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

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Abstract

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

Capsule

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

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

1. Introduction

Portmán Bay is a heavily impacted area resulting from decades of metal mine tailings disposal that lasted until 1990. Minerals extracted in the Portmán mining district were mainly pyrite (FeS_2), galena (PbS) and sphalerite (ZnS), which were mechanically treated for concentration of metals, with about 95% of mine tailings waste generated (Martínez-Sánchez et al. 2008, Oyarzun et al. 2013). About 60 Mt of tailings were dumped into the sea, moving the shoreline seaward about 500-600 m and reaching the continental shelf off Portmán Bay (Manteca et al. 2014). The mine tailings deposit has a maximum thickness of about 14 m and is composed of fine sediments highly enriched with metals (mainly Fe, Zn, As and Pb, with metal concentrations 10 to 60 times higher than coastal sediments in the Mediterranean Sea). Above the deposit there is a thin layer of approximately 10-20 cm of coarse sediments reworked by natural (waves) and anthropogenic (bottom trawling) processes, with metal concentrations 10 to 20 times higher than unpolluted sediments (Cerdà-Domènech et al., in prep).

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

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

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

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

2. Materials and methods

2.1. Sediments resuspension experiment and sampling sites

In the summer of 2014, the MIDAS-Portmán research cruise was conducted in Portmán Bay and in its adjacent marine area (Murcia, SE Spain) on board of the Spanish research vessels R/V Ángeles Alvariño and R/V Ramon Margalef. Transects of bottom trawling off Portmán Bay (Fig. 1) were carried out to resuspend the sediments and originate plumes, being usually less than 10 m in height, with a variable though relatively quick decline and limited dispersal, and a maximum tracking time for a given plume of about 4 hours (Canals et al. 2016). Before the resuspension events, a transplant monitoring experiment was carried out with caged mussels *M. galloprovincialis* obtained from a mussel farm (Cademar) located on the Ebro Delta. Mussels (length 5.0-6.5 cm; width 1.7-3.5cm; wet weight 20-37g) were deployed at about 3 m above the seafloor in the following three sites (Fig. 1): off the mine tailings deposit area (Mooring_UPM2, hereafter "O", 37° 32.713' N 0° 50.684' W, 57 m); on the mine tailings deposit without the influence from the resuspension plumes (Mooring_UPM3, hereafter "B", 37° 34.553' N 0° 51.563' W, 17 m); under the influence of the artificially generated resuspension plumes (Mooring_UPM1, hereafter "P", 37° 34.177' N 0° 51.386' W, 42 m). After 6 days of exposure, cages were retrieved on board and mussels from each site were immediately dissected and tissues (gills, digestive gland and mantle) were flash frozen and preserved at -80 °C for chemical and biomarker analyses. Surface sediment samples (top 1 cm) were also collected at the same sites and frozen at -20 °C until further analysis.

2.2. Sediments grain size analyses

Sediment grain size was determined using a Coulter LS230 Laser Diffraction Particle Size Analyser. Samples were oxidized with a 10% H_2O_2 solution to remove organic matter, and one subsample analysed and another treated with 1M HCl to remove carbonates. The total and non-biogenic grain size distribution determined and data analysed with GradiStat®.

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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_3 = 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).

SOD activity was determined by the reduction of cytochrome c by the xanthine oxidase/hypoxanthine system at 550 nm (molar extinction coefficient (ε) of -50 M^{-1} cm^{-1}; McCord and Fridovich 1969) and the results are expressed in U mg^{-1} of total protein. CAT activity was determined as the decrease in absorbance for 1 min after the H_2O_2 consumption at 240 nm (ε = -40 M^{-1} cm^{-1}; Greenwald 1985) with results being expressed as µmol min^{-1} mg^{-1} of total protein. GPx activities were assessed by following for 5 min the NADPH oxidation in the presence of excess glutathione reductase, reduced glutathione and cumene hydroperoxide (Se-I GPx) or H_2O_2 (Se-D GPx) as substrate at 340 nm (ε = -6.22 mM^{-1} cm^{-1}; Flohe and Gunzler 1984 and adapted to a microplate reader by McFarland et al. 1999). Total GPx activity refers to the sum of Se-I GPx and Se-D GPx activities and results are expressed as nmol min^{-1} mg^{-1} of total protein. GST activity was measured by following the conjugation of reduced glutathione (GSH) with 1-chloro 2,4 dinitrobenzene (CDNB) at 340 nm for 1 min (ε = -9.6 mM^{-1} cm^{-1}; Habig et al. 1974) and results are expressed as µmol min^{-1} mg^{-1} of total protein.

