

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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56

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_2), 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% H₂O₂ 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:HNO₃ = 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 (ϵ) of $-50 \text{ M}^{-1} \text{ cm}^{-1}$
179 $^{-1}$; McCord and Fridovich 1969) and the results are expressed in U mg^{-1} of total protein.
180 CAT activity was determined as the decrease in absorbance for 1 min after the H_2O_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 H_2O_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 M ; 5 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 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 min and centrifuged for 45 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 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 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 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[®] 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 EC₅₀ (g L⁻¹) 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_w). 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_C) is calculated following the equation below
245 where an average RTR_w was obtained for all of the parameters with RTR ≤ 1 (i.e. below
246 normative limit), while the RTR_w 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 ≥ 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 (Σ) 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 (Σ) 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 (Σ) 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[®] 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 EC₅₀ ($r=0.99$, 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

521 **Acknowledgements**

522 This work was developed under the MIDAS project (Managing impacts of deep-sea
523 resource exploitation), funded by the European Commission 7th Framework Programme
524 under the theme “Sustainable management of Europe’s deep sea and sub-seafloor
525 resources” (Grant Agreement 603418). This work was also supported by: 1) the
526 Portuguese Science Foundation (FCT) through the grant UID/MAR/00350/2013
527 attributed to CIMA, University of Algarve; 2) the EU Horizon2020 project MERCES
528 (Grant agreement n. 689518); 3) the Spanish Ministry on Economy and Competitiveness
529 through research project NUREIEV (New challenges on submarine canyon research:
530 indicators of environmental status and spatiotemporal variability - The role of sea storms
531 - CTM2013-44598-R) allocated to the University of Barcelona (UB), which included ship
532 time; 4) the Government of Catalonia for sustaining the activities of GRC Geociències

533 Marines (grant 2014 SGR 1068) of its excellence research groups program at UB. We
534 further thank the captain and the crew of the R/V Ángeles Alvariño and R/V Ramon
535 Margalef.

536

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669

670

671 **Table captions**

672 **Table 1.** Metal concentrations in sediments ($\mu\text{g g}^{-1}$) total (T D₅₀) and non-biogenic (NB
673 D₅₀) sediments grain size and toxicity (EC₅₀ 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. EC₅₀ - concentration of sediment at which 50 % of the bacteria
679 *Vibrio fischeri* luminescence decreased after 15 minutes (Microtox[®] 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
Ag	0.5 ± 0.1	1.4 ± 0.2	1.6 ± 0.2	-	-	-
As	31 ± 2	321 ± 35	299 ± 48	35	70	280
Au	bdl	38 ± 4	44 ± 8	-	-	-
Cd	0.9 ± 0.1	5.6 ± 0.5	5.8 ± 0.8	1.2	2.4	9.6
Cr	24 ± 2	48 ± 5	46 ± 7	140	340	1000
Cu	7 ± 1	11 ± 2	22 ± 3	70	168	675
Hg	0.2 ± 0.01	0.1 ± 0.01	0.2 ± 0.02	0.35	0.71	2.84
Ni	8 ± 1	32 ± 4	29 ± 4	30	63	234
Pb	148 ± 28	1259 ± 302	822 ± 164	80	218	600
Sb	bdl	28 ± 3	22 ± 3	-	-	-
Zn	335 ± 37	3772 ± 717	4054 ± 892	205	410	1640
Fe	2.1 ± 0.3 × 10 ⁴	14.0 ± 2.5 × 10 ⁴	12.5 ± 1.9 × 10 ⁴	-	-	-
T D₅₀ (µm)	60.3	207.4	42.7	-	-	-
NB D₅₀ (µm)	42.3	190.9	48.4	-	-	-
EC₅₀ 15 min (g L⁻¹)	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)	
Off deposit	Moderate	Absent	Absent	Absent (DG)	Absent (DG)	Slight	MODERATE		SLIGHT		ABSENT	
				Absent (G)	Absent (G)							
				Absent (M)	Absent (M)							
On deposit	Severe	Severe	Moderate	Absent (DG)	Slight (DG)	Absent	MODERATE		MODERATE		MODERATE	
				Slight (G)	Moderate (G)							
				Slight (M)	Absent (M)							
Plume	Severe	Severe	Slight	Absent (DG)	Moderate (DG)	Slight	MAJOR		MAJOR		MODERATE	
				Slight (G)	Major (G)							
				Slight (M)	Moderate (M)							

