:** sediment resuspension; bioaccumulation; biomarkers; *Mytilus*  
54 *galloprovincialis*; Portmán Bay.

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## 57 **1. Introduction**

58 Portmán Bay is a heavily impacted area resulting from decades of metal mine tailings  
59 disposal that lasted until 1990. Minerals extracted in the Portmán mining district were  
60 mainly pyrite (FeS<sub>2</sub>), galena (PbS) and sphalerite (ZnS), which were mechanically treated  
61 for concentration of metals, with about 95% of mine tailings waste generated (Martínez-  
62 Sánchez et al. 2008, Oyarzun et al. 2013). About 60 Mt of tailings were dumped into the  
63 sea, moving the shoreline seaward about 500-600 m and reaching the continental shelf  
64 off Portmán Bay (Manteca et al. 2014). The mine tailings deposit has a maximum  
65 thickness of about 14 m and is composed of fine sediments highly enriched with metals  
66 (mainly Fe, Zn, As and Pb, with metal concentrations 10 to 60 times higher than coastal  
67 sediments in the Mediterranean Sea). Above the deposit there is a thin layer of  
68 approximately 10-20 cm of coarse sediments reworked by natural (waves) and  
69 anthropogenic (bottom trawling) processes, with metal concentrations 10 to 20 times  
70 higher than unpolluted sediments (Cerdà-Domènech et al., in prep).

71 The resuspension of contaminated sediments may alter their physical and chemical  
72 characteristics, such as redox potential, pH, dissolved oxygen, potentially triggering  
73 desorption and remobilizing contaminants, affecting their mobility, bioavailability and  
74 increasing the risk of negative effects to marine fauna and ecosystem health (Bocchetti et  
75 al. 2008, Ondiviela et al. 2012). Therefore, the assessment of the impact of contaminated  
76 marine areas, such as mine tailings deposits should be investigated in different  
77 environmental matrices (sediment, water and biota) combining information from the  
78 chemistry and ecotoxicological impact, integrating data from bioavailability,  
79 bioaccumulation and biomarker responses and from ecotoxicological bioassays on  
80 bioindicator species (Viarengo et al. 2007). Biomarkers are known as important early  
81 warning signals of adverse effects, usually responding in the sub-lethal toxicity range of  
82 single or mixture of contaminants (Cajaraville et al. 2000, Annicchiarico et al. 2007,  
83 Taylor and Maher 2016). Nevertheless, it is acknowledged that confounding factors, such  
84 as seasonality or reproductive cycle, may affect the biomarkers sensitivity, highlighting  
85 the importance of the adequate selection of bioindicator species individuals and  
86 experimental design (including controls) to allow comparability and meaningfulness of  
87 results. The integration of different quality Descriptors to assess the impact on biota and  
88 ecosystem functioning is required by the Descriptors 8 and 9 of the Marine Strategy  
89 framework directive (European Commission 2008). The quantitative weight of evidence  
90 (WOE) model (Sediqualsoft), is considered to be a promising tool to assess the  
91 environmental hazards and ecological risks since it integrates data from the sediment  
92 chemistry, bioaccumulation, biomarkers responses and toxicity bioassays (Piva et al.  
93 2011, Benedetti et al. 2012, 2014, Regoli et al. 2014, Bebianno et al. 2015).

94 The ore type exploited in the Portmán mining district, for a certain extent, is similar to  
95 that present in mid-ocean ridges and hydrothermal vent sites (ISA 2002, Martínez-  
96 Sánchez et al. 2008, Oyarzun et al. 2013, Canals et al. 2016). Also, the hydrodynamics of  
97 the bay are low energy, somehow similar to the deep sea, being a suitable shallow-water  
98 analogue to investigate the potential impacts of deep-sea mining (Canals et al. 2016). In

99 this sense, it is a unique place to conduct sediment resuspension experiments on a deposit  
100 of sulphide mining by-products, investigating the chemical and physical behaviour of  
101 metal loaded sediments and their ecotoxicological effects to marine organisms.

102 In the present study, a transplant experiment was carried out to assess the short-term  
103 effects of sediment resuspension on caged mussels (*Mytilus galloprovincialis*). Metal  
104 accumulation and biomarkers responses were analysed in mussel tissues and combined  
105 with the results from the sediment chemistry and toxicity bioassay. These were then  
106 integrated in the WOE elaboration to provide specific hazard indices for each typology  
107 of data before their overall integration to classify the hazard for the different areas and  
108 assess the impact of sediments resuspension in Portmán Bay.

109

## 110 **2. Materials and methods**

### 111 *2.1. Sediments resuspension experiment and sampling sites*

112 In the summer of 2014, the MIDAS-Portmán research cruise was conducted in Portmán  
113 Bay and in its adjacent marine area (Murcia, SE Spain) on board of the Spanish research  
114 vessels R/V Ángeles Alvariño and R/V Ramon Margalef. Transects of bottom trawling  
115 off Portmán Bay (Fig. 1) were carried out to resuspend the sediments and originate  
116 plumes, being usually less than 10 m in height, with a variable though relatively quick  
117 decline and limited dispersal, and a maximum tracking time for a given plume of about 4  
118 hours (Canals et al. 2016). Before the resuspension events, a transplant monitoring  
119 experiment was carried out with caged mussels *M. galloprovincialis* obtained from a  
120 mussel farm (Cademar) located on the Ebro Delta. Mussels (length 5.0-6.5 cm; width 1.7-  
121 3.5cm; wet weight 20-37g) were deployed at about 3 m above the seafloor in the  
122 following three sites (Fig. 1): off the mine tailings deposit area (Mooring\_UPM2,  
123 hereafter “O”, 37° 32.713' N 0° 50.684' W, 57 m); on the mine tailings deposit without  
124 the influence from the resuspension plumes (Mooring\_UPM3, hereafter “B”, 37° 34.553'  
125 N 0° 51.563' W, 17 m); under the influence of the artificially generated resuspension  
126 plumes (Mooring\_UPM1, hereafter “P”, 37° 34.177' N 0° 51.386' W, 42 m). After 6 days  
127 of exposure, cages were retrieved on board and mussels from each site were immediately  
128 dissected and tissues (gills, digestive gland and mantle) were flash frozen and preserved  
129 at -80 °C for chemical and biomarker analyses. Surface sediment samples (top 1 cm) were  
130 also collected at the same sites and frozen at -20 °C until further analysis.

131

### 132 *2.2. Sediments grain size analyses*

133 Sediment grain size was determined using a Coulter LS230 Laser Diffraction Particle  
134 Size Analyser. Samples were oxidized with a 10% H<sub>2</sub>O<sub>2</sub> solution to remove organic  
135 matter, and one subsample analysed and another treated with 1M HCl to remove  
136 carbonates. The total and non-biogenic grain size distribution determined and data  
137 analysed with GradiStat®.