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

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

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

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

2.5. Sediment bioassays

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

sample containing toxic materials. During the test the *V. fischeri* are in direct contact with the sample particles, increasing the probability for the measurement of the responses to particle bound and marginally soluble toxicants. Each test consists of 2 controls and 13 sample serial dilutions in duplicate, luminescence data is analysed with the MicrotoxOmni software (Azur Environmental). The toxicity endpoint is the luminescence inhibition EC_{50} (g L^{-1}) at 15 min (Azur Environmental 1998).

2.6. Statistical analyses

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

2.7. Weight of evidence elaboration (WOE) model

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

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

The Hazard Quotient for chemistry (HQ_C) is calculated following the equation below where an average RTR_W was obtained for all of the parameters with RTR ≤ 1 (i.e. below normative limit), while the RTR_W was individually added into the summation Σ for those with RTR > 1 (Eq. 1; Piva et al. 2011).

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

Eq.1

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

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

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

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

Eq. 2

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

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

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

Eq. 3

The hazard level related to the LOE4 – ecotoxicological bioassays is determined by the cumulative hazard quotient ($HQ_{Battery}$). $HQ_{Battery}$ is calculated by the summation (Σ) of the weighted effects ($Effect_W$), that correspond to the variations measured for each test compared to specific thresholds (corrected for the statistical significance of the difference (w)), the biological importance of the endpoint of each test and the exposure conditions ($w2$; Eq. 4).

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

Eq. 4

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

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

3. Results and discussion

3.1. Sediment analyses

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

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

3.2. Bioaccumulation of metals in mussels

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

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

3.3. Biomarkers

SOD activity was significantly lower in the gills of mussels from the mine tailings deposit (B) when compared to the area off the mine tailings (O), while in the mantle SOD was significantly lower in P when compared to O ($p<0.05$; Fig. 3). A significantly higher CAT activity was noticed in the gills in P when compared to B, while in the mantle a significantly higher CAT activity was noted in P when compared to O ($p<0.05$). In the digestive gland, a significant induction of Se-I GPx activity in P was noted when compared to both O and B, while in the gills and mantle a significant increase was observed in P only when compared to B ($p<0.05$). P induces a significantly higher Se-D 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 EC_{50} (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 (< 63µm; 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

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

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

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

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

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

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

4. Conclusions

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

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Table captions

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

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

Figure captions

Figure 1. Contour map off Portman Bay. The dashed line delineates the submarine extension of the mine tailings deposit, defined after the analysis of high-resolution multibeam and seismic reflection data. Contours are every 5 m. The position of the moored cages are shown: Mooring_UPM2 was deployed off the mine tailings deposit area (referred as "O" in the text), Mooring_UPM3 was deployed on the mine tailings deposit without influence from resuspension plumes (referred as "B" in the text), and Mooring_UPM1 was deployed under the influence of resuspension plumes (referred as "P" in the text). Trawling transects are also shown.

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

Figure 3. SOD, CAT, Se-I GPx, Se-D GPx, Total GPx, MT, GST and LPO in the different mussel tissues (gills, digestive gland and mantle) after deployment in 3 different sites in Portmán Bay. O - off the mining deposit area; B - on the mining deposit without the influence from the sediment plume; P - under the influence of the artificially generated sediment plume. Different capital and lower case letters indicate significant differences between tissues within the same site and for the same tissue 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	$2.1 \pm 0.3 \times 10^4$	$14.0 \pm 2.5 \times 10^4$	$12.5 \pm 1.9 \times 10^4$	-	-	-
T D_{50} (µm)	60.3	207.4	42.7	-	-	-
NB D_{50} (µm)	42.3	190.9	48.4	-	-	-
EC_{50} 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)	
Off deposit	Moderate	Absent	Absent	Absent (DG) Absent (G) Absent (M)	Absent (DG) Absent (G) Absent (M)	Slight	MODERATE		SLIGHT		ABSENT	
On deposit	Severe	Severe	Moderate	Absent (DG) Slight (G) Slight (M)	Slight (DG) Moderate (G) Absent (M)	Absent	MODERATE		MODERATE		MODERATE	
Plume	Severe	Severe	Slight	Absent (DG) Slight (G) Slight (M)	Moderate (DG) Major (G) Moderate (M)	Slight	MAJOR		MAJOR		MODERATE	

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724

725

Fig. 1.