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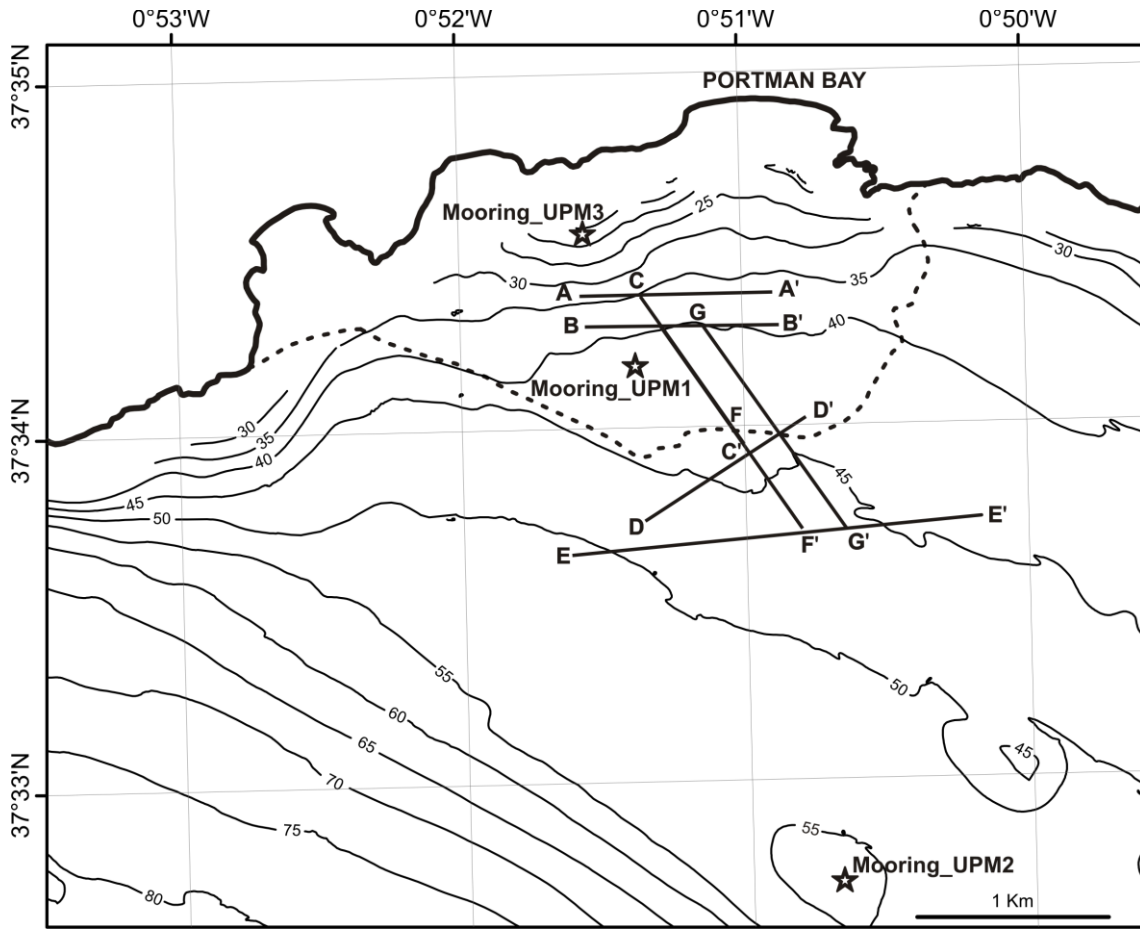
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726 **Fig. 1.**