138

### 139 2.3. Trace metals analyses in sediments and mussels

140 The concentrations of trace elements (Ag, As, Au, Cd, Cr, Cu, Fe, Hg, Ni, Pb, Sb, Zn) in  
141 the sediment were determined after acid digestion as follows: ca. 0.5 g of dried sediment  
142 (n=3) were transferred in Teflon vessels, added with 5 mL fluoridric acid and 1 mL of  
143 “aqua regia” (i.e. HCl:HNO<sub>3</sub> = 3:1) and, then, incubated at 150 °C for 90 min. At the end  
144 of the incubation, 5 mL of 10% boric acid were added and the extracts were analyzed by  
145 inductively coupled plasma-atomic emission spectrometry (ICP-AES). Mercury was  
146 determined by ICP-AES exciting the element to form volatile hydride in a hydrides  
147 generation reactor, according to previously published procedure (Pohl 2004). Standard  
148 curves were prepared in the same acid matrix used for the sediment samples. Caution was  
149 used in preparing and analysing samples to minimize contamination from air, glassware,  
150 and reagents, all of which were of Suprapur quality. Replicated measures of certified  
151 reference material (PACS-2, marine sediment reference material) and reagent blanks  
152 were used to assess precision and contamination. The analytical accuracy was routinely  
153 between 5 and 6%, and never higher than 10%. With the exception of Au, the  
154 concentrations of the same elements, were also determined in mussels tissues (gills,  
155 digestive gland and mantle) dissected from 15 individuals at each sampling site. Mussels  
156 tissues (about 0.3 g) were dried at 50°C and digested with 5 ml nitric acid and 1 ml  
157 hydrogen peroxide in a microwave digestion system. Quality assurance and quality  
158 control were done by processing blank samples and certified reference material (CRM  
159 278, mussel tissue). The values obtained for the certified reference materials were always  
160 within the 95% confidence interval of certified values.

161

### 162 2.4. Biomarkers analyses

163 From each site, five pools with tissues (gills, digestive gland and mantle), each of them  
164 obtained from three *M. galloprovincialis* individuals, were prepared for the analysis of  
165 the following biomarkers: oxidative stress (superoxide dismutase – SOD, catalase – CAT,  
166 glutathione peroxidase – GPx), metal exposure (metallothioneins – MT),  
167 biotransformation (glutathione-S-transferase – GST) and oxidative damage (lipid  
168 peroxidation – LPO).

169 Antioxidant enzymes activities (SOD, CAT, total GPx, Se-I GPx and Se-D GPx) and GST  
170 were measured by spectrophotometric methods in the cytosolic fraction of gills, digestive  
171 gland and mantle. Tissues were homogenized in 0.02 M Tris-HCl buffer, pH 7.6,  
172 containing 1 mM of EDTA, 0.5 M of sucrose, 0.15 M of KCl and 1 mM of DTT, in an  
173 ice bath for 2 min (wet weight of tissue: buffer volume ratio of 1:5). The homogenates  
174 were centrifuged at 500 g for 15 min, at 4 °C. The cytosolic fraction was obtained after a  
175 second centrifugation of the supernatant for 45 min at 4 °C and 12 000 g (e.g. Rocha et  
176 al. 2015).

177 SOD activity was determined by the reduction of cytochrome c by the xanthine  
178 oxidase/hypoxanthine system at 550 nm (molar extinction coefficient ( $\epsilon$ ) of  $-50 \text{ M}^{-1} \text{ cm}^{-1}$   
179  $^{-1}$ ; McCord and Fridovich 1969) and the results are expressed in  $\text{U mg}^{-1}$  of total protein.  
180 CAT activity was determined as the decrease in absorbance for 1 min after the  $\text{H}_2\text{O}_2$   
181 consumption at 240 nm ( $\epsilon = -40 \text{ M}^{-1} \text{ cm}^{-1}$ ; Greenwald 1985) with results being expressed  
182 as  $\mu\text{mol min}^{-1} \text{ mg}^{-1}$  of total protein. GPx activities were assessed by following for 5 min  
183 the NADPH oxidation in the presence of excess glutathione reductase, reduced  
184 glutathione and cumene hydroperoxide (Se-I GPx) or  $\text{H}_2\text{O}_2$  (Se-D GPx) as substrate at  
185 340 nm ( $\epsilon = -6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ ; Flohe and Gunzler 1984 and adapted to a microplate reader  
186 by McFarland et al. 1999). Total GPx activity refers to the sum of Se-I GPx and Se-D  
187 GPx activities and results are expressed as  $\text{nmol min}^{-1} \text{ mg}^{-1}$  of total protein. GST activity  
188 was measured by following the conjugation of reduced glutathione (GSH) with 1-chloro  
189 2,4 dinitrobenzene (CDNB) at 340 nm for 1 min ( $\epsilon = -9.6 \text{ mM}^{-1} \text{ cm}^{-1}$ ; Habig et al. 1974)  
190 and results are expressed as  $\mu\text{mol min}^{-1} \text{ mg}^{-1}$  of total protein.

191 For each site, five additional pools with tissues of three specimens were prepared for MTs  
192 and LPO. Samples were homogenized at  $4 \text{ }^\circ\text{C}$  in a Tris-HCl ( $0.02 \text{ M}$ ;  $5 \text{ mL}$  per g of tissue)  
193 buffer with butylated hydroxytoluene (BHT,  $10 \mu\text{L mL}^{-1}$ ), pH 8.6. The homogenate was  
194 separated in soluble and insoluble fractions by centrifugation ( $30\,000 \text{ g}$ ,  $45 \text{ min}$ ,  $4 \text{ }^\circ\text{C}$ )  
195 and a part of the supernatant was used for the measurement of LPO and total protein  
196 content. The other part was heat-treated at  $80 \text{ }^\circ\text{C}$  for  $10 \text{ min}$  and centrifuged for  $45 \text{ min}$   
197 at  $4 \text{ }^\circ\text{C}$  and  $30\,000 \text{ g}$ , with the resulting supernatant used for MTs measurements.

198 MTs concentration was determined by differential pulse polarography ( $\mu\text{Autolab II}$   
199 potentiostat/galvanostat) following the method by Bebianno and Langston (1989). The  
200 standard addition method was used to calibrate MT concentration, using the MT standard  
201 of rabbit liver (Sigma-Aldrich). Results are expressed as  $\text{mg g}^{-1}$  of total protein.

202 LPO was assessed by measuring the concentration of two sub-products of  
203 polyunsaturated fatty acid peroxidation: malondialdehyde (MDA) and 4-hydroxyalkenals  
204 (4-HNE). The method proposed by Erdelmeier et al. (1998) was followed, with a maximal  
205 absorbance at  $586 \text{ nm}$ , and using malondialdehyde bis-(dimethyl acetal; Sigma-Aldrich)  
206 as standard. Results are expressed as  $\text{nmol of MDA} + 4\text{-HNE mg}^{-1} \text{ prot.}$

207 Total protein concentration of the cytosolic fraction was measured by the Bradford  
208 method (Bradford 1976, adapted to a microplate reader), using Bovin Serum Albumin  
209 (Sigma-Aldrich) as a standard. Protein concentration is expressed as  $\text{mg g}^{-1}$  of tissue wet  
210 weight.