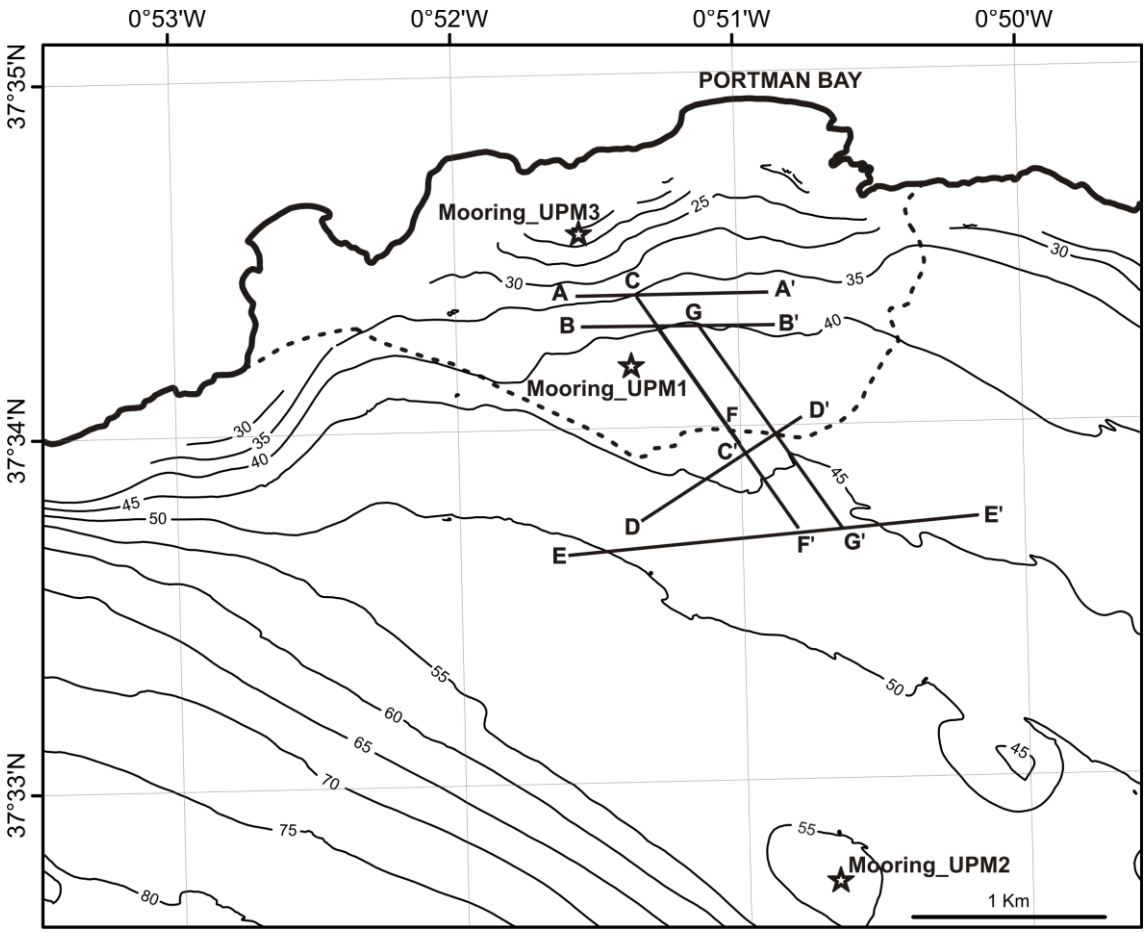

0°53'W
0°52'W
0°51'W
0°50'W
37°35'N
37°34'N
37°33'N
PORTMAN BAY
Mooring_UPM3
Mooring_UPM1
Mooring_UPM2
A
A'
B
B'
C
C'
D
D'
E
E'
F
F'
G
G'
25
30
35
40
45
50
55
60
65
70
75
80
1 Km

