Are shallow-water shrimps proxies for hydrothermal-vent shrimps to assess the impact of deep-sea mining?

N.C. Mestre[a*], M. Auguste[a], L.C. de Sá[a], T.G. Fonseca[a], C. Cardoso[a], A. Brown[b], D. Barthelemy[c], N. Charlemagne[d], C. Hauton[b], J. Machon[d], J. Ravaux[d], B. Shillito[d], S. Thatje[b], M.J. Bebianno[a]

[a]Centre for Marine and Environmental Research (CIMA), Universidade do Algarve, Campus Universitário de Gambelas, 8005-139 Faro, Portugal.

[b]Ocean and Earth Science, University of Southampton, National Oceanography Centre, Southampton, SO14 3ZH, UK.

[c]Océanopolis, Port de Plaisance du Moulin Blanc BP 91039, 29210 Brest Cedex1, France

[d]Sorbonne Universités, UPMC Univ Paris 06, UMR CNRS 7208, MNHN/IRD-207/UCN/UA, Biologie des Organismes et Ecosystèmes Aquatiques", 7 Quai St Bernard, 75252 Cedex 5 Paris, France.

[*]Corresponding author at: N.C. Mestre, Tel.: +351 289800100; Fax: + 351 289800069.

E-mail address: neliamestre@gmail.com; ncmestre@ualg.pt

ABSTRACT

Polymetallic seafloor massive sulphide deposits are potential targets for deep-sea mining, but high concentrations of metals (including copper - Cu) may be released during exploitation activities, potentially inducing harmful impact. To determine whether shallow-water shrimp are suitable ecotoxicological proxies for deep-sea hydrothermal vent shrimp the effects of waterborne Cu exposure (3 and 10 days at 0.4 and 4 μM concentrations) in *Palaemon elegans, Palaemon serratus*, and *Palaemon varians* were compared with *Mirocaris fortunata*. Accumulation of Cu and a set of biomarkers were analysed. Results show different responses among congeneric species indicating that it is not appropriate to use shallow-water shrimps as ecotoxicological proxies for deep-water shrimps. During the evolutionary history of these species they were likely subject to different chemical environments which may have induced different molecular/biochemical adaptations/tolerances. Results highlight the importance of

analysing effects of deep-sea mining *in situ* and in local species to adequately assess ecotoxicological effects under natural environmental conditions.

Keywords: deep-sea mining; ecotoxicology; biomarkers; *Mirocaris fortunata*; *Palaemon*.

1. Introduction

The worldwide consumption of mineral raw materials is increasing and many mineral elements are essential components of low carbon technologies (Moss et al. 2011, Kopf et al. 2012). Recycling is not yet available at sufficient scale to meet manufacturing demands and therefore pressure exists to find new exploitable resources. Deep-sea mineral deposits (seafloor massive sulphides, polymetallic nodules and ferromanganese crusts) are now considered to have significant potential for technologically and economically viable exploitation (Kopf et al. 2012). However, any economic cost-benefit analysis of deep-sea resource exploitation needs to constrain the scale of environmental impact to accurately quantify and value the ecosystem services that might be compromised, as well as identify potential mitigation measures that may be implemented.

Besides removing the habitat locally where the mining operations will take place, localized sediment plumes of complex mixtures of potentially toxic elements are likely to form, exposing local fauna to metals released into the water column, either in mineral form or as dissolved metal ions (Simpson and Spadaro 2016). In addition, dewatering ore slurry may have impacts on the euphotic zone, midwater or near the seafloor, depending on the discharge depth of the waste produced, affecting the ecosystem services provided by the different water column layers (Hauton et al. 2017, Drazen et al. 2019). Moreover, natural environmental conditions of the deep sea, where high hydrostatic pressures and low temperatures prevail, are crucial considerations when assessing the ecotoxicological impacts from deep-sea resource exploitation, limiting the usefulness of toxicity thresholds already found for shallow-water species (Mestre et al. 2014, Brown et al. 2017a, Mevenkamp et al. 2017). Current knowledge regarding ecotoxicological thresholds, life cycle or connectivity of deep-sea species, or on deep-sea ecosystem functioning is scarce, as is knowledge of how at risk ecosystem services will be managed and/or regulated. Nonetheless, it is acknowledged that species' resilience to impacts will be influenced by

65 their evolved physiological capacity to resist toxic element exposures (Gollner et al.
66 2017), highlighting the need to understand toxic mechanism in appropriate high-pressure
67 adapted physiologies (e.g. Brown et al. 2018).

68 Copper is one of the most abundant metals in seafloor massive sulphides, reaching
69 over 20 % of their composition in some sites (e.g. German et al. 2016). Therefore, it is
70 likely that dissolved Cu will increase in the areas adjacent to mining activities. When total
71 dissolved metal concentration increases in the aquatic environment, metal uptake rates by
72 organisms increase (Rainbow 1998). Although Cu naturally occurs in cells and tissues
73 and is a cofactor of some enzymes, it is a known toxicant when in excess in organisms
74 (e.g. Gaetke and Chow 2003). Increased uptake is accompanied by the formation of
75 reactive oxygen species (ROS) in cells leading to the activation of different cellular
76 mechanisms. For example, the antioxidant defence may be stimulated, comprising
77 enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione
78 peroxidase (GPx), which are able to constrain ROS levels and thus prevent oxidative
79 damage (Di Giulio et al. 1995, Gaetke and Chow 2003). When metal levels result in ROS
80 formation exceeding antioxidant capacity, lipid peroxidation (LPO) of polyunsaturated
81 fatty acids is expected to occur (Halliwell and Gutteridge 1984). Similarly, metal-binding
82 proteins such as metallothioneins (MTs) may be induced, which can counteract metal
83 accumulation in cells.

84 Metal accumulation and toxicity have been investigated in deep-water fauna from
85 the naturally occurring high-metal concentration hydrothermal vent environment, such as
86 in the mussel *Bathymodiolus azoricus* and in the shrimp *Rimicaris exoculata* (Company
87 et al. 2004, 2006a,b, 2007, 2008, Bebianno et al. 2005). Metal exposure experiments with
88 the deep-sea holothurian *Amperima sp.* have also been conducted *in situ* (Brown et al.
89 2017b), while other studies have analysed metal toxicity of deep-sea species under
90 laboratory-controlled conditions including high-pressure (Company et al. 2006a, Auguste
91 et al. 2016, Martins et al. 2017). Experiments were also conducted at surface pressure for
92 some deep-sea species such as the cold-water coral *Dentomoricea meteor* (Martins et al.
93 2018) and the eurybathic brittle star *Amphipholis squamata* (Black et al. 2015). Other
94 experiments were conducted at deep-sea and/or surface pressures for shallow-water
95 relatives of deep-sea fauna as an attempt to identify proxy shallow-water species that
96 reflect the effects of their deep-water counterparts (Brown et al. 2017a,b, Mevenkamp et
97 al. 2017, Brown & Hauton 2018). However, it is difficult to compare these studies, and

extract common patterns in terms of ecotoxicological effects given the phylogenetic distance, physiological differences, or different exposure conditions. Thus, it seems pertinent to investigate ecotoxicological effects among a close phylogenetic group, which include both shallow-water and deep-sea species, using similar exposure conditions as an attempt to identify common patterns and/or key physiological traits responsible for identified differences.

The aim of this study was to assess and compare the effects of waterborne Cu (0.4 and 4 µM Cu) exposure in the deep-sea hydrothermal vent shrimp *M. fortunata* and in the shallow-water shrimp *P. elegans, P. serratus* and *P. varians*. For this, the accumulation of Cu in different tissues (gills, hepatopancreas and muscle) as well as a set of biomarkers – oxidative stress (superoxide dismutase - SOD, catalase - CAT, glutathione peroxidase - GPx), metal exposure (metallothioneins), biotransformation (glutathione-S-transferases - GST) and oxidative damage (lipid peroxidation - LPO) – were analysed after 3 and 10 days of exposure. The selected Cu concentrations (0.4 µM = 25 µg L^{-1}; 4 µM = 254 µg L^{-1}) are in the range of the levels obtained for dissolved Cu released after 30 min in field-based and lab-based elutriate tests performed with fragments of deep-sea massive sulphide deposits as part of the environmental impact study of Solwara 1 mining project at Papua New Guinea (Nautilus EIS, Simpson et al. 2008). The gills, hepatopancreas and muscle tissues were chosen to enable a comparison with previous studies, including Auguste et al. 2016, but also because different tissues are sensitive to the accumulation of metals in different ways and some metals can be translocated to different tissues (e.g. White and Rainbow 1982, Pourang et al. 2004). The natural habitat distribution depth, of the investigated species, has been recorded between 840 - 3875 m for *M. fortunata* (Desbruyères et al. 2000), from the surface down to 20 and 40 m for *P. elegans* (Kotta and Kuprigenov 2012) and *P. serratus* (Holthuis et al. 1980) respectively, and in shallow brackish waters of coastal lagoons for *P. varians* (Barnes et al. 1994).