211

## 212 2.5. Sediment bioassays

213 The toxicity of the sediments was analysed using the solid phase Microtox<sup>®</sup> bioassay.  
214 This test is a quantitative and functional test measuring the changes in luminescence (a  
215 by-product of cellular respiration) by about one million non-pathogenic naturally  
216 luminescent marine bacteria (*Vibrio fischeri*) upon exposure to a toxic substance or

217 sample containing toxic materials. During the test the *V. fischeri* are in direct contact with  
218 the sample particles, increasing the probability for the measurement of the responses to  
219 particle bound and marginally soluble toxicants. Each test consists of 2 controls and 13  
220 sample serial dilutions in duplicate, luminescence data is analysed with the  
221 MicrotoxOmni software (Azur Environmental). The toxicity endpoint is the luminescence  
222 inhibition EC<sub>50</sub> (g L<sup>-1</sup>) at 15 min (Azur Environmental 1998).

223

## 224 2.6. Statistical analyses

225 Significant differences were assessed using the non-parametric multiple-comparisons  
226 Kruskal Wallis test. Significant differences are for  $p < 0.05$ .

227

## 228 2.7. Weight of evidence elaboration (WOE) model

229 A quantitative WOE approach was used to assess the impact posed by the sediments and  
230 sediment plume (O, B and P) using a Sediqualsoft model (Piva et al. 2011). WOE  
231 elaborates data from the sediments chemistry (Line Of Evidence – LOE 1) in relation to  
232 different sediment quality guidelines (SQGs), and results were integrated with those of  
233 bioaccumulation in mussel tissues (LOE 2), biomarkers (LOE 3) and sediment bioassays  
234 (LOE 4). Details about the model concept, calculations and thresholds, are described  
235 elsewhere (Piva et al. 2011, Benedetti et al. 2012, 2014, Regoli et al. 2014, Bebianno et  
236 al. 2015).

237 The hazard level related to the LOE1 – sediment chemistry is determined by the  
238 calculation, for each parameter, of the ratio between the measured concentrations and that  
239 indicated by various sediment quality guidelines, or Ratio to Reference (RTR). In order  
240 to consider if the contaminant is a “priority” or “priority and hazardous” (EC Directive  
241 2008/105), the RTR value is corrected by a specific weight (RTR<sub>w</sub>). The SQGs used here  
242 were the 3 action levels (A, B, C) of the Spanish normative guidelines on dredged  
243 sediments (CIEM 2015).

244 The Hazard Quotient for chemistry (HQ<sub>C</sub>) is calculated following the equation below  
245 where an average RTR<sub>w</sub> was obtained for all of the parameters with RTR ≤ 1 (i.e. below  
246 normative limit), while the RTR<sub>w</sub> was individually added into the summation Σ for those  
247 with RTR > 1 (Eq. 1; Piva et al. 2011).

248

$$249 \quad HQ_C = \frac{\sum_{j=1}^N RTR_W(j)_{RTR(j) \leq 1}}{N} + \sum_{k=1}^M RTR_W(k)_{RTR(k) > 1}$$

250 Eq.1

251  $N$  and  $M$  are the number of parameters with RTR respectively ≤ or >1, while  $j$  and  $k$  are  
252 indices allowing to repeat the calculation for  $N$  or  $M$  times.

253 The values of  $HQ_C$  are then assigned to one of six classes of chemical hazard identified  
 254 according to different colours: absent/white <0.7; negligible/green 0.7–<1.3; slight/azure  
 255 1.3–<2.6; moderate/yellow 2.6–<6.5; major/red 6.5–<13; severe/black  $\geq 13$  (Piva et al.  
 256 2011).

257 The LOE2 – bioaccumulation hazard in the different mussel tissues is based on the  
 258 calculation of the RTR for each parameter measured in tissues of exposed compared to  
 259 control organisms (Piva et al. 2011). The  $RTR_W$  is calculated according to the weighting  
 260 of the pollutants and each chemical parameter was directly assigned to one of five classes  
 261 of hazard, considering the natural variability of contaminants in tissues. The hazard for a  
 262 single parameter ranged from absent to slight if the  $RTR_W$  was < 2.6 (i.e. less than a two-  
 263 fold increase of tissue concentration for a non-priority-and-hazardous pollutant),  
 264 moderate for  $2.6 \leq RTR_W < 6.5$ , major for  $6.5 \leq RTR_W < 13$ , and severe for  $RTR_W \geq 13$   
 265 (i.e. a 10-fold increase in a priority and hazardous pollutant). The cumulative Hazard  
 266 Quotient for bioavailability ( $HQ_{BA}$ ) does not consider parameters with  $RTR_W < 1.3$  (hazard  
 267 absent), calculates the average for those with  $RTR_W$  ranging between 1.3 and 2.6 and sums  
 268 ( $\Sigma$ ) all those with  $RTR_W \geq 2.6$  (Eq. 2).

269

$$271 \quad HQ_{BA} = \frac{\sum_{n=1}^j RTR_W(n)_{1.3 \leq RTR_W < 2.6}}{j} + \sum_{n=1}^k RTR_W(n)_{RTR_W \geq 2.6}$$

270 Eq. 2

272 The hazard level of cumulative  $HQ_{BA}$  is then classified from Absent to Severe, depending  
 273 on the distribution of analysed chemicals within the different classes of effect (Benedetti  
 274 et al. 2012; Piva et al. 2011).

275 The biomarkers hazard – LOE3 integrates a large set of biomarker responses where each  
 276 is assigned a “weight”, taking into account the relevance of the biological endpoint, and  
 277 a “threshold” for changes of biological relevance, considering tissue differences, and the  
 278 possibility of both induction and/or inhibition for biomarkers potentially showing  
 279 biphasic responses (Piva et al. 2011). The measured variation in each biomarker is  
 280 compared to the threshold (effect), then corrected for the weight of the response and the  
 281 statistical significance of the difference compared to controls. Variations of each  
 282 biomarker were assigned to one of five classes of hazard (absent, slight, moderate, major,  
 283 severe) depending on the calculated effects. The Hazard Quotient for biomarkers ( $HQ_{BM}$ )  
 284 does not consider the contribution of responses with an effect <1 (lower than threshold),  
 285 calculates the average for biomarkers with an effect up to two-fold compared to the  
 286 threshold, adding the summation ( $\Sigma$ ) for the responses with variations greater than 2-fold  
 287 to the respective threshold (Eq. 3; Piva et al. 2011).