Fig. 2A

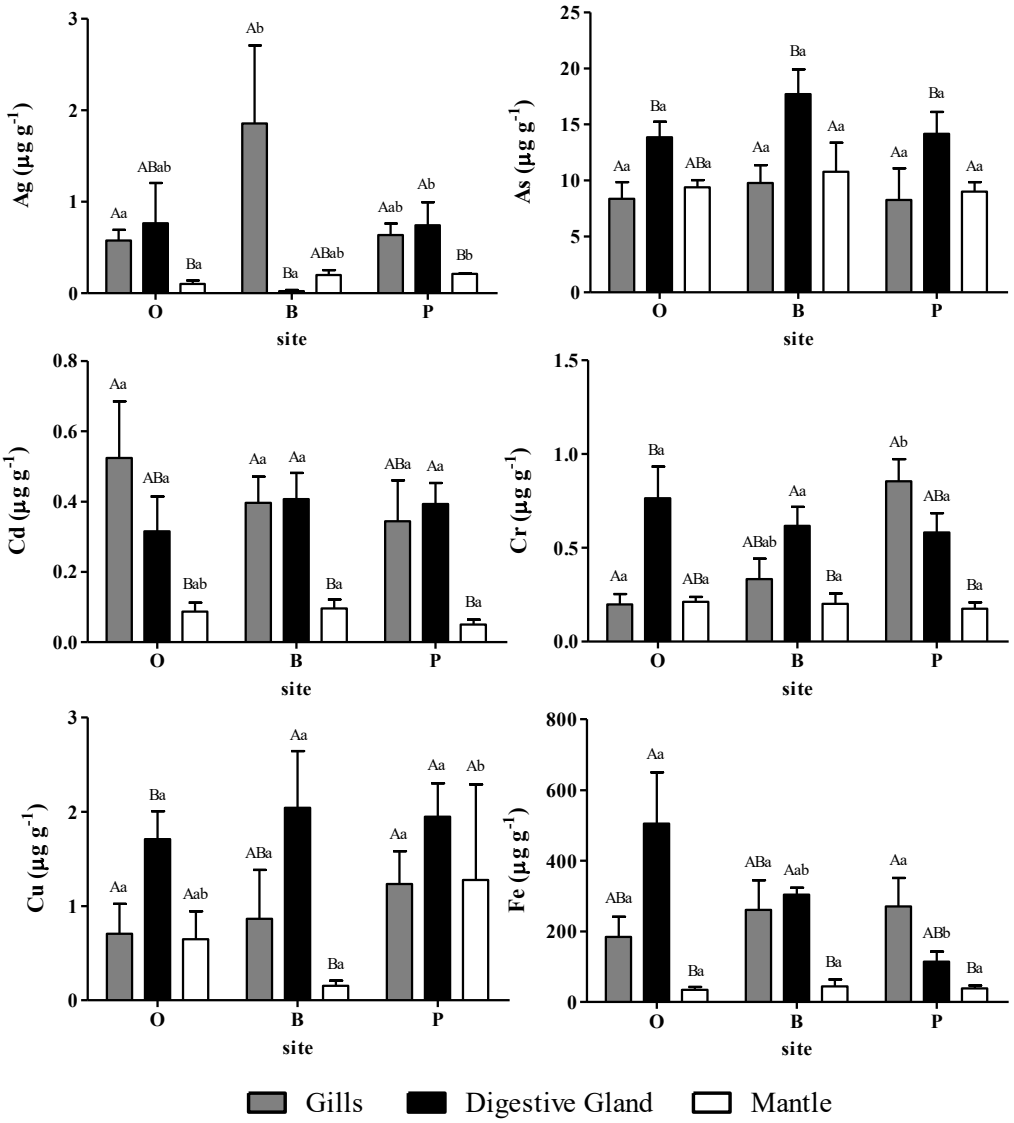

Ag (µg g-1)
As (µg g-1)
Cd (µg g-1)
Cr (µg g-1)
Cu (µg g-1)
Fe (µg g-1)
site
O
B
P
Gills
Digestive Gland
Mantle