2. Materials and methods

2.1. Sample collection and maintenance

Sampling of *M. fortunata* specimens (2.1 – 2.5 cm body length) took place in 2013 during the Biobaz cruise, on board the oceanographic ship "Pourquoi Pas?", using the Remotely Operated Vehicle (ROV) Victor 6000 (IFREMER) at the Lucky Strike vent

field (MAR, 37°17'N, ~ 1750 m depth). Specimens were sampled using a suction device operated by the hydraulic arm on the submersible. Immediately after recovery on board the ship, the shrimps were transferred to tanks of approximately 5 – 10 L of aerated seawater in a cold room (5 – 9 °C) at surface pressure, in groups of a few individuals (< 5). At the end of the cruise, shrimps were landed in the Azores (Horta, Portugal) and further shipping of the animals to Océanopolis aquarium (Brest, France) was achieved by air freight by "Flying Sharks" (Lisbon, Portugal), a company specialized in the transport of live marine fauna. The shrimps were stored in groups of 20 – 25 in sealed plastic bags containing seawater and pure oxygen. The journey lasted about 24 hours (Shillito et al. 2015). Once at Océanopolis, shrimps' husbandry was performed by aquariology staff members of the aquarium. The shrimps were maintained at atmospheric pressure in a dark room (10 °C) in groups of around 50 in flow-through 80 L tanks, each equipped with one 24 °C heating element. This heater was placed near the surface to avoid water temperature homogenization by convection, therefore providing a local "hotspot" with respect to the surrounding 10 °C environment. Shrimps were kept for >1 year at 10 ºC and 0.1 MPa in these aquaria, and were fed every 4 – 5 days with Liptoaqua food pellets (Liptosa, Madrid, Spain; Shillito et al. 2015).

P. elegans (2.5 – 3.4 cm body length) were collected by hand nets in the coastal waters near Brest (France; 48°23'N, 4°25'W), and kept at Oceanopolis for 2 months before exposure, at 10 ºC and 0.1 MPa in flow-through 80 L tanks, in light:dark 12h:12h cycle, and fed every 3 days with Liptoaqua food pellets (Liptosa, Madrid, Spain).

P. varians (4 – 5 cm body length) were collected by hand net from Lymington salt marshes (Hampshire, UK; 50°45'N, 1°32'W) in May 2015. *P. serratus* (4.5 – 6.0 cm body length) were collected by hand net from Calshot (Hampshire, UK; 50°81'N, 1°32'W) during low tide on the same day. Shrimps were maintained at the National Oceanography Centre Southampton (NOCS) in a flow-through system with controlled salinity (~32) and temperature (15 °C), in a light:dark 12h:12h cycle for at least 1 month, and fed with excess food three times per week with Tetra Goldfish flakes. Seven days before exposure shrimps were transferred to 10 L PVC tanks with artificial seawater, continuous aeration and at 10 ºC and 0.1 MPa, with partial water changes every 3 days, and were starved for 3 days before exposure.

2.2. Cu exposure experiments

All shrimps were exposed to three treatments at 10 ºC and surface pressure (0.1 MPa): control (seawater only), 0.4 µM of Cu and 4 µM of Cu. Before the exposure (day 0), specimens were sampled and dissected (gills, hepatopancreas and muscle; n = 5 for *M. fortunata* and *P. elegans*; n = 6 for *P. varians* and *P. serratus*). The Cu exposure experiments were divided into 3 experiments, with experiment 1 and 2 performed at the Oceanopolis, Brest, France, while experiment 3 was performed at NOC, Southampton, UK.

Experiment 1 – M. fortunata and *P. elegans* (n = 10 per species and per treatment) were exposed for 3 days inside 40 L tanks, 1 tank per treatment, with 50% water renewal in all treatments every day.

Experiment 2 – M. fortunata and *P. elegans* (n = 10 per species and per treatment) were exposed for 10 days inside 40 L tanks, 1 tank per treatment, with 50% water renewal in all treatments every day.

Experiment 3 – P. varians and *P. serratus* (n = 6 per species and per treatment) were incubated inside 6 L PVC plastic barrels in the high-pressure aquarium (IPOCAMP) (Shillito et al. 2014) at surface pressure (0.1 MPa) for 3 days following the protocol of Auguste and colleagues (2016). In all treatments 100% of water was changed every 12 h.

Shrimp survival was nearly 100% throughout the exposure duration, with only one *P. elegans* specimen found dead at day 9 in the control and one specimen found dead at day 8 in the 0.4 µM Cu exposure, and one *M. fortunata* specimen found dead at day 6 in the 4 µM Cu exposure. At the end of exposure, shrimps were dissected to separately preserve gills, hepatopancreas and muscle and flash frozen in liquid nitrogen and stored at -80 ºC until further analyses.

2.3. Tissue preparation

Individual tissue samples were weighed and homogenized at 4 °C in a Tris-HCl (0.02 M, 5 mL g^{-1} soft tissue) buffer with butylated hydroxytoluene (BHT, 10 µl mL^{-1}), pH 8.6. The homogenate (3 mL) was separated into soluble and insoluble fractions by centrifugation (30 000*g*, 30 min, 4 °C), and the remaining homogenate (~2 mL) was preserved at -20 ºC for later determination of metal concentrations. After centrifugation, a part of the supernatant was preserved at -80ºC for posterior measurement of LPO and total protein content. A second centrifugation (30 000*g*, 30 min, 4 °C) separated the low

195 molecular weight proteins, and the supernatant was preserved at -20 ºC for
196 metallothionein analysis (MT) (adapted from Bebianno and Langston 1989).

197 A further set of individual tissue samples were prepared for antioxidant enzyme
198 analysis by homogenizing in 50 mM Tris-HCl buffer, pH 7.6, containing sucrose (250
199 mM), $MgCl_2$ (5 mM) and DTT (1 mM). After 10 min incubation, the homogenates were
200 centrifuged at 1 000*g* for 10 min at 4 °C and the cytosolic fraction was kept at -80 ºC until
201 analysed (e.g. Auguste et al. 2016).

202

203 **2.4. Cu analysis**

204 Tissue homogenates reserved for Cu concentration determination were weighed,
205 dried (80 °C, 48 h), and submitted to wet acid digestion with 67% nitric acid on a hot
206 plate (80 °C, 2 h). Copper was analysed by graphite furnace absorption spectrometry
207 (AAS, AAnalyst 800- PerkinElmer). Accuracy of the analytical method was confirmed
208 by analysing certified reference material TORT-2 (NRC-CNRC) (lobster
209 hepatopancreas). Measured values (106.0 ± 10.4 µg g^{-1}, n=18) were in agreement with
210 the certified values of the reference material (106 ± 10 µg g^{-1}). Values were expressed as
211 µg g^{-1} of dry weight of tissue (d.w.). The gills of *M. fortunata* were not analysed given
212 the small size of the tissues.

213

214 **2.5. Biomarker analysis**

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

219 Spectrophotometric methods were used to analyse the antioxidant (SOD, CAT,
220 GPx) and biotransformation (GST) enzyme activities in the cytosolic fraction of gills,
221 hepatopancreas and muscle. The activity of SOD was determined by the reduction of
222 cytochrome c by the xanthine oxidase/hypoxanthine system at 550 nm (McCord and
223 Fridovich 1969), with results expressed as U mg^{-1} of total protein. CAT activity was
224 determined by the decrease in absorbance for 1 min after H_2O_2 consumption at 240 nm
225 (Greenwald 1985), with results expressed as µmol min^{-1} mg^{-1} of total protein. GPx

activity was assessed by following for 5 min the NADPH oxidation in the presence of excess glutathione reductase, reduced glutathione and cumene hydroperoxide as substrate at 340 nm (Flohe and Gunzler, 1984; adapted to a microplate reader by McFarland et al. 1999), with results expressed as nmol min^{-1} mg^{-1} of total protein. GST activity was assessed by following the conjugation of reduced glutathione (GSH) with 1-chloro 2,4 dinitrobenzene at 340 nm for 1 min (Habig et al. 1974), with results expressed as µmol min^{-1} mg^{-1} of total protein.

Differential pulse polarography using a µAutolab II potentiostat/galvanostat was used to determine MTs concentration 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.

The concentration of two sub-products of polyunsaturated fatty acid peroxidation: malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) provided the LPO data, using the method by Erdelmeier et al. (1998), with 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} of total protein.

2.9. Statistical analysis

Significant differences were assessed using the non-parametric Kruskal Wallis ANOVA with multiple-comparisons test. Results were considered significantly different when $p<0.05$. Principal component analysis (PCA) was used to evaluate the relationship between the shrimp species and the analysed variables in the hepatopancreas (Cu accumulation and biomarkers) for the different treatments and exposure period.