288

$$290 \quad HQ_{BM} = \left( \frac{\sum_{j=1}^N Effect_W(j)_{1 < Effect(j) \leq 2}}{num\ biomark_{1 < Effect(j) \leq 2}} + \sum_{k=1}^M Effect_W(k)_{Effect(j) > 2} \right)$$

289

291 Eq. 3

292 The hazard level related to the LOE4 – ecotoxicological bioassays is determined by the  
293 cumulative hazard quotient ( $HQ_{Battery}$ ).  $HQ_{Battery}$  is calculated by the summation ( $\Sigma$ ) of the  
294 weighted effects ( $Effect_W$ ), that correspond to the variations measured for each test  
295 compared to specific thresholds (corrected for the statistical significance of the difference  
296 ( $w$ )), the biological importance of the endpoint of each test and the exposure conditions  
297 ( $w_2$ ; Eq. 4).

$$298 \quad HQ_{Battery} : \sum Effect_W(k) w_2$$

299 Eq. 4

300  $HQ_{Battery}$  is then normalized to a scale from 0 to 10, where 1 is the battery threshold (when  
301 all the measured bioassays exhibit an effect equal to the threshold), and where 10 is when  
302 all the assays exhibit 100% of effect. The  $HQ_{Battery}$  results are then assigned to one of the  
303 five classes of hazard.

304 Results from individual LOEs were elaborated with a classical weight of evidence  
305 approach which integrates and gives a different weight to various typologies of data.  
306 Scales used within the different LOEs to calculate the class of  $HQ$  were normalized to a  
307 common scale setting for  $HQ_C$  (that could theoretically reach unlimited values) a  
308 saturation limit of 13, i.e. the value corresponding to the beginning of the severe class of  
309 hazard. The obtained values were multiplied by 1.0 (for  $HQ_C$  and  $HQ_{BM}$ ) and by 1.2 (for  
310  $HQ_{BA}$  and  $HQ_{Battery}$ ) thus giving a greater weighting to data on bioavailability compared  
311 to the presence of chemicals in the sediments, and to acute effects compared to sub-lethal  
312 responses at the cellular level. An overall WOE level of environmental hazard for each  
313 condition analysed was then calculated and assigned to one of the 5 hazard levels, i.e.,  
314 from absent to severe (Piva et al. 2011).

315

### 316 **3. Results and discussion**

#### 317 *3.1. Sediment analyses*

318 Metal concentrations in the sediments are reported in Table 1. Lower concentrations were  
319 measured in the area outside of the mine tailings deposit (O), with the exception of Hg,  
320 which showed similar concentrations outside of the deposit (O) and in the mine tailings  
321 deposit site affected by the resuspension plumes (P). In general, sediments from the mine  
322 tailings deposit area (B) and those under the influence of the plumes (P) were  
323 characterized by a similar chemical composition, although concentrations of As, Cr, Fe,  
324 Ni, Pb and Sb were slightly higher in B. The concentrations of As, Pb and Zn in the  
325 sediments from the mine tailings deposit with (P) and without (B) the influence of the  
326 resuspension plumes exceeded the limit values of action level C, while Cd was higher  
327 than the action level B limits for Spanish sediment quality criteria of dredged materials  
328 (CIEM 2015, Table 1). According to such normative guidelines, sediments containing

329 metal concentrations above level C are considered highly contaminated and dredged  
330 material must be isolated into confined areas or subjected to specific treatments before  
331 considering dumping it at sea (CIEM 2015). Overall, the mine tailings deposit in Portmán  
332 Bay has such high concentrations of contaminants that the tailings should have never been  
333 dumped at sea.

334

### 335 *3.2. Bioaccumulation of metals in mussels*

336 On average for all caged mussels from different locations, the metals concentration, from  
337 the higher to the lower, was the following: Fe > Zn > As > Pb > Cu > Ag > Sb > Cr > Cd  
338 > Ni > Hg (Fig. 2A-B). The gills of mussels from the mine tailings deposit affected by  
339 the resuspension plumes (P) showed significantly higher concentrations for Cr and Ni  
340 when compared to the other sites ( $p<0.05$ ). Significantly higher concentrations of Ag  
341 were found in the gills of mussels deployed on the mine tailings deposit area without the  
342 influence from the plume (B) when compared to the area outside the mine tailings deposit  
343 (O) ( $p<0.05$ ), while in the digestive gland significantly lower concentrations of Ag were  
344 found in B when compared to P ( $p<0.05$ ). Fe and Pb presented significantly higher  
345 concentrations in the digestive gland from site O when compared to P, while Ni was  
346 significantly higher in B when compared to P ( $p<0.05$ ). In the mantle of mussels exposed  
347 to the plume (P) Cu was significantly higher when compared to the mine tailings deposit  
348 (B) ( $p<0.05$ ). No significant differences were found between sites for the accumulation  
349 of As, Hg, Sb and Zn in the three tissues analysed ( $p>0.05$ ; Fig.2A-B).

350 Metal accumulation was tissue specific (Fig. 2A-B). Mantle was the tissue with  
351 significantly lower concentrations of Ag, Cd, Fe, Hg, Ni, Pb, Zn, in all sites, when  
352 compared to both gills and digestive gland ( $p<0.05$ ). Significantly higher concentrations  
353 of As were observed in the digestive gland for all sites ( $p<0.05$ ) when compared to mantle  
354 and gills. Gills are the first target of metals present in the seawater, hence the high levels  
355 of accumulation, while the metal accumulation in the digestive gland may also be linked  
356 to metal metabolism and detoxification (Marigómez et al. 2002).

357

### 358 *3.3. Biomarkers*

359 SOD activity was significantly lower in the gills of mussels from the mine tailings deposit  
360 (B) when compared to the area off the mine tailings (O), while in the mantle SOD was  
361 significantly lower in P when compared to O ( $p<0.05$ ; Fig. 3). A significantly higher CAT  
362 activity was noticed in the gills in P when compared to B, while in the mantle a  
363 significantly higher CAT activity was noted in P when compared to O ( $p<0.05$ ). In the  
364 digestive gland, a significant induction of Se-I GPx activity in P was noted when  
365 compared to both O and B, while in the gills and mantle a significant increase was  
366 observed in P only when compared to B ( $p<0.05$ ). P induces a significantly higher Se-D  
367 GPx activity in the gills when compared to O, while in the digestive gland in P the activity

368 was significantly higher than in B ( $p<0.05$ ). Total GPx activity was significantly higher  
369 in P in all tissues when compared to B ( $p<0.05$ ).

370 MTs significantly increased in the gills of mussels exposed to the resuspension plume (P)  
371 when compared to the area off the mine tailings deposit ( $p<0.05$ ). GST in the digestive  
372 gland was significantly lower in P when compared to B ( $p<0.05$ ), while in the mantle it  
373 was significantly higher in P when compared to O. Oxidative damage (LPO) was higher  
374 in the gills of mussels exposed to the plume, although no significant difference was found  
375 ( $p>0.05$ ). The levels of SOD, CAT, GST and LPO were significantly higher in the gills  
376 from all areas, when compared to the mantle ( $p<0.05$ ).