Fig. 2B

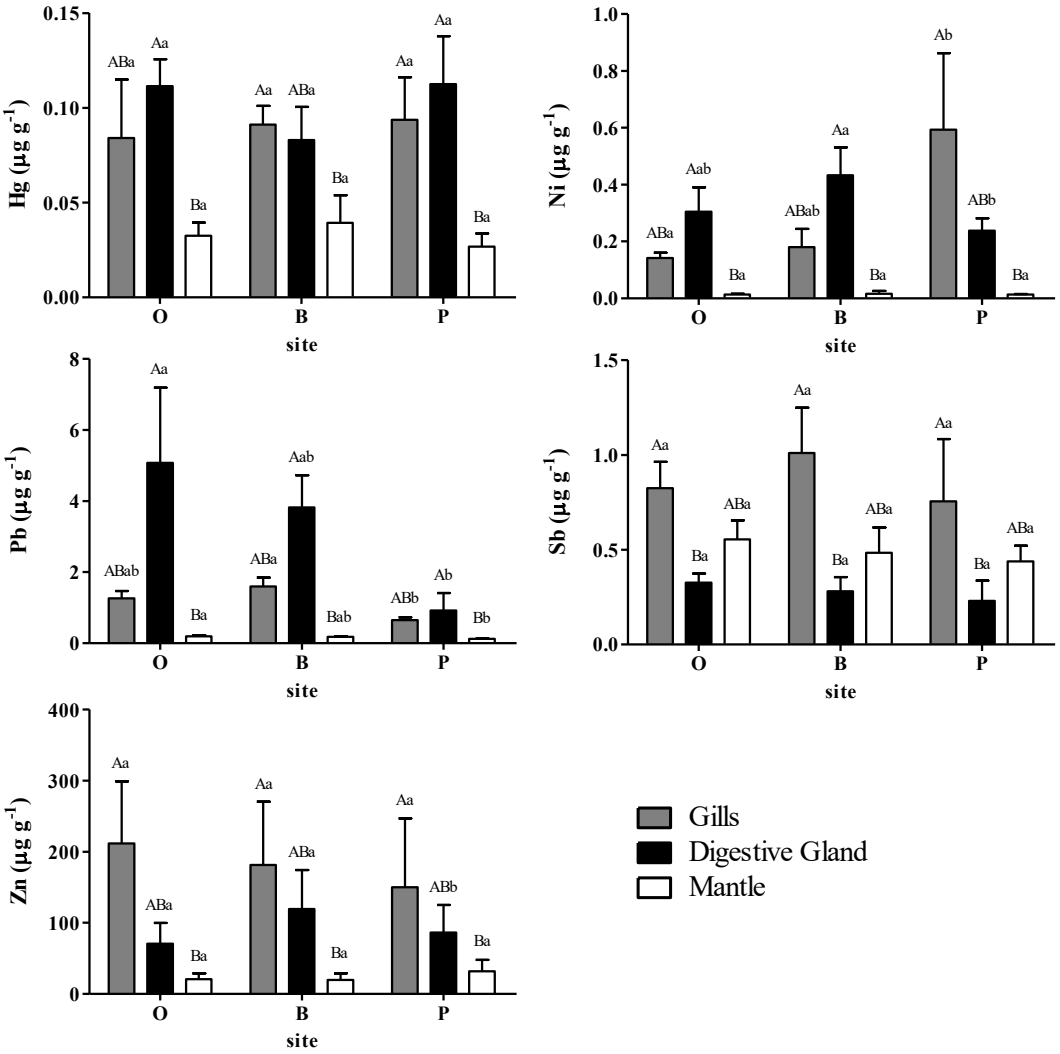
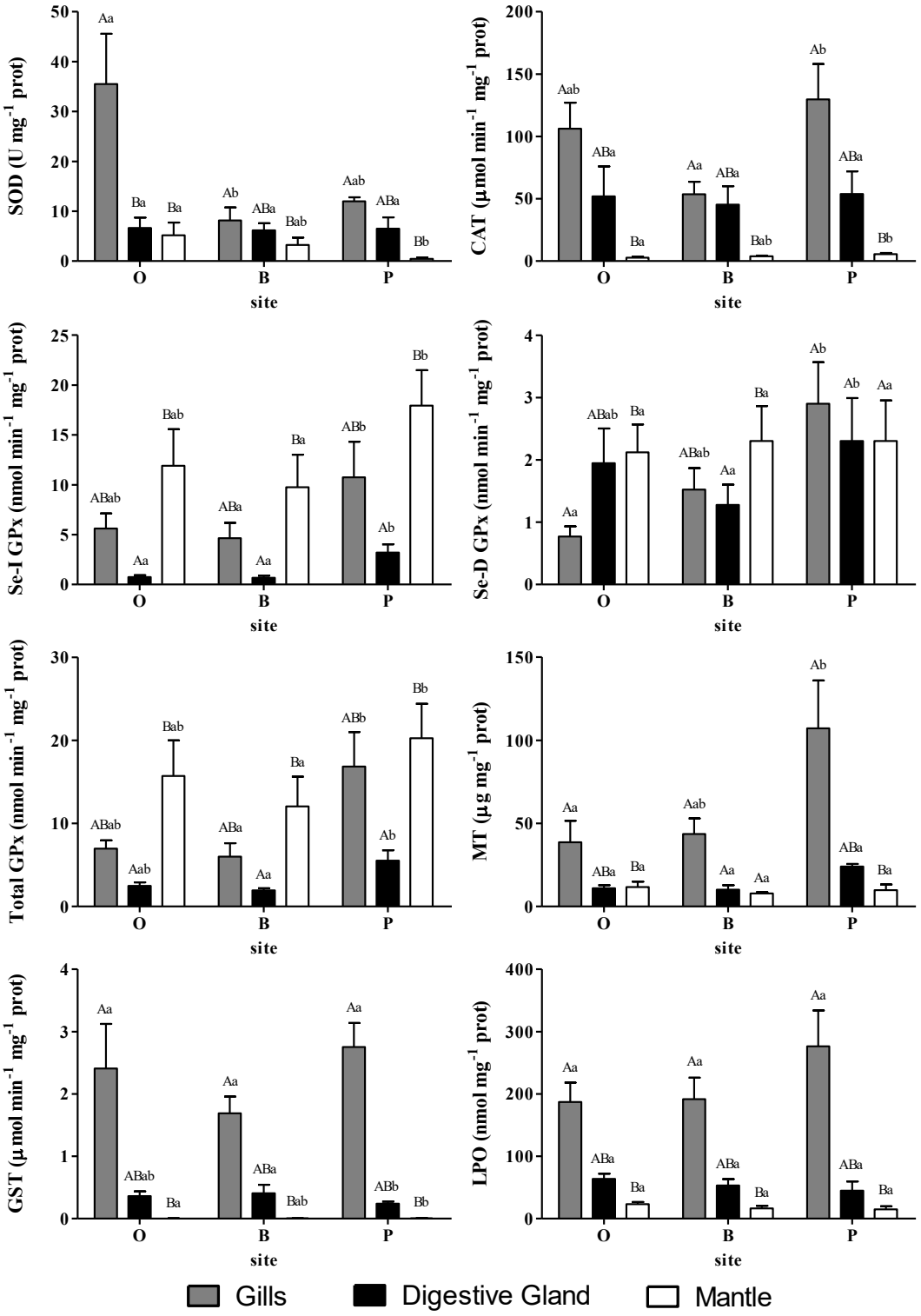

