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► To cite this version:

M. Auguste, N.C. Mestre, T.L. Rocha, C. Cardoso, V. Cueff-Gauchard, et al.. Development of an ecotoxicological protocol for the deep-sea fauna using the hydrothermal vent shrimp *Rimicaris exoculata*. *Aquatic Toxicology*, 2016, 175, pp.277-285. 10.1016/j.aquatox.2016.03.024 . hal-03800831

HAL Id: hal-03800831

<https://hal.science/hal-03800831>

Submitted on 6 Oct 2022

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1 **Development of an ecotoxicological protocol for the deep-sea fauna using**
2 **the hydrothermal vent shrimp *Rimicaris exoculata***

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18 **ABSTRACT**

19 In light of deep-sea mining industry development, particularly interested in
20 massive-sulphide deposits enriched in metals with high commercial value, efforts are
21 increasing to better understand potential environmental impacts to local fauna. The aim
22 of this study was to assess the natural background levels of biomarkers in the
23 hydrothermal vent shrimp *Rimicaris exoculata* and their responses to copper exposure at
24 *in situ* pressure (30 MPa) as well as the effects of depressurization and pressurization of
25 the high-pressure aquarium IPOCAMP. *R. exoculata* were collected from the chimney
26 walls of the hydrothermal vent site TAG (Mid Atlantic Ridge) at 3630 m depth during
27 the BICOSE cruise in 2014. Tissue metal accumulation was quantified in different
28 tissues (gills, hepatopancreas and muscle) and a battery of biomarkers was measured:
29 metal exposure (metallothioneins), oxidative stress (catalase, superoxide dismutase,
30 glutathione-S-transferase and glutathione peroxidase) and oxidative damage (lipid
31 peroxidation). Data show a higher concentration of Cu in the hepatopancreas and a

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32 slight increase in the gills after incubations (for both exposed groups). Significant
33 induction of metallothioneins was observed in the gills of shrimps exposed to 4 μM of
34 Cu compared to the control group. Moreover, activities of enzymes were detected for
35 the *in situ* group, showing a background protection against metal toxicity. Results
36 suggest that the proposed method, including a physiologically critical step of
37 pressurizing and depressurizing the test chamber to enable the seawater exchange
38 during exposure to contaminants, is not affecting metal accumulation and biomarkers
39 response and may prove a useful method to assess toxicity of contaminants in deep-sea
40 species.

41
42 *Keywords:* Deep-sea mining; hydrothermal vents; biomarkers; IPOCAMP; copper;
43 TAG.

44 45 46 **1. Introduction**

47 Hydrothermal vents are massive sulphide deposits with high concentration of
48 various metals (e.g. Cu, Ni, Zn, Mn, Co, Ag, Au) with potential economic interest to
49 mining industries (Hannington et al. 2011). Recently, the Government of Papua New
50 Guinea has given exploration license to Nautilus Minerals Corporation to explore this
51 type of mineral deposits in the Manus basin (Gena 2013). However, it is likely that the
52 impact of human activities will somehow change the natural equilibrium of this
53 particular environment (Van Dover 2014). The living fauna will likely be exposed to
54 variations in environmental metal composition, hence the need to further explore the
55 toxicity of chemicals associated with deep-sea exploitation. For this it is considered
56 essential to adapt the existing ecotoxicological protocols from shallow-water to the
57 deep-sea conditions to enable a better understanding of potential anthropogenic impact
58 on deep-sea fauna (Mestre et al. 2014).

59 The Trans-Atlantic Geotraverse (TAG) hydrothermal vent field is located on the
60 Mid-Atlantic Ridge and its hot fluids are rich in various metals, including copper with
61 concentrations ranging between 120 and 150 μM (Edmonds et al. 1996). Copper is an
62 essential trace element found in a variety of cells and tissues and used as cofactor of
63 several enzymes. However, free Cu ions (Cu^+ and Cu^{2+}) can participate in the formation

64 of reactive oxygen species (ROS) (Gaetke and Chow 2003). As invertebrates possess
65 the ability to concentrate certain metals from the surrounding medium, reflecting the
66 variation in metal concentration in the environment (White and Rainbow 1985), they are
67 useful tools to monitor change of metal amounts as a result of anthropogenic activities.