3. Results

3.1. Cu accumulation in shrimp species

The baseline Cu concentration, before exposure to Cu, was similar in the gills of the three shallow-water *Palaemon* species (*P. elegans* 185.5 ± 74.2 µg g^{-1} d.w.; *P. serratus* 245.9 ± 119.0 µg g^{-1} d.w.; *P. varians* 148.4 ± 33.2 µg g^{-1} d.w.; $p>0.05$) (Fig. 1).

After 3 days of exposure to 4 µM of Cu there was a significant increase in Cu concentration in the gills of *P. elegans* ($p<0.05$), but after 10 days the Cu concentration was similar to pre-exposure ($p>0.05$). In the gills of *P. serratus* exposed to 0.4 µM of Cu there was a significant increase when compared to 4 µM Cu treatment at day 3 ($p<0.05$). For all treatments Cu concentration in the gills of *P. varians* was similar before and after Cu exposure ($p>0.05$) (Fig. 1). No data are available for the gills of *M. fortunata* given the small size of the gills and the small number of individuals available.

Cu concentration in the hepatopancreas before exposure (day 0) was lowest in *P. varians* (54.6 ± 5.8 µg g^{-1} d.w.), followed by *P. elegans* (305.3 ± 26.7 µg g^{-1} d.w.), *P. serratus* (1238.6 ± 982.6 µg g^{-1} d.w.) and *M. fortunata* (1990.5 ± 907.6 µg g^{-1} d.w.) (Fig. 2). Cu concentration was similar before and after exposure in the hepatopancreas of all treatments for *P. serratus* and *P. varians* ($p>0.05$). In the hepatopancreas of *P. elegans* exposed to 4 µM of Cu there was a significant increase in Cu concentration with time of exposure ($p<0.05$). In *M. fortunata* hepatopancreas no significant differences were noted ($p>0.05$).

The concentration of Cu in the muscle was similar in all species (*M. fortunata* 55.8 ± 0.5 µg g^{-1} d.w.; *P. elegans* 58.5 ± 4.0 µg g^{-1} d.w.; *P. serratus* 40.4 ± 13.4 µg g^{-1} d.w.; *P. varians* 40.5 ± 20.9 µg g^{-1} d.w.; $p>0.05$) and no significant increment in Cu concentration was observed over time or with exposure to Cu ($p>0.05$) (Fig. 3). Of the three tissues analysed, the highest concentration of Cu was measured in the hepatopancreas, followed by gills and muscle in *P. elegans*, *P. serratus* and *M. forunata*. In *P. varians*, higher Cu concentration was observed in the gills, followed by hepatopancreas and muscle.

3.2. Oxidative stress

No significant effect of Cu exposure on SOD activity was noted in the gills of *P. elegans*, *P. serratus* and *P. varians* after 3 days exposure ($p>0.05$) (Fig. 1). SOD activity in the gills of *P. elegans* after 10 days exposure to 4 µM of Cu was significantly higher when compared to control and 0.4 µM Cu treatments at day 10 ($p<0.05$) (Fig. 1). The activity of SOD was higher in the hepatopancreas of *M. fortunata* and *P. elegans* when compared to the two other species ($p<0.05$) (Fig. 2). No significant effect of Cu exposure on SOD activity was noted in the hepatopancreas of all species when compared to controls

289 of the same time, or to pre-exposure conditions (p>0.05) (Fig 2). A species-specific
290 response in SOD activity in the muscle was noted (Fig. 3). No significant effects of Cu
291 exposure on SOD activity in the muscle of *M. fortunata* were detected (p>0.05). In the
292 muscle of *P. serratus* a significant decrease in SOD was noted in the 4 µM Cu treatment
293 after 3 days of exposure when compared to pre-exposure. In *P. varians*, a significant
294 increase of SOD was noted in the 4 µM Cu exposure when compared to both pre-exposure
295 and the other treatments after 3 days (p<0.05) (Fig. 3). In the muscle of *P. elegans* a
296 significant decrease in both 0.4 and 4 µM Cu was observed after 3 and 10 days exposure
297 when compared to pre-exposure (p<0.05).

298 The activity of CAT in the gills remained similar throughout the exposure period
299 and between all treatments in all species (p>0.05) (Fig. 1). In *P. varians* a significant
300 decrease in CAT activity in the hepatopancreas after 3 days exposure to 0.4 and 4 µM Cu
301 treatments when compared to control (p<0.05) (Fig. 2). In the hepatopancreas of *P.*
302 *elegans*, CAT activity in the 0.4 µM Cu treatment significantly increased with exposure
303 time (p<0.05). After 10 days exposure, the activity of CAT was higher in hepatopancreas
304 exposed to 0.4 µM Cu when compared to control, for both *P. elegans* and *M. fortunata*
305 (p<0.05). The exposure to Cu had no significant effect in the muscle of the shallow-water
306 shrimps (p>0.05), while in *M. fortunata* a significant decrease in CAT activity was noted
307 after 3 days in 4 µM Cu treatment (p<0.05), followed by a return to pre-exposure activity
308 after 10 days (Fig. 3). In addition, after 10 days of exposure to 0.4 µM Cu there was a
309 significant decrease in CAT activity in the muscle of *M. fortunata* when compared to the
310 other exposure times (p<0.05). In all species the activity of CAT was higher in the
311 hepatopancreas, followed by gills and muscle.

312 GPx activity in the gills of *P. elegans* was lower when compared to the two other
313 *Palaemon* species. In the gills of *P. elegans* a significant decrease in GPx was noted after
314 3 days exposure to 4 µM Cu when compared to the two other treatments (p<0.05),
315 returning to pre-exposure activity at day 10. The activity of GPx in the gills of *P. varians*
316 was significantly higher after 3 days of exposure to 0.4 µM and 4 µM Cu when compared
317 to control and pre-exposure (p<0.05). No significant differences were noted in the gills
318 of *P. serratus* (p>0.05) (Fig. 1). Cu exposure had no significant effects on GPx activity
319 in the hepatopancreas of *M. fortunata*, *P. serratus* and *P. varians* (p>0.05), which was
320 similar (Fig. 2). Overall GPx activity was lower in *P. elegans* hepatopancreas than in the
321 other species. However, significantly higher GPx activity was noted in *P. elegans* after 3

322 days of exposure to the 0.4 µM Cu treatment when compared to control and 4 µM Cu
323 treatment, and to the other exposure times (p<0.05) (Fig. 2). A significant increase was
324 observed in GPx activity in the muscle of *M. fortunata* after 3 days exposure to 4 µM Cu
325 when compared to both control and 0.4 µM Cu within the same time, and to the other
326 exposure times (p<0.05). Higher GPx activity was also noted in the muscle of *P. elegans*
327 after 3 days exposure to 0.4 and 4 µM Cu when compared to control (p<0.05). In this
328 tissue, significantly lower GPx activity was observed in *P. elegans* (0.5 ± 0.1 µg g^{-1} d.w.)
329 and *P. serratus* (0.3 ± 0.1 µg g^{-1} d.w.) pre-exposure than in the other species (*M. fortunata*
330 8.5 ± 2.0 µg g^{-1} d.w.; *P. varians* 13.1 ± 4.8 µg g^{-1} d.w.; p<0.05) (Fig. 3). Unfortunately,
331 samples were lost in the process of analysis and no data are available for GPx in the
332 muscle of *P. varians* exposed to Cu.

333

334 **3.3. Metallothioneins**

335 No significant differences in levels of MTs were observed in the gills of *P.*
336 *serratus* (p>0.05) (Fig. 4). In the gills of *P. varians*, lower levels of MTs were noted in
337 all treatments on day 3 when compared to pre-exposure levels (p<0.05) (Fig. 4).
338 Significantly higher MTs levels were noted in the gills of *P. elegans* after 10 days of
339 exposure to 0.4 µM Cu when compared to pre-exposure (p<0.05). No significant
340 differences in levels of MTs were observed in the hepatopancreas of *P. elegans* and *P.*
341 *varians* (p>0.05) (Fig. 5). Significantly higher levels of MTs were noted in the
342 hepatopancreas of *P. serratus* after 3 days of exposure to 0.4 and 4 µM Cu treatments
343 when compared to control (p<0.05), although these values were similar to pre-exposure
344 values (p>0.05). In the hepatopancreas of *M. fortunata* a significant increase in MTs
345 levels in all treatments was noted after 10 days of exposure when compared to previous
346 exposure times (p<0.05). No significant differences in levels of MTs were observed in
347 the muscle of *P. elegans* and *P. varians* (p>0.05) (Fig. 6). Significantly higher levels of
348 MTs were noted in the muscle of *M. fortunata* exposed to 0.4 and 4 µM Cu when
349 compared to control after 3 days of exposure and other exposure times (p<0.05).
350 Unfortunately, samples were lost in the process of analysis and no data are available for
351 MTs in the muscle of *P. serratus* exposed to Cu.