377 Metals can induce the production of reactive oxygen species (ROS), inducing oxidative  
378 stress what may trigger the action of the antioxidant system composed by several enzymes  
379 (such as those analysed here) which in turn counteract the effects of ROS (e.g. Di Giulio  
380 et al. 1989). While MTs can play a role in the detoxification of metals they can also be  
381 active as an antioxidant defence mechanism (e.g. Roesijadi 1992). Depending on the  
382 concentration and metal mixtures, the exposure period, the organism health status or other  
383 environmental stressors, both antioxidant enzymes activity, detoxification and  
384 biotransformation processes (GST) can be enough to counteract the potential toxic effects  
385 of metals and little or no oxidative damage is observed (e.g. Di Giulio et al. 1989). Given  
386 the overall effects of the mine tailings deposit resuspension plume on the biomarkers  
387 analysed in the different tissues, the mussel gills were the most affected during the 6 days  
388 of exposure and an increase in oxidative damage was noted, although not significant. This  
389 indicates that the mussel gills are more susceptible to ROS generation, antioxidant  
390 capacity changes and oxidative stress induced by metals from resuspension plumes  
391 generated on a mine tailings deposit site. Nevertheless, a better understanding of the  
392 ecotoxicological effects of the resuspension plumes would benefit from a prolonged  
393 exposure period.

394

#### 395 3.4. Sediment bioassays

396 Sediments toxicity assessed with the Microtox<sup>®</sup> bioassay indicated that the least toxic  
397 location was on the deposit without the influence from the resuspension plume (B), while  
398 the other locations (O and P) were more toxic (Table 1). The sediments toxicity was  
399 negatively correlated with both total and non-biogenic grain size, i.e. the lower the grain  
400 size, the lower the EC<sub>50</sub> ( $r=0.99$ , for  $p<0.05$ ).

401 It has been previously reported that the solid-phase Microtox bioassay results can be  
402 biased when sediments have a high content of silt-clay ( $< 63\mu\text{m}$ ; Ringwood et al. 1997).  
403 This issue is due to the fact that a portion of the bacteria can adsorb on to these smaller  
404 particles, which are retained in the filter, and will not be present in the filtrate where  
405 bioluminescence is measured. This may result in the potential erroneous classification of  
406 finer-grained sediments as being more toxic than they actually are (Ringwood et al. 1997).  
407 Still, the Microtox bioassay is a screening bioassay with interesting qualities (fast, low  
408 amount of sediment is required, etc.) and the inherent errors, that any type of bioassay

409 usually have, can be minimized when other indicators of toxicity are used in parallel, as  
410 done in this study (e.g. chemistry, bioaccumulation, biomarkers). Nevertheless, in future  
411 studies, it should be considered the inclusion of additional whole-sediment bioassays,  
412 including a dietary exposure route (e.g. Campana et al. 2012), with endpoints such as  
413 survival, reproduction, larval development, etc., using organisms such as amphipods,  
414 copepods, polychaetes, bivalves, etc. (reviewed by Simpson and Kumar 2016, Simpson  
415 et al. 2017).

416

### 417 *3.5. Weight of evidence elaboration*

418 For each of the 3 sites (O, B and P) a WOE approach was elaborated with sediment  
419 chemistry (LOE1), metal accumulation in gills, digestive gland and mantle (LOE2),  
420 biomarkers in gills, digestive gland and mantle (LOE3) and bioassay (LOE4; based only  
421 on Microtox data). After obtaining the results on individual hazard indices for specific  
422 LOEs (see supplementary material), the overall WOE approach was generated for the 3  
423 sites, combining the hazards of various LOEs (for full details on each of the LOEs see  
424 supplementary data in Appendix A) in a final WOE risk index (Table 2).

425 LOE1: The model output for sediment chemistry provides the elaboration with weighted  
426 criteria toward the 3 action levels (A, B, C) of the Spanish normative guidelines for  
427 dredged sediments (CIEM 2015). The chemical hazard elaborated for the off mine tailings  
428 deposit site (O) was moderate (action level A) or absent (levels B, C), on the mine tailings  
429 deposit site without the influence of the resuspension plumes (B) was severe (levels A,  
430 B) or moderate (level C) and for the mine tailings deposit site under the influence of the  
431 resuspension plumes was severe (levels A, B) or slight (level C) for the plumes (P; Table  
432 2). Sites B and P represented a major environmental hazard associated with the high  
433 concentrations of As, Cd, Pb and Zn previously noted above (Table 1) and that exceed  
434 the Spanish action level C (or B for cadmium) and for what a dredged material with these  
435 characteristics could not be dumped at sea.

436 The LOE1 is directly compared to sediment quality guidelines for dredged material  
437 according to the specific country regulations and values are based on total metal  
438 concentrations. However, only a fraction of the total concentration in sediments will be  
439 bioavailable to organisms and associated to toxicity. For instance, Simpson and Spadaro  
440 (2016) assessed the bioavailability and toxicity of sediments spiked with a range of  
441 sulphide minerals and noted that the dilute-acid extractable metal concentration was more  
442 reliable to predict toxicity than the total concentration. It is further advised that future  
443 studies could include this analysis when assessing sediments derived from mining and  
444 new guidelines for sediment quality based on the dilute-acid extractable metal  
445 concentration are suggested (Simpson and Spadaro 2016; Simpson et al. 2016).

446 LOE2: Compared to the off mine tailings deposit site (O), a generally limited (and quite  
447 comparable) metal accumulation was observed in mussels deployed on the mine tailings  
448 deposit without (B) and under the influence of the resuspension plumes sites (P). In both  
449 cases (B and P), the accumulation was absent for digestive gland, and slight in gills and

450 mantle. The accumulation was higher in the gills of mussels exposed to the plume (P)  
451 than in those deployed on the mine tailings deposit. These results confirm the increased  
452 bioavailability of some metals under investigated resuspension conditions.

453 LOE3: Compared to the area outside of the mine tailings deposit (O), biomarkers response  
454 was greater in mussels exposed to the plume (P) rather than in those deployed on the  
455 mining deposit site (B). In the mussels from B, the hazard was slight in digestive gland,  
456 moderate in the gills and absent in mantle, while in mussels from P the hazard was major  
457 in gills, moderate in digestive gland and mantle. These elaborations confirm that the gills  
458 were the most affected by the release of contaminants due to sediment resuspension  
459 (Table 2).

460 LOE4: This was based on a single bioassay, which conclusions may be rather limited, as  
461 usually a battery of 2-4 bioassays is used to take in better consideration of the potential  
462 variability and sensitivity of the assays. An additional test that can be performed in future  
463 studies is the acute 10-day survival sediment toxicity test with marine amphipods (e.g.  
464 ASTM 2014). Still, additional or alternative whole-sediment bioassays, with different  
465 endpoints (e.g. survival, reproduction, larval development) using organisms such as  
466 amphipods, copepods, polychaetes, bivalves, etc., can be an option (reviewed by Simpson  
467 and Kumar 2016, Simpson et al. 2017). In light of deep-sea mining novel bioassays with  
468 local fauna will have to be developed in the future. Nevertheless, considering only *V.*  
469 *fischeri*, the elaborated hazard was slight for outside the mine tailings deposit (O) and for  
470 the resuspension plume (P) and absent for the mine tailings deposit site (B; Tables 1 and  
471 2).