68 Increasing total dissolved metal concentration will lead to a higher metal uptake
69 rate (Rainbow 1998). To prevent the increase of metal accumulation in cells, organisms
70 possess defence mechanisms such as specific proteins which play a role in binding
71 metals, as for example the metallothioneins (MTs). These cysteine-rich non-enzymatic
72 proteins are vital for various biological functions like storage, transport,
73 compartmentalization of essential and non-essential metals (detoxification mechanisms)
74 and can also act as oxyradicals scavengers protecting cells against ROS (Roesijadi
75 1992). In the presence of certain metals and/or high-metals concentrations, ROS
76 formation will induce an antioxidant defence mechanism. This mechanism consists in
77 the action of specific enzymatic proteins, such as superoxide dismutase (SOD), catalase
78 (CAT), glutathione peroxidase (GPx) and glutathione-s-transferase (GST). These
79 enzymes will limit the ROS levels and prevent oxidative damage (Di Giulio et al. 1995).
80 Nevertheless, if the action of these enzymes is not sufficient, the free radicals will reach
81 the cellular membranes and cause oxidative damage, such as lipid peroxidation (LPO),
82 which is the oxidation of polyunsaturated fatty acids (PUFAs; Halliwell and Gutteridge
83 1984).

84 Previous studies have already looked into metal accumulation and antioxidant
85 responses in the deep-sea hydrothermal vent mussel *Bathymodiolus azoricus* from
86 Menez-Gwen, Lucky Strike and Rainbow vent sites (Company et al. 2004, 2006a,b,
87 2007, 2008, Bebianno et al. 2005). A few studies have looked into metal accumulation
88 (Kádár et al. 2006, Geret et al. 2002), antioxidant enzymes activities (Gonzalez-Rey et
89 al. 2008), MTs and oxidative damage (Geret et al. 2002, Gonzalez-Rey et al. 2007) in
90 the vent shrimp *Rimicaris exoculata*. Nevertheless, only one study has investigated the
91 toxicity of metal exposure under laboratory controlled deep-sea conditions of high
92 pressure and low temperature (Company et al. 2006a) using one of the few available
93 equipment that enable these kind of studies, the IPOCAMP (Incubateur Pressurisé pour
94 l'Observation et la Culture d'Animaux Marins Profonds; Shillito et al. 2014).

95 The chimney walls at TAG are covered by dense swarms (1200-3000 indiv.m²)
96 of the vent shrimp *R. exoculata*, also known to be one of the most abundant invertebrate

1 97 species in the Mid-Atlantic Ridge (Desbruyères et al. 2000, Schmidt et al. 2008). *R.*
2 98 *exoculata* is also one of the most studied deep-sea species, making it a good model for
3 99 investigating deep-sea environmental changes (e.g. Van Dover et al. 1988, Gebruk et al.
4 100 1993, Vereshchaka 1997, Rieley et al. 1999, Allen et al. 2001, Geret et al. 2002, Ravaux
5 101 et al. 2003, Zbinden et al. 2004, 2008, Copley et al. 2007, Gonzalez-Rey et al. 2007,
6 102 2008, Anderson et al. 2008, Corbari et al. 2008, Schmidt et al. 2008, 2009, Cottin et al.
7 103 2010, Ponsard et al. 2013, Jan et al. 2015).

8 104 Elutriate toxicity tests with weathered vent chimney material were conducted
9 105 during the environmental impact studies at Solwara 1, Papua New Guinea. The
10 106 concentration of Cu released after 30 min in the field-based elutriate tests was 15-20
11 107 $\mu\text{g.L}^{-1}$ and in the laboratory-based tests was 38-140 $\mu\text{g.L}^{-1}$ (Simpson et al. 2008). The
12 108 selected copper concentrations used in the present study reflect these results.