352

353 **3.4. Biotransformation**

No significant differences between treatments or times were found in GST activity in the gills of *P. elegans* and *P. varians* ($p>0.05$) (Fig. 4). GST activity in the gills of *P. serratus* significantly increased after 3 days of exposure to 0.4 and 4 µM Cu when compared to control and pre-exposure ($p<0.05$). In the hepatopancreas of the different species, exposure to 0.4 and 4 µM Cu did not affect GST activity when compared to controls ($p>0.05$) (Fig. 5). In *P. elegans,* significantly lower levels of GST in the hepatopancreas were noted before the exposure when compared to the other exposure times and for all treatments ($p<0.05$). In the muscle of the shallow-water species, no significant differences in GST were observed between Cu exposed treatments and controls within the same exposure time ($p>0.05$) (Fig. 6). The activity of GST in the muscle of *M. fortunata* was significantly higher in 4 µM Cu treatment after 10 days of exposure when compared to other treatments within the same time, and to pre-exposure and day 3 ($p<0.05$).

3.5. Oxidative damage

Significantly higher LPO levels in the gills of *P. serratus* exposed to 4 µM Cu were noted on day 3 when compared to both control and 0.4 µM Cu treatments ($p<0.05$) although levels were similar to pre-exposure ($p>0.05$) (Fig. 4). The levels of LPO in the gills of *P. elegans* exposed to 0.4 µM Cu significantly decreased after 3 days, remaining at these levels until the end of exposure ($p<0.05$). In contrast, after decrease on day 3, LPO returned to pre-exposure levels in *P. elegans* exposed to 4 µM Cu. In the hepatopancreas for all species analysed no significant differences were found between LPO levels of the different treatments and exposure times ($p>0.05$) (Fig. 5). Similarly, no significant differences in LPO levels between the different treatments and times were found in the muscle of *M. fortunata* ($p>0.05$) (Fig. 6). In *P. elegans* muscle tissues, LPO levels after 3 days of exposure were significantly higher than control at the same exposure time ($p<0.05$). The LPO levels in the muscle of *P. serratus* after 3 days of exposure were higher in control and 0.4 µM Cu when compared to pre-exposure ($p<0.05$), while in *P. varians* were significantly higher in 0.4 µM Cu when compared to pre-exposure levels ($p<0.05$).

3.6. Species specific biomarker patterns

386 The data on Cu accumulation and biomarkers for the hepatopancreas of the four
387 shrimp species for the different treatments and exposure periods were used to elaborate
388 the PCA (Fig. 7). The overall PCA shows a clear separation between the deep-sea species
389 *M. fortunata* and the shallow-water species (*P. elegans*, *P. serratus* and *P. varians*). On
390 the PC1 axis, Cu accumulation, SOD, GST, MT and LPO are positively related with *M.*
391 *fortunata* while CAT and GPx are positively related to the shallow-water species. The
392 two principal components represent 74 % of total variance in the hepatopancreas (PC1 =
393 53 %, PC2 = 21 %).

394

395 **4. Discussion**

396 The effects of exposure to Cu at dissolved concentrations that may be available to
397 biota during deep-sea mining activities (Simpson et al. 2008) were studied in four
398 caridean shrimp species: three congeneric shallow-water species and one deep-sea
399 hydrothermal-vent endemic shrimp. The Cu exposures employed (0.4 µM and 4 µM) can
400 be considered as sub-lethal concentrations.

401 Cu accumulation and biomarker responses to Cu exposure were tissue and species
402 specific. Significant increase in Cu was observed in the 4 µM treatment of *P. elegans*: in
403 the gills after 3 days exposure and in the hepatopancreas after 10 days exposure (Figs. 1,
404 2). A significant reduction in GPx levels occurred in the gills of *P. elegans* after 3 days
405 exposure to 4 µM Cu, presumably as a response to Cu accumulation, while no significant
406 biomarker changes in the hepatopancreas were observed after 10 days. However,
407 exposure to 0.4 and 4 µM Cu did not produce significant responses in the other analysed
408 biomarkers or in other species. This may be an indication that all four species can tolerate
409 Cu without apparent significant negative effect during these short durations and at surface
410 pressure. Nonetheless, the effects of longer duration exposures to these Cu concentrations
411 and also effects on other life stages (e.g. in brooding females and larvae) should be
412 investigated to confirm that 4 µM of Cu is tolerated / regulated by these shrimp species.

413 In recent years, significant progress has been made in increasing knowledge on
414 the ecotoxicological effects of metal exposure on deep-water fauna. Gathered evidence
415 point that hydrothermal vent species are able to regulate dissolved Cu at concentrations
416 similar to those potentially released during mining according to elutriate test studies
417 (Simpson et al. 2008). In addition, although some shallow-water species can tolerate high-

418 hydrostatic pressure, hydrostatic pressure may increase the negative effect of some
419 metals. For example, hydrostatic pressure increases sensitivity to Cu in *P. varians* but has
420 no apparent effect on sensitivity to Cd, whilst sensitivity to a mixture of Cu and Cd is
421 magnified by hydrostatic pressure (Brown et al. 2017a).

422 Although there are few experimental assessments of the impact of decreased
423 hydrostatic pressure sensitivity to toxicants in deep-sea fauna, there is evidence of
424 significant metabolic effects of decreased hydrostatic pressure in e.g. *M. fortunata*.
425 Metabolic rate was significantly lower at 0.1 MPa than at 17 MPa in shrimp sampled at
426 1700 m depth (Shillito et al. 2006), and metabolic rate appears to decline further with
427 sustained exposure to surface pressure (cf. Shillito et al. 2006, Smith et al. 2013). Shifts
428 in metabolic rate may affect capacity to respond to toxicants. For example, the pre-
429 exposure Cu levels in the hepatopancreas of *M. fortunata* (1990±908 μg g^{-1} d.w., Fig. 2)
430 that were acclimatized for over 1 year at surface pressure in aquaria at Oceanopolis, Brest,
431 France, were 4 times higher than the Cu concentrations measured in *M. fortunata* after
432 collection from Rainbow (400±100 μg g^{-1} d.w.) or from Lucky Strike (500±200 μg g^{-1}
433 d.w.; Kádár et al. 2006). However, the Cu concentration in the muscle (around 55.8±0.5
434 μg g^{-1} d.w.) were comparable (Rainbow site: 200±60 μg g^{-1} d.w. Lucky Strike site: 40±10
435 μg g^{-1} d.w., Kádár et al. 2006). This high hepatopancreas Cu concentration is quite
436 puzzling, since Cu levels at Oceanopolis public aquarium are regularly checked, and are
437 consistently below 1 μg L^{-1}. It may indicate a regulation mechanism that preferentially
438 eliminates Cu, but which accumulates Cu in specific tissues beyond a critical threshold
439 concentration. Such a mechanism may compensate the usually higher environmental Cu
440 levels at hydrothermal vents. A similar regulation mechanism has been proposed for *P.*
441 *elegans* (White and Rainbow 1982) up to an environmental concentration of 100 μg L^{-1},
442 after which accumulation reflects the environmental levels. Results in the present study
443 are consistent with the proposed mechanism. Cu was elevated in the gills of *P. elegans*
444 exposed to 4 μM after 3 days, but reduced after 10 days exposure whilst Cu in the
445 hepatopancreas increased after 10 days exposure, suggesting Cu may have been
446 translocated to the hepatopancreas (Figs. 1, 2; White and Rainbow 1982, Pourang et al.
447 2004). Higher Cu concentration was also found in the hepatopancreas of *R. exoculata*
448 when compared to the gills, and it was suggested that this was caused by the presence of
449 high amounts of haemocyanin, Cu-containing granules and MTs (Auguste et al. 2016).
450 Haemocyanins are synthesized in the hepatopancreas (up to 50% of total protein

synthesized) and are responsible for oxygen transport (Viarengo and Nott 1993). If the high Cu concentration found in this tissue is associated with an increase in haemocyanin concentration, this may alternatively be an indication of an increased metabolic demand in *P. elegans* after 10 days exposure. The existence of such regulation mechanisms increase the complexity involved in predicting the ecotoxicological effects of metal mixtures such as those potentially found in sediment plumes of deep-sea mining.

Similar results to those presented here were obtained for the hydrothermal vent shrimp *R. exoculata*, collected from TAG vent Field (3630 m depth), Mid-Atlantic Ridge (MAR), exposed to the same concentrations of dissolved Cu but under high-pressure (30 MPa) and low temperature (10ºC) representative of *in situ* conditions (Auguste et al. 2016). Auguste et al. (2016) reported no accumulation of Cu in the different tissues, when compared to *in situ* and control conditions, and only a significant increase in MTs was noted in the gills of shrimps exposed to 4 µM for 72 h. In contrast, *P. varians* exposed to 100 µg L^{-1} (~1.6 µM) of Cu caused a significant increase in both SOD and GPx after 96 h exposure at 10ºC and 10 MPa (Brown et al. 2017a). However, at surface pressure (0.1 MPa) significant biomarker responses were only observed in the 1000 µg L^{-1} (~16 µM) Cu treatment (Brown et al. 2017a). Consequently, Brown et al. (2017a) suggested that sensitivity and responses to toxicants may not differ between *P. varians* and *R. exoculata* at a common temperature at native hydrostatic pressures.