472 The overall WOE elaboration for the 3 sites (Table 2) indicated the specific hazard levels  
473 elaborated for individual LOEs and their final WOE integration. When using the Spanish  
474 action level B sediment quality guidelines (SQGs) to derive the Chemistry (LOE1) risk  
475 quotient, the WOE risk was slight outside the mine tailings deposit (O), moderate on the  
476 mine tailings deposit (B) and major on the mine tailings deposit site under the influence  
477 of the resuspension plume (P). If the Spanish action level C SQG was applied instead of  
478 level B, the hazard is absent for O, and moderate for B and P. These results show that the  
479 mine tailings deposit (B) and the resuspension plume (P) sites have a worst environmental  
480 condition compared to the site off the mine tailings deposit (O). This WOE model results  
481 are consistent with the observed high levels of metals concentrations in suspended  
482 particles for several hours after each trawling event (Canals et al. 2016). This approach  
483 appeared particularly useful for integrating heterogeneous datasets in a synthetic  
484 evaluation easy to understand for environmental managers and political decision-makers.

485 In addition to the presented case study, the application of this WOE model has already  
486 been validated to classify environmental hazards in different conditions characterized by  
487 greater complexity of contaminant mixtures, origin, typology and intensity of pollution.  
488 Such scenarios included highly and moderately contaminated sites from industrial areas,  
489 harbours, brackish environments, shallow-natural seepage and the recent Costa  
490 Concordia shipwreck (Piva et al., 2011; Benedetti et al., 2012, 2014; Regoli et al., 2014;  
491 Bebianno et al., 2015). The weighted criteria described in this work for elaboration and

492 integration of data on chemical and ecotoxicological characterization of sediments have  
493 been included in the new Italian Law on characterization and management of dredged  
494 sediments (DM 173, 15/07/2016). Despite the choice of more appropriate LOEs depends  
495 on local objectives and specificities, WOE procedures always provided an added value to  
496 quality characterization based on the use of single LOEs. WOE studies have been  
497 increasingly adopted for assessing the ecological status as required by actual European  
498 Directives, like the Water Framework Directive (WFD) and the Marine Strategy  
499 Framework Directive (MSFD).

500

#### 501 **4. Conclusions**

502 The mine tailings deposit off Portmán Bay has very high concentrations of metals of  
503 concern as As, Cd, Pb and Zn. The resuspension experiment of these sediments  
504 demonstrated that chemical contaminants are released from the sediments inducing  
505 ecotoxicological impact in mussels moored 3 meters above the seafloor. The integrated  
506 approach used in this study is useful to detect and quantify the environmental hazard  
507 posed by a mine tailings deposit, especially in case of sediment resuspension. The gills  
508 are in direct contact with seawater, being directly exposed to the toxic effects posed by  
509 the resuspension plumes, and are probably the most suitable tissue to investigate the short-  
510 term effects of exposure to the contaminated plume. Considering that Portmán Bay mine  
511 tailings deposit are a by-product of sulphide mining, and that polymetallic sulphides are  
512 important target for the deep-sea mining, it is likely that the plumes derived from mining  
513 activities will have a significant ecotoxicological impact on exposed marine fauna.  
514 However, prolonged field studies are needed to provide a more accurate assessment of  
515 the environmental hazards generated by deep-sea mining exploitation scenario, as the  
516 present study is not able to reveal the cumulative impact (more than 6 days) of exposure  
517 to resuspension plumes. Nevertheless, a similar approach to that used in this study could  
518 be applied in future deep-sea test mining in order to assess the environmental hazard for  
519 each exploitation area.

520

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536

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669

670

671 **Table captions**

672 **Table 1.** Metal concentrations in sediments ( $\mu\text{g g}^{-1}$ ) total (T D<sub>50</sub>) and non-biogenic (NB  
673 D<sub>50</sub>) sediments grain size and toxicity (EC<sub>50</sub> 15 min) from the 3 sites investigated in  
674 Portmán Bay. In addition, the limit values for the concentration of metals of concern for  
675 levels A-C in Spain established to allow dredged material to be dumped at sea are also  
676 provided (CIEM 2015). O - off the mine tailings deposit area; B - on the mine tailings  
677 deposit without the influence from the resuspension plume; P - under the influence of  
678 resuspension plume. EC<sub>50</sub> - concentration of sediment at which 50 % of the bacteria  
679 *Vibrio fischeri* luminescence decreased after 15 minutes (Microtox<sup>®</sup> bioassay). Mean  
680 (n=3) and standard deviations ( $\pm$ ) for the different metals are reported. bdl = below  
681 detection limits (detection limit for Au is  $10 \mu\text{g g}^{-1}$  and for Sb is  $5 \mu\text{g g}^{-1}$ ).

682

683 **Table 2.** Classification of environmental hazards at different sites in the Portmán Bay  
684 area according to the weight of evidence (WOE). Levels are summarized for the  
685 different lines of evidence (LOEs) and for their overall WOE integration. Spanish  
686 sediment quality guidelines (SQGs) have been considered for the elaborations (Action  
687 Levels A, B and C) G: Gills; DG: Digestive Gland; M: Mantle.

688

689

690 **Figure captions**

691 **Figure 1.** Contour map off Portman Bay. The dashed line delineates the submarine  
692 extension of the mine tailings deposit, defined after the analysis of high-resolution  
693 multibeam and seismic reflection data. Contours are every 5 m. The position of the  
694 moored cages are shown: Mooring\_UPM2 was deployed off the mine tailings deposit  
695 area (referred as “O” in the text), Mooring\_UPM3 was deployed on the mine tailings  
696 deposit without influence from resuspension plumes (referred as “B” in the text), and  
697 Mooring\_UPM1 was deployed under the influence of resuspension plumes (referred as  
698 “P” in the text). Trawling transects are also shown.

699

700 **Figure 2A-B.** Metal concentration of different mussel tissues (gills, digestive gland and  
701 mantle) after deployment in 3 different sites in Portmán Bay. O - off the mine tailings  
702 deposit area; B - on the mine tailings deposit without the influence from the  
703 resuspension plume; P - under the influence of resuspension plume. Different capital  
704 and lower case letters indicate significant differences between tissues within the same  
705 site and for the same tissue between sites, respectively ( $p>0.05$ ).

706

707 **Figure 3.** SOD, CAT, Se-I GPx, Se-D GPx, Total GPx, MT, GST and LPO in the  
708 different mussel tissues (gills, digestive gland and mantle) after deployment in 3  
709 different sites in Portmán Bay. O - off the mining deposit area; B - on the mining  
710 deposit without the influence from the sediment plume; P - under the influence of the  
711 artificially generated sediment plume. Different capital and lower case letters indicate  
712 significant differences between tissues within the same site and for the same tissue  
713 between sites, respectively ( $p>0.05$ ).