13 109 This study assesses the biomarkers (MTs, antioxidant enzymes and LPO)
14 110 response in different tissues (gills, hepatopancreas and abdominal muscle) in *R.*
15 111 *exoculata* exposed to 0.4 μM (25 $\mu\text{g.L}^{-1}$) and 4 μM (254 $\mu\text{g.L}^{-1}$) of copper at *in situ*
16 112 pressure and temperature (30 MPa, 10°C). It also compares the metal accumulation (Cu,
17 113 Zn, Fe, Mn, Co, Cd) and biomarkers baseline levels under natural conditions (isobaric
18 114 sampling) and after maintenance in a high-pressure aquarium IPOCAMP (no metal
19 115 exposure). Finally, this study also compares the effects of depressurization and
20 116 pressurization of the test chamber (conditions necessary for the Cu enriched seawater
21 117 and oxygen renewal during the test).

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24 120 **2. Materials and Methods**

25 121 **2.1. Sample collection**

26 122 *Rimicaris exoculata* were sampled in February 2014 from the TAG vent field
27 123 (26° 08.2' N, 44° 49.5' W) in the Mid-Atlantic Ridge, during the oceanographic cruise
28 124 BICOSE, aboard the research vessel *Pourquoi pas?*. The organisms were collected
29 125 using the slurp gun of the ROV VICTOR 6000 (IFREMER), and using the PERISCOP
30 126 for isobaric and isothermal recovery (Shillito et al. 2008). The shrimps were taken from
31 127 the main black smoker on the wall near the main spire.

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129 **2.2. Cu exposure experiments**

130 A total of 24 shrimps were used in this study, divided into 4 groups. The first
131 group (n=6) called *in situ* (IS) was dissected under sterile conditions and flash frozen in
132 liquid nitrogen straight after collection and stored at -80°C until further use. The other
133 shrimps were directly transferred into the pressurized aquarium IPOCAMP for a Cu
134 exposure experiment at 10°C. A copper solution was prepared using CuSO₄ (analytical
135 grade, Sigma). Shrimps (n= 6 *per* condition) were isolated in 3.8 L boxes for: control
136 (C; only seawater); 0.4 µM of Cu (Cu1); and 4 µM of Cu (Cu2). The experiment was
137 carried out at 30 MPa and 10°C for 72 h. For seawater and oxygen renewal, the
138 IPOCAMP was depressurized every 12 h, and the viability of the animals was checked.
139 At the end of the exposure period (72 h), survival of the shrimp was 100%, although
140 one of the animals of the Cu2 group appeared less responsive to stimulation. The tissues
141 were dissected (gills, hepatopancreas and muscle; without gut or exoskeleton) and
142 immediately frozen in liquid nitrogen and stored at -80°C until further analyses.

144 **2.3. Tissue preparation**

145 Tissues were individually homogenized at 4°C in a Tris-HCl (0.02 M, 5 ml.g⁻¹
146 soft tissue) buffer with butylated hydroxytoluene (BHT, 10 µl.mL⁻¹), pH 8.6. The
147 homogenate (3 ml) was separated in soluble and insoluble fractions by centrifugation
148 (30000×g, 30 min, 4°C) and the remaining homogenate was used for determination of
149 metal concentrations. After centrifugation, a part of the supernatant was used for the
150 measurement of LPO and total protein content. A second centrifugation (30000×g, 30
151 min, 4°C) separated the low molecular weight proteins, and the supernatant was used
152 for MT measurements.

153 For antioxidant enzyme analysis, tissues were individually homogenized in 50
154 mM Tris-HCl buffer, pH 7.6, containing sucrose (250 mM), MgCl₂ (5 mM), DTT (1
155 mM). After 10 min of incubation, the homogenates were centrifuged at 1000×g for 10
156 min at 4°C and the cytosolic fraction was kept for analysis.