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Fig. 2A

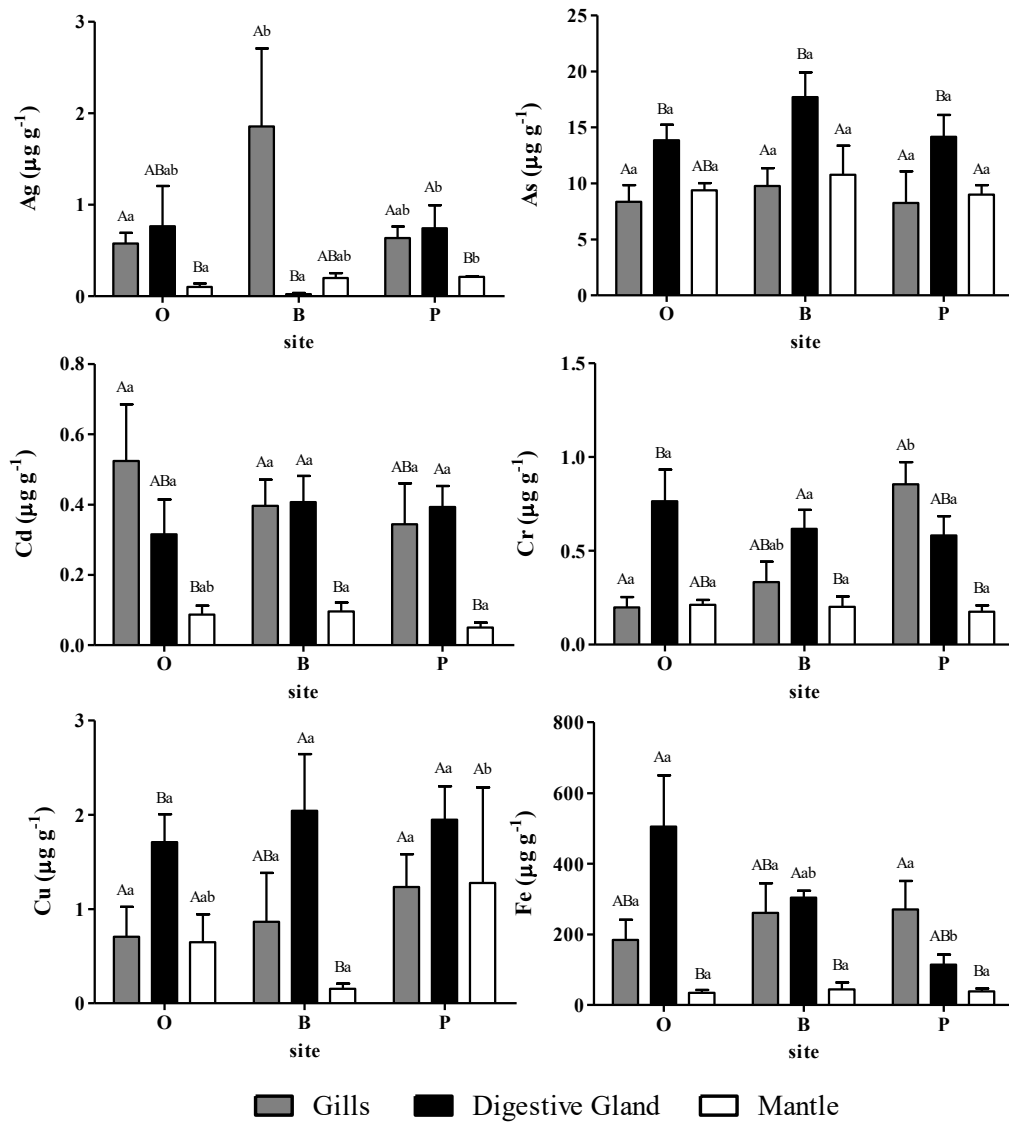


Fig. 2B

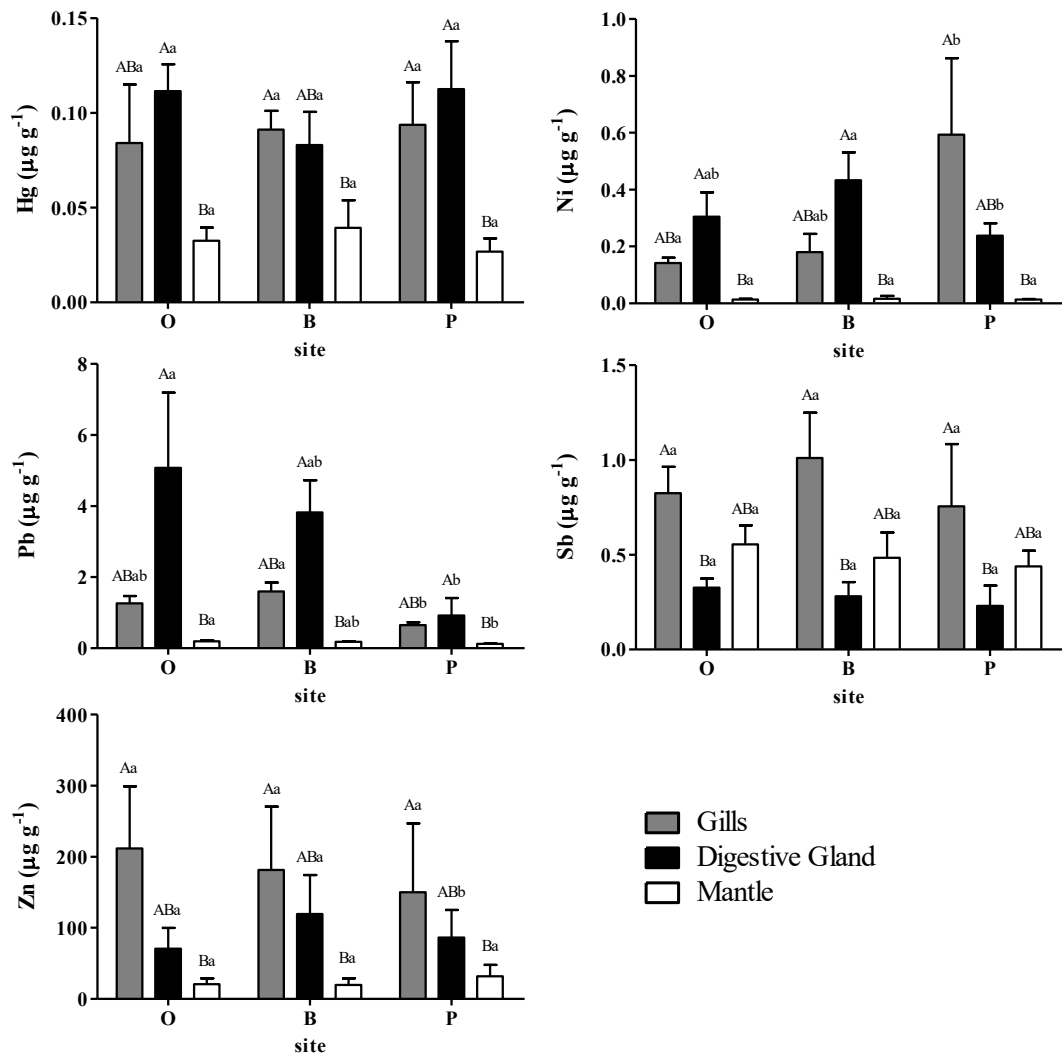


Fig. 3