714 **Table 1.**

715

	Sites			Spanish legislation for dredged material		
	O	B	P	Level A	Level B	Level C
<b>Ag</b>	0.5 ± 0.1	1.4 ± 0.2	1.6 ± 0.2	-	-	-
<b>As</b>	31 ± 2	321 ± 35	299 ± 48	35	70	280
<b>Au</b>	bdl	38 ± 4	44 ± 8	-	-	-
<b>Cd</b>	0.9 ± 0.1	5.6 ± 0.5	5.8 ± 0.8	1.2	2.4	9.6
<b>Cr</b>	24 ± 2	48 ± 5	46 ± 7	140	340	1000
<b>Cu</b>	7 ± 1	11 ± 2	22 ± 3	70	168	675
<b>Hg</b>	0.2 ± 0.01	0.1 ± 0.01	0.2 ± 0.02	0.35	0.71	2.84
<b>Ni</b>	8 ± 1	32 ± 4	29 ± 4	30	63	234
<b>Pb</b>	148 ± 28	1259 ± 302	822 ± 164	80	218	600
<b>Sb</b>	bdl	28 ± 3	22 ± 3	-	-	-
<b>Zn</b>	335 ± 37	3772 ± 717	4054 ± 892	205	410	1640
<b>Fe</b>	2.1 ± 0.3 × 10 <sup>4</sup>	14.0 ± 2.5 × 10 <sup>4</sup>	12.5 ± 1.9 × 10 <sup>4</sup>	-	-	-
<b>T D<sub>50</sub> (µm)</b>	60.3	207.4	42.7	-	-	-
<b>NB D<sub>50</sub> (µm)</b>	42.3	190.9	48.4	-	-	-
<b>EC<sub>50</sub> 15 min (g L<sup>-1</sup>)</b>	2.1	35.0	2.8	-	-	-

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720 **Table 2.**

Sample	LOE1 (A)	LOE1 (B)	LOE1 (C)	LOE2	LOE3	LOE4	WOE (Level A)		WOE (Level B)		WOE (Level C)	
							WOE (Level A)		WOE (Level B)		WOE (Level C)	
<b>Off deposit</b>	Moderate	Absent	Absent	Absent (DG)	Absent (DG)	Slight	MODERATE		SLIGHT		ABSENT	
				Absent (G)	Absent (G)							
				Absent (M)	Absent (M)							
<b>On deposit</b>	Severe	Severe	Moderate	Absent (DG)	Slight (DG)	Absent	MODERATE		MODERATE		MODERATE	
				Slight (G)	Moderate (G)							
				Slight (M)	Absent (M)							
<b>Plume</b>	Severe	Severe	Slight	Absent (DG)	Moderate (DG)	Slight	MAJOR		MAJOR		MODERATE	
				Slight (G)	Major (G)							
				Slight (M)	Moderate (M)							

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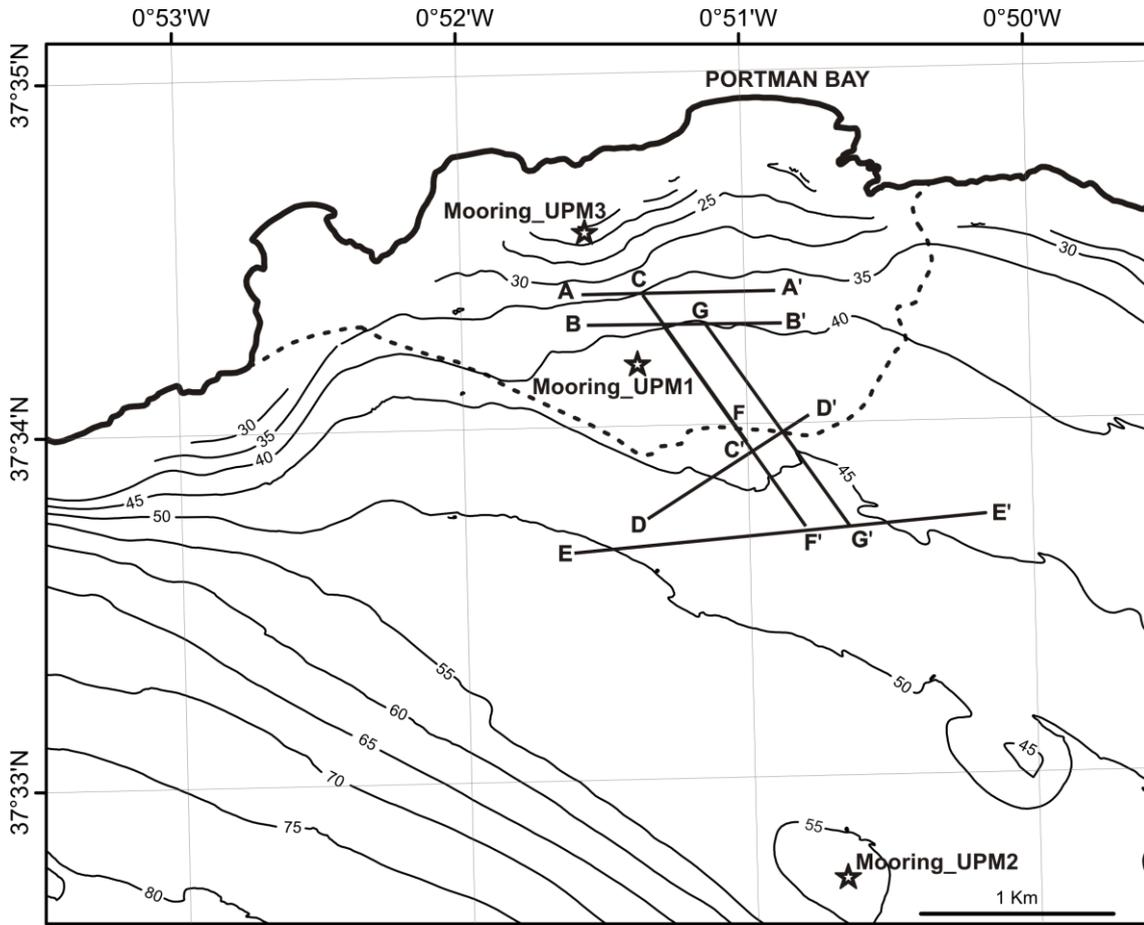
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726 **Fig. 1.**