The hydrothermal vent endemic mussel *Bathymodiolus azoricus* collected from Lucky Strike (MAR) also displayed no significant effect on antioxidant enzyme activities (SOD, GPx and CAT) after exposure up to 300 µg L^{-1} of dissolved Cu (same range as in the present study, 254 µg L^{-1}) at native hydrostatic pressure (17.5 MPa) and 10ºC, but GPx was significantly lower at higher Cu concentrations (800 and 1600 µg L^{-1} of Cu; Martins et al. 2017). However, the cold-water octocoral *Dentomuricea meteor* collected from the Condor seamount (MAR) at depths around 200 m appears to be more sensitive to dissolved Cu exposure, with a 96 h LC_{50} of 137 µg L^{-1} at 0.1 MPa and 13°C (Martins et al. 2018). Still, the eurybathic brittle star *A. squamata* was observed to be even more sensitive to Cu exposure with a 96 h LC_{50} value for Cu at 25ºC of 46 µg L^{-1} (Black et al. 2015). Nevertheless, the phylogenetic and physiological distance of the different taxa, as well as the different experimental exposure conditions do not help to identify patterns for ecotoxicological effects of Cu.

Results of the multiple biomarkers analysed showed differences in enzyme activities between species, such as a 5-fold higher levels of GST in the hepatopancreas observed in the vent shrimp when compared to the shallow-water shrimps (Fig. 5). Such differences discourage the use of proxy species for assessing the effects of exposure to contaminants in species from contrasting ecological settings, at least when considering the experimental conditions of this study (i.e. Cu concentration, exposure duration, and biomarkers analysed). Important differences in acute thermal and hyperbaric tolerances have already been noted among the congeneric shrimp species *P. varians* and *P. serratus* where it has been suggested that differences in these species' evolutionary environments may have contributed to differing physiological stress tolerances (Pallareti et al. 2018). Similarly, *P. elegans, P. serratus*, and *P. varians* may have been exposed to different chemical environments during their evolutionary history which may have led to different molecular/biochemical adaptations/tolerances observed here in the differences in biomarker baselines and responses among species (Fig. 7). Indeed, although phylogeny may constrain physiological tolerances, the chemical composition of a species habitat appears to be crucial in determining physiological thresholds, leading to different antioxidant baselines and responses among phylogenetically related species (Faria et al. 2018). Differential accumulation of metals and toxicity thresholds among phylogenetically close crustaceans has been demonstrated previously (reviewed by Rainbow 1998). Thus, the present study contributes to growing evidence that *in situ* ecotoxicological experiments using local fauna (therefore at native environmental conditions such as hydrostatic pressure and temperature) provide more reliable knowledge on the ecotoxicological environmental hazards posed by deep-sea mining than using shallow-water proxy species. Similarly, while it is recognised that dissolved metal phases are more toxic than particulates (Simpson and Spadaro 2016), under deep-sea mining conditions there will be concomitant presence of dissolved and particulate metal phases which are difficult to mimic simultaneously under laboratory controlled conditions, particularly given that mineral composition is ore deposit dependent. Although it is rather expensive to conduct ecotoxicity experiments in the deep sea, future studies should focus on *in situ* experiments incorporating a range of toxicity indicators to better understand the effects of deep-sea mining or deep-sea mine tailings disposal on deep-sea fauna (Mestre et al. 2017).

5. Conclusions

Results suggest that different chemical environments during the evolutionary history of phylogenetically proximate species cause different molecular/biochemical adaptations/tolerances, such as those observed in the differences in Cu accumulation patterns and biomarker baselines and responses among the studied species. In addition, environmental variables such as low temperature and high pressure likely influence sub-lethal effects in deep-water species. The use of shallow-water proxy species related to deep-water relatives does not appear to provide adequate inferences. Future studies should therefore focus on *in situ* experiments with local species, mimicking deep-sea mining activity scenarios, such as a sediment plume, to provide the most accurate information on the biological impacts to local fauna.

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Competing interests

The authors have no competing interests to declare.

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739

740 **Figure captions**

741

742 **Figure 1.** Copper concentration and superoxide dismutase (SOD), catalase (CAT) and
743 total glutathione peroxidase (GPx) activities (mean ± SD) in the **gills** of *Palaemon*
744 *elegans, P. serratus* and *P. varians* for the different treatments. Different capital and
745 lower case letters indicate significant differences between treatments within the same day
746 and between exposure days for the same treatment, respectively ($p < 0.05$). Treatments:
747 control (white bars); 0.4 µM (grey bars); 4 µM (black bars).

748

749 **Figure 2.** Copper concentration and superoxide dismutase (SOD), catalase (CAT) and
750 total glutathione peroxidase (GPx) activities (mean ± SD) in the **hepatopancreas** of
751 *Mirocaris fortunata*, *Palaemon elegans, P. serratus* and *P. varians* for the different
752 treatments. Different capital and lower case letters indicate significant differences
753 between treatments within the same day and between exposure days for the same
754 treatment, respectively ($p < 0.05$). Treatments: control (white bars); 0.4 µM (grey bars);
755 4 µM (black bars).

756

757 **Figure 3.** Copper concentration and superoxide dismutase (SOD), catalase (CAT) and
758 total glutathione peroxidase (GPx) activities (mean ± SD) in the **muscle** of *Mirocaris*
759 *fortunata*, *Palaemon elegans, P. serratus* and *P. varians* for the different treatments.
760 Different capital and lower case letters indicate significant differences between treatments
761 within the same day and between exposure days for the same treatment, respectively ($p <$
762 0.05). Treatments: control (white bars); 0.4 µM (grey bars); 4 µM (black bars).

763

764 **Figure 4.** Metallothioneins (MTs), glutathione-S-transferase (GST) and lipid
765 peroxidation (LPO) (mean ± SD) in the **gills** of *Palaemon elegans, P. serratus* and *P.*
766 *varians* for the different treatments. Different capital and lower case letters indicate
767 significant differences between treatments within the same day and between exposure
768 days for the same treatment, respectively ($p < 0.05$). Treatments: control (white bars); 0.4
769 µM (grey bars); 4 µM (black bars).

770

771

772 **Figure 5.** Metallothioneins (MTs), glutathione-S-transferase (GST) and lipid
773 peroxidation (LPO) (mean ± SD) in the **hepatopancreas** of *Mirocaris fortunata*,
774 *Palaemon elegans, P. serratus* and *P. varians* for the different treatments. Different
775 capital and lower case letters indicate significant differences between treatments within
776 the same day and between exposure days for the same treatment, respectively ($p < 0.05$).
777 Treatments: control (white bars); 0.4 µM (grey bars); 4 µM (black bars).