Hg (µg g-1)
0.15
0.10
0.05
0.00
ABa
Aa
Ba
Aa
ABa
Ba
Aa
Aa
Ba
O
B
P
site
Ni (µg g-1)
1.0
0.8
0.6
0.4
0.2
0.0
ABa
Aab
Ba
ABab
Aa
Ba
Ab
ABb
Ba
Pb (µg g-1)
8
6
4
2
0
ABab
Aa
Ba
ABa
Aab
Bab
ABb
Ab
Bb
Sb (µg g-1)
1.5
1.0
0.5
0.0
Aa
Ba
ABa
Aa
Ba
ABa
Aa
Ba
ABa
Zn (µg g-1)
400
300
200
100
0
Aa
ABa
Ba
Aa
ABa
Ba
Aa
ABb
Ba
Gills
Digestive Gland
Mantle

Fig. 3


SOD (U mg-1 prot)
CAT (µmol min-1 mg-1 prot)
Se-I GPx (nmol min-1 mg-1 prot)
Se-D GPx (nmol min-1 mg-1 prot)
Total GPx (nmol min-1 mg-1 prot)
MT (µg mg-1 prot)
GST (µmol min-1 mg-1 prot)
LPO (nmol mg-1 prot)
site
O
B
P
Gills
Digestive Gland
Mantle