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**Fig. 2A**

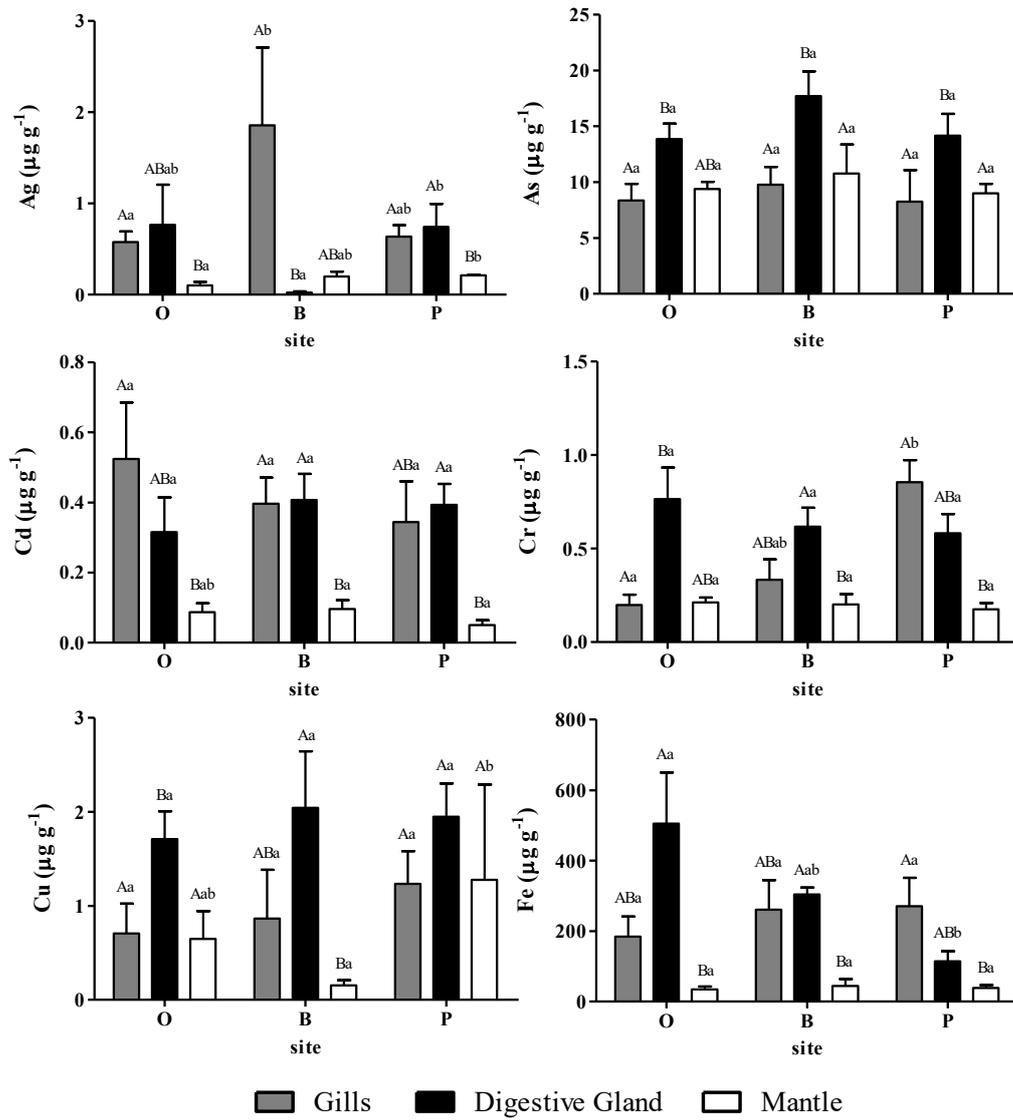
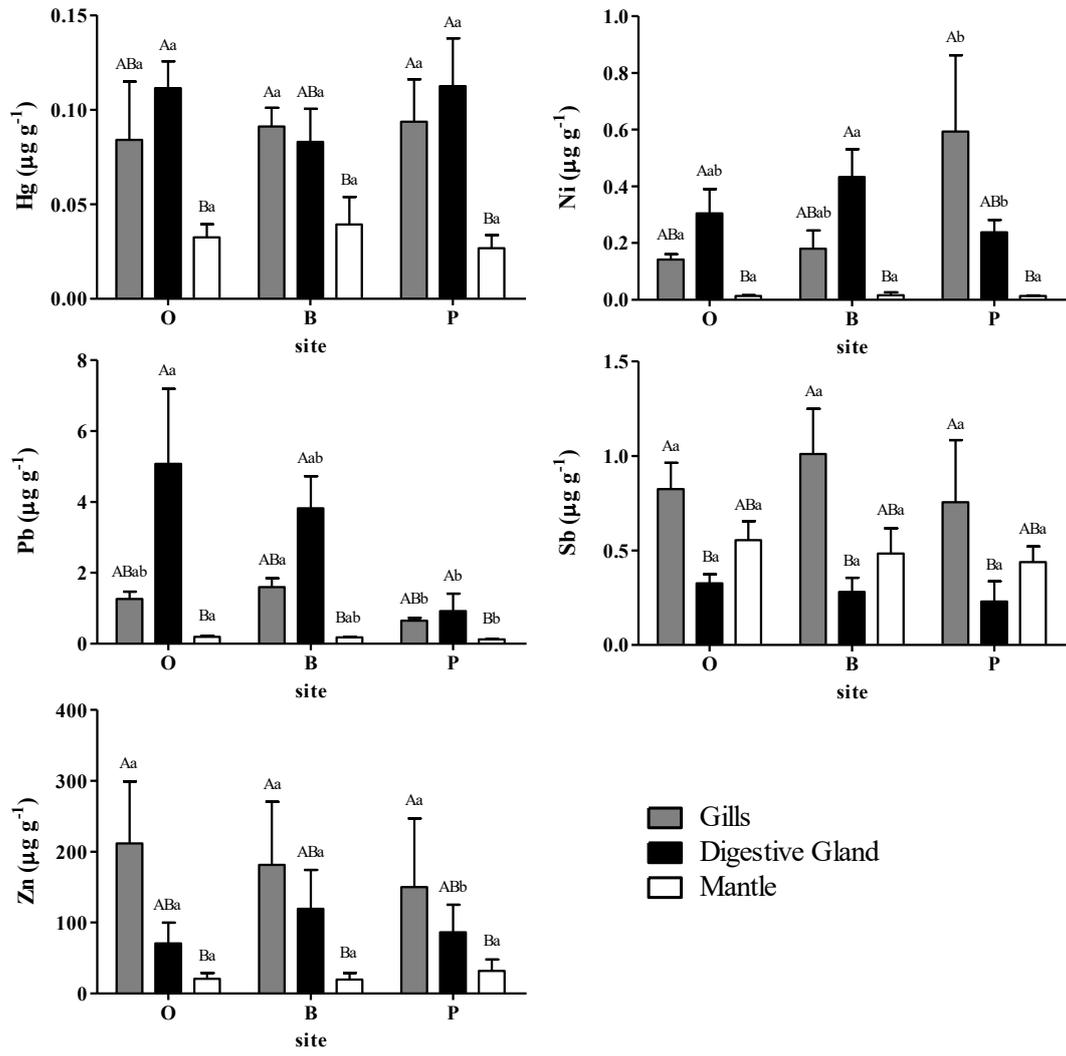


Fig. 2B



**Fig. 3**