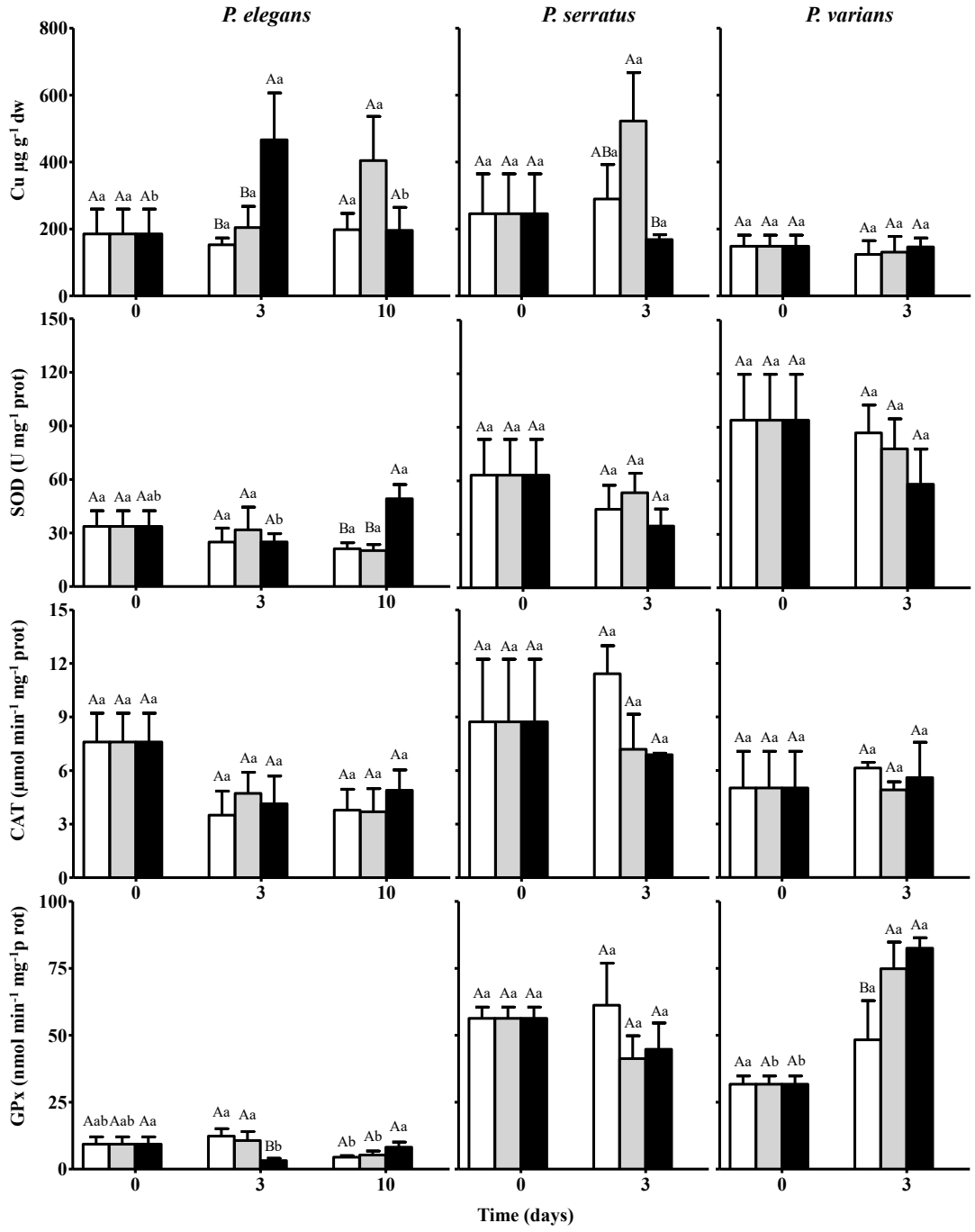
778

779 **Figure 6.** Metallothioneins (MTs), glutathione-S-transferase (GST) and lipid
780 peroxidation (LPO) (mean ± SD) in the **muscle** of *Mirocaris fortunata*, *Palaemon*
781 *elegans, P. serratus* and *P. varians* for the different treatments. Different capital and
782 lower case letters indicate significant differences between treatments within the same day
783 and between exposure days for the same treatment, respectively ($p < 0.05$). Treatments:
784 control (white bars); 0.4 µM (grey bars); 4 µM (black bars).

785

786 **Figure 7.** Principal component analysis (PCA) of copper content (Cu) and biomarkers
787 (SOD, CAT, GPx, MT, GST and LPO) in the hepatopancreas of the four shrimp species
788 (*Mirocaris fortunata*, *Palaemon elegans*, *P. serratus* and *P. varians*) over the duration of
789 the experiment (T0 = pre-exposure, T3 = 3rd day of exposure and T10 = 10th day of
790 exposure) for the different treatments (C = control, 0.4 = 0.4 µM of Cu, 4 = 4 µM of Cu).
791 Variables are marked with a red cross.

792 **Figure 1.**



793

794

Figure 2.

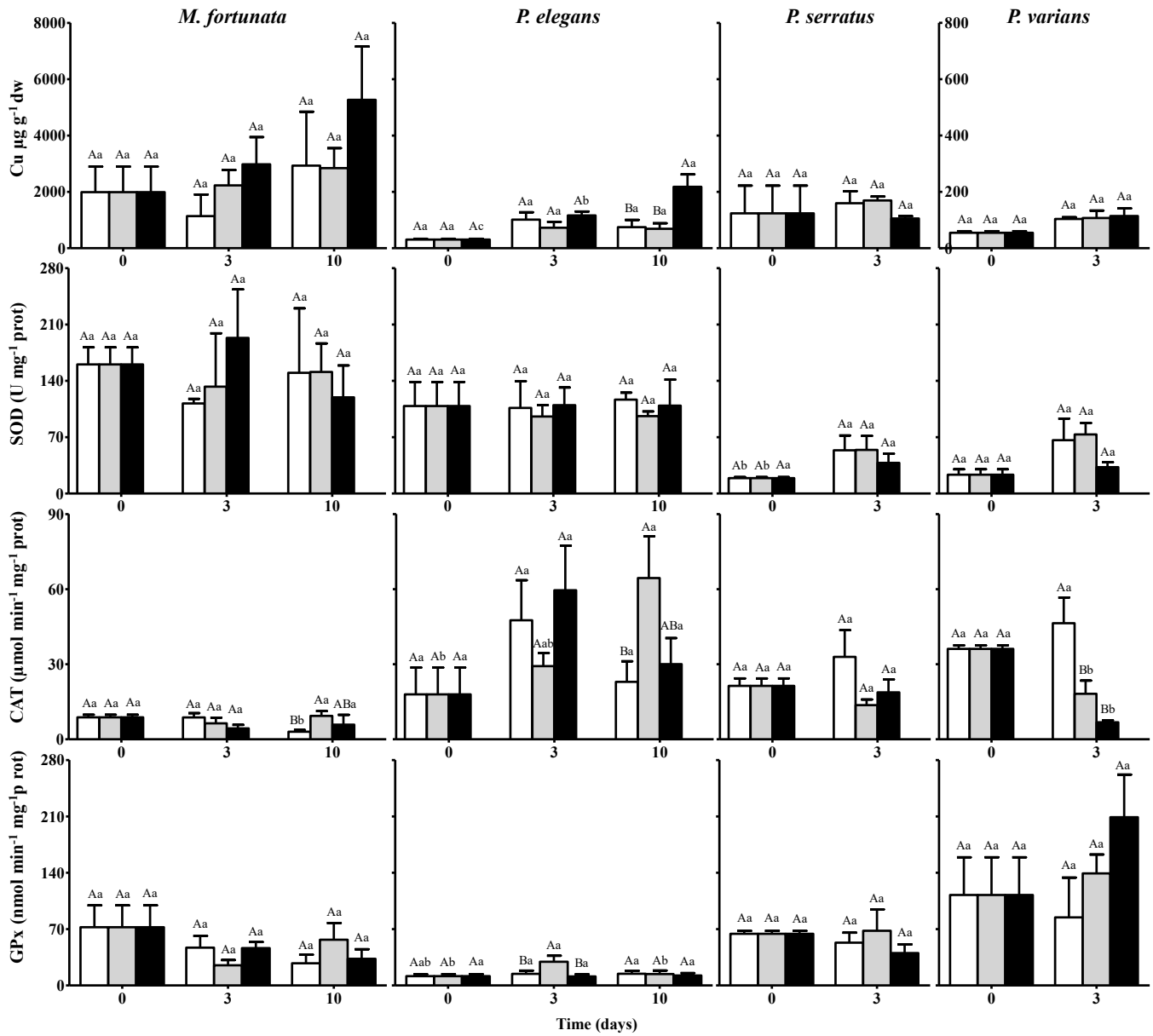
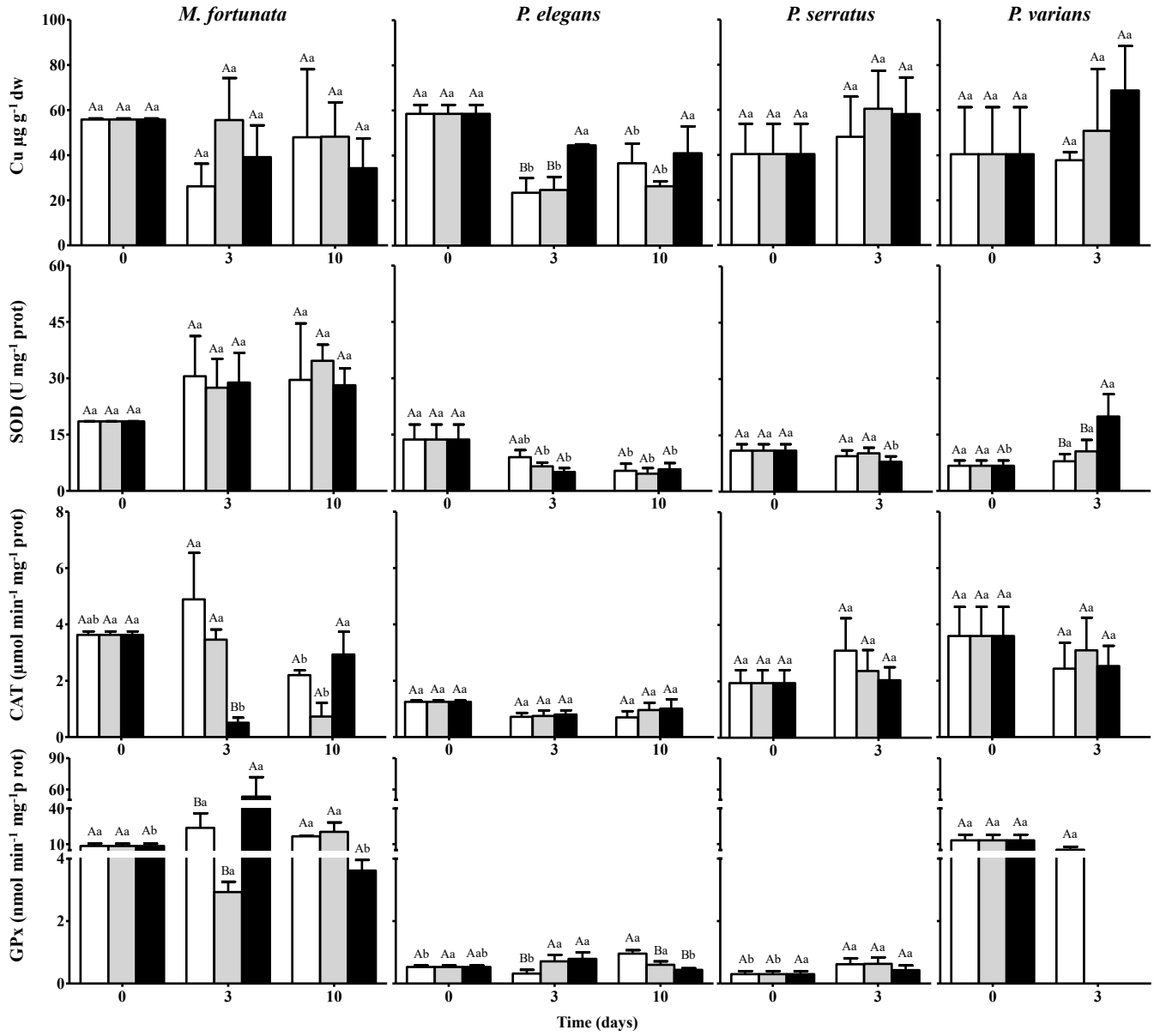
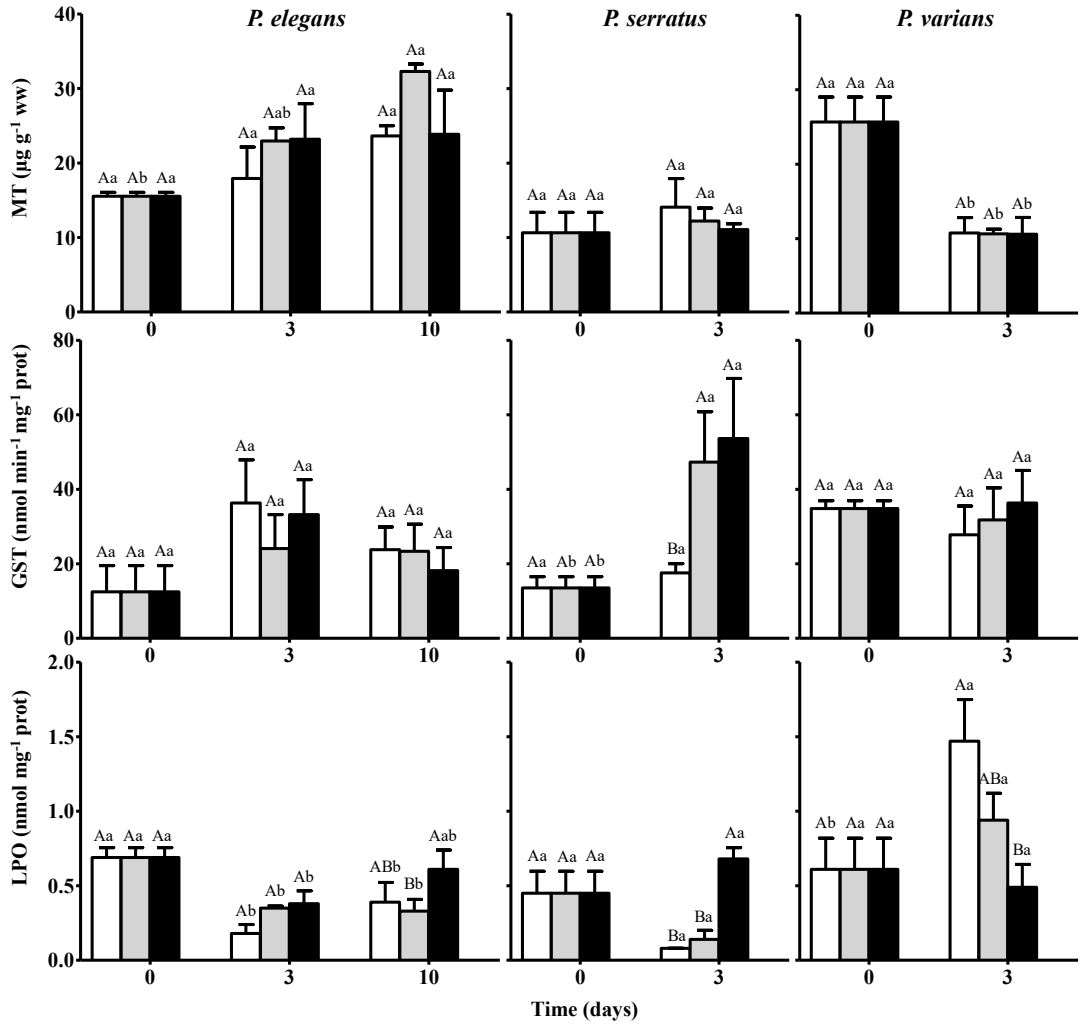

M. fortunata
P. elegans
P. serratus
P. varians
Cu µg g-1 dw
SOD (U mg-1 prot)
CAT (µmol min-1 mg-1 prot)
GPx (nmol min-1 mg-1p rot)
Time (days)

Figure 3.

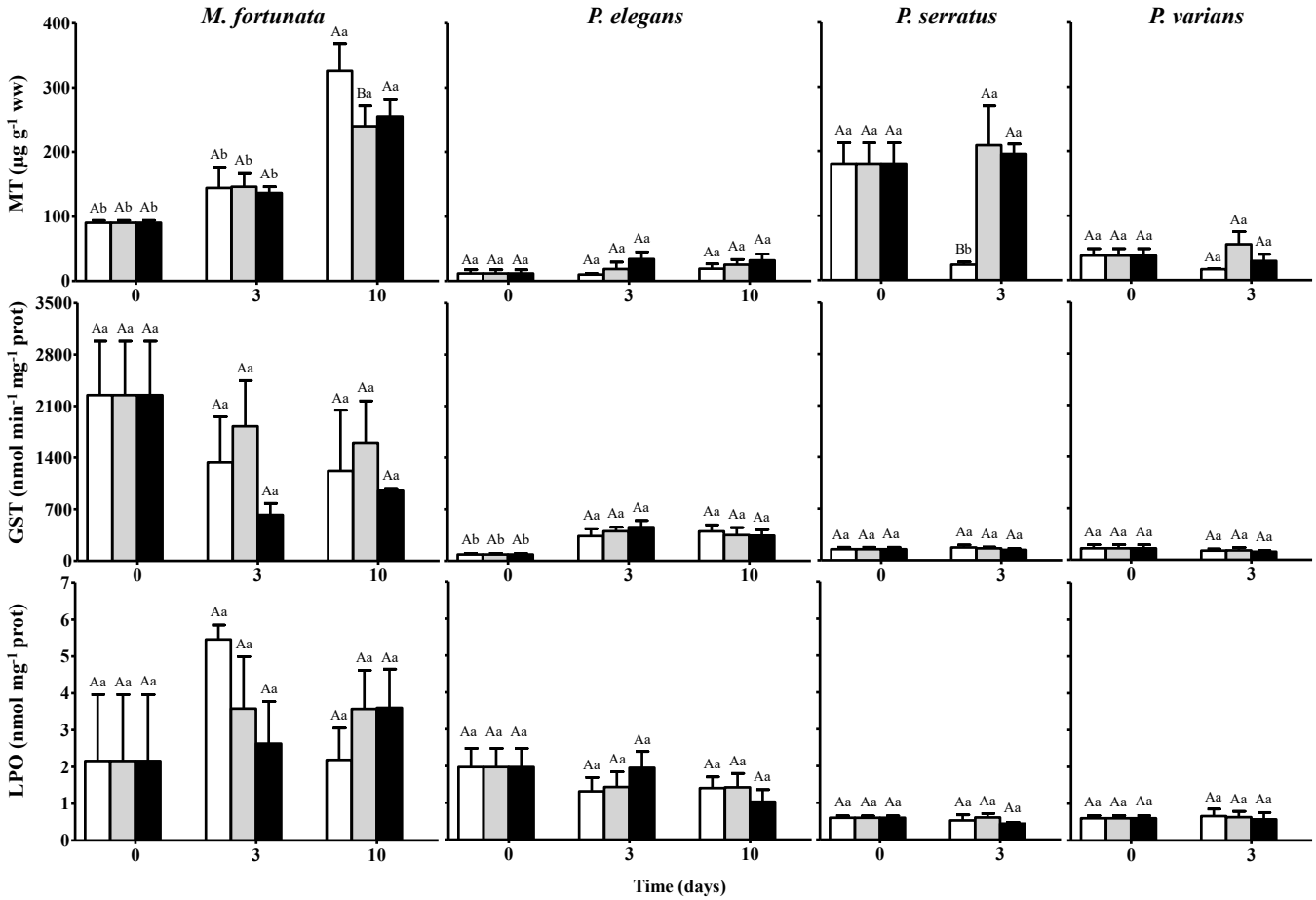

M. fortunata
P. elegans
P. serratus
P. varians
Cu µg g-1 dw
SOD (U mg-1 prot)
CAT (µmol min-1 mg-1 prot)
GPx (nmol min-1 mg-1p rot)
Time (days)

801 **Figure 4.**



802

803 **Figure 5.**



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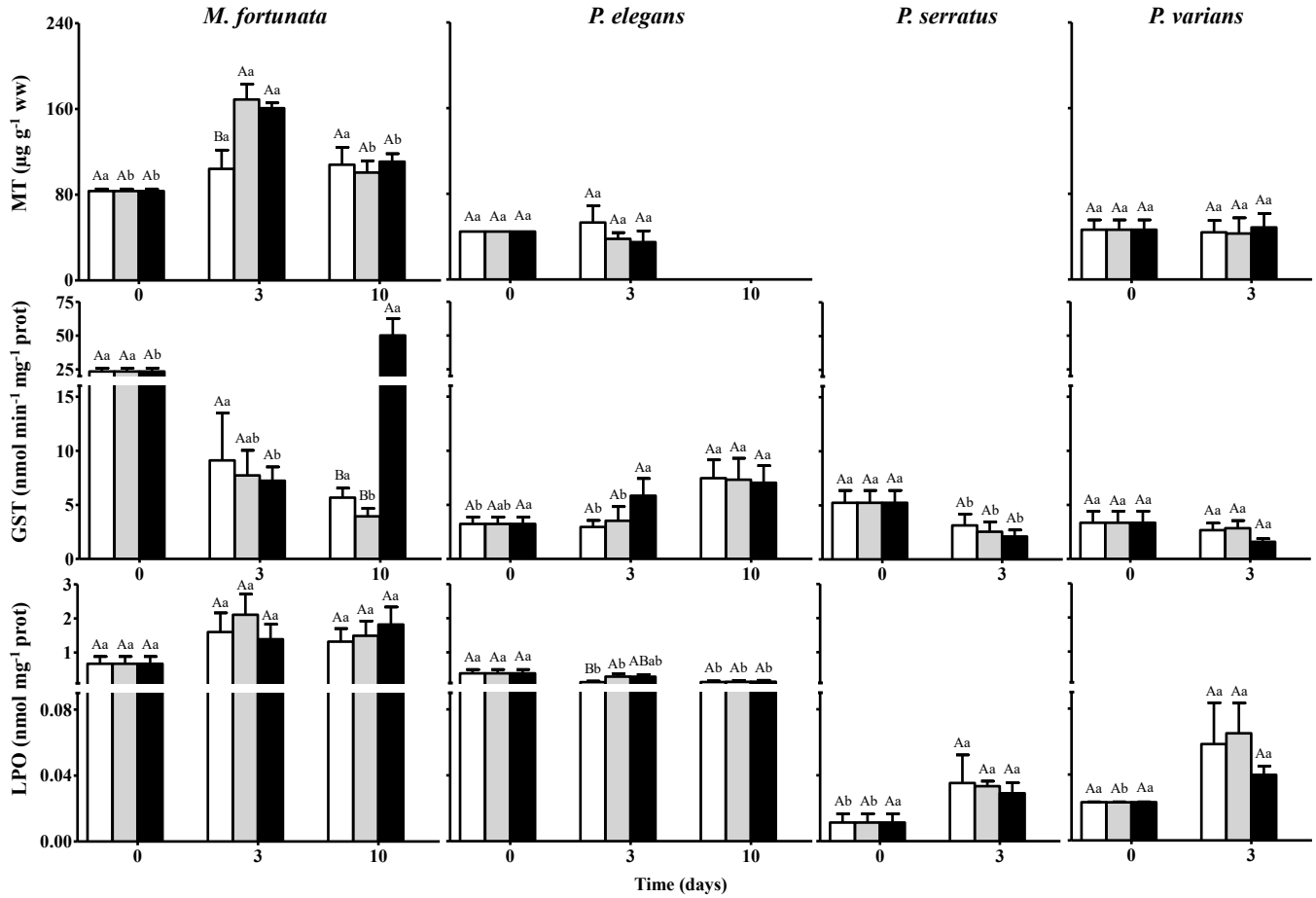
805

806 **Figure 6.**

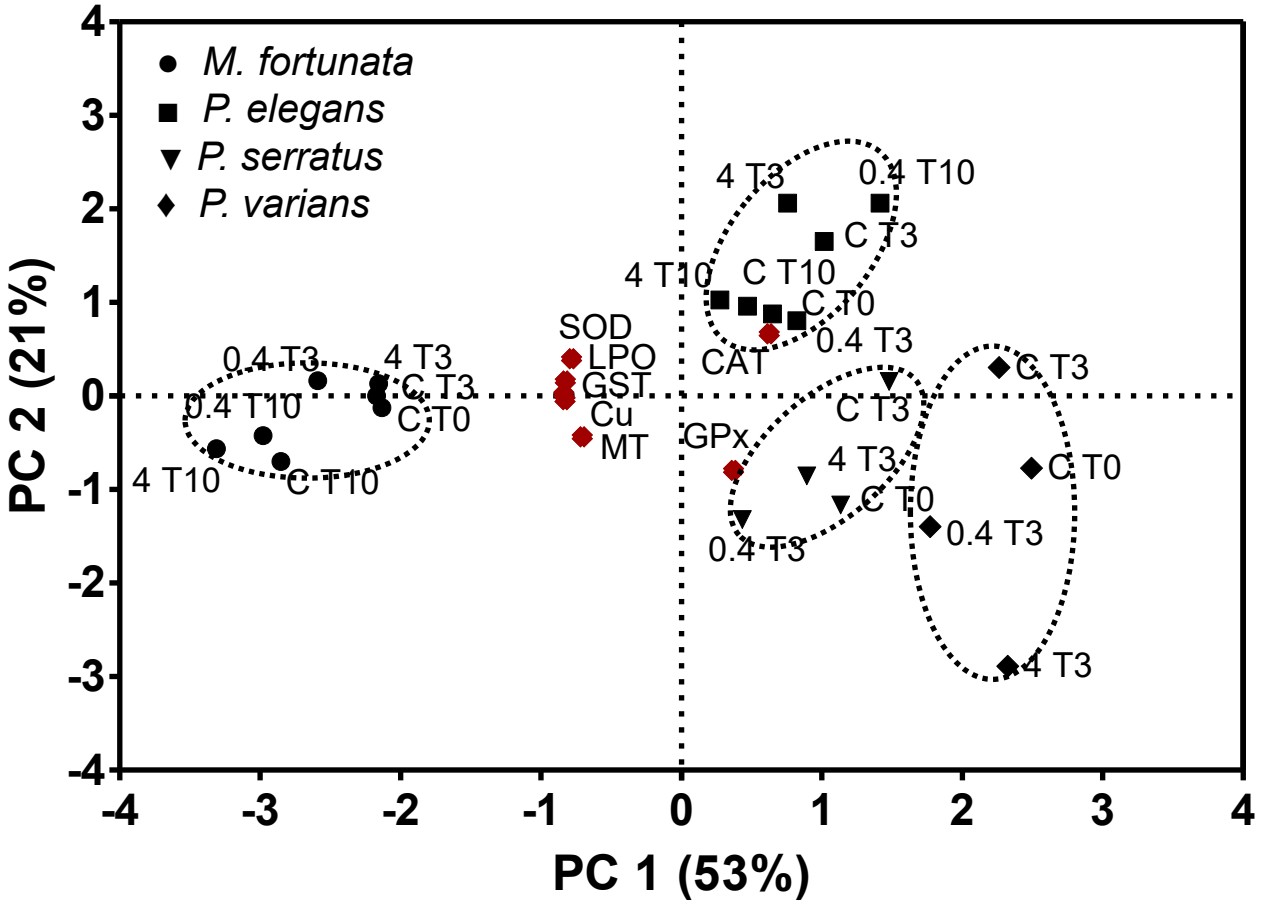


807

808

809 **Figure 7.**

810



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