158 **2.4. Metal analysis**

159 For metal concentration determination, homogenates were weighed, dried (80°C,
160 48 h) and submitted to wet acid digestion with 67% nitric acid in a hot plate (80°C, 2 h).
161 Six metals (Cu, Zn, Fe, Mn, Co, Cd) were analysed by graphite furnace absorption
162 spectrometry (AAS, AAnalyst 800- PerkinElmer). Accuracy of the analytical method
163 was checked by analysing certified reference material TORT-2 (NRC-CNRC) (lobster
164 hepatopancreas), measured values ($99 \pm 17 \mu\text{g.g}^{-1}$ Cu , 138 ± 28 Fe, 124 ± 52 Zn, 14 ± 2
165 Mn, 0.49 ± 0.08 Co, 33 ± 3 Cd) were within the values of certified values in the
166 reference material ($106 \pm 10 \mu\text{g.g}^{-1}$ Cu, 105 ± 13 Fe, 180 ± 6 Zn, 13.6 ± 1.2 Mn,
167 0.51 ± 0.09 Co, 26.7 ± 0.6 Cd). Data expressed as $\mu\text{g.g}^{-1}$ of dry tissue weight (d.w.). The
168 values for total soft tissues correspond to the sum of gills, hepatopancreas and muscle,
169 taking into the account the contribution of the weight of each tissue.

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171 **2.5. Metallothioneins (MTs)**

172 MTs were determined in the heat-stable supernatant by differential normal pulse
173 polarography (Bebianno and Langston 1989), using an Eco Chemie mAutolab III
174 potentiostat and a Methrom 663VA stand with a static mercury drop electrode (SMDE)
175 and GPES 4.9 software (Eco Chemie). In the absence of a shrimp MT standard, a rabbit
176 MT standard was used. MTs levels are expressed in $\mu\text{g g}^{-1}$ of wet tissue weight (w.w.).

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178 **2.6. Total proteins**

179 Total proteins were measured by the method of Bradford et al. (1976) adapted to
180 a microplate reader (Infinite® 200 PRO-Tecan), using Bovine Serum Albumin (Sigma-
181 Aldrich) as a standard. The proteins concentration is expressed as mg.g^{-1} of wet tissue
182 weight.

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184 **2.7. Antioxidant enzymes activities**

185 Superoxide dismutase (SOD) activity was determined by measuring the
186 reduction of cytochrome c by the xanthine oxidase/hypoxanthine system at 550 nm
187 (McCord and Fridovich 1969). One unit of SOD is defined as the amount of enzyme
188 that inhibits the reduction of cytochrome c by 50%. SOD activity is expressed in U.mg^{-1}
189 prot.

190 Catalase (CAT) activity was determined according to Greenwald (1985). The
191 activity was determined as the decrease in absorbance during 1 min at 240 nm due to
192 H₂O₂ consumption in buffer of monobasic and dibasic potassium, pH 7.5. CAT activity
193 is expressed as $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ prot.

194 Glutathione peroxidation (GPx) activity was measured by following NADPH
195 oxidation at 340 nm in the presence of excess glutathione reductase, reduced glutathione
196 and corresponding peroxide (Flohe and Gunzler 1984). The GPx activities were
197 measured by using cumene hydroperoxide as substrate. GPx activity is expressed as
198 $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ prot.

199 Glutathione-S-transferase (GST) activity was determined by following the
200 conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB;
201 Habig et al. 1974). A buffer containing KH₂PO₄/ K₂HPO₄ (0.2 M), pH 7.9, was used.
202 The change in absorbance was recorded at 340 nm for 1 min. The activity was
203 calculated as nmol of CDNB formed per $\text{min}^{-1}\cdot\text{mg}^{-1}$ prot (using a molar extinction
204 coefficient of $9.6\text{ mM}^{-1}\cdot\text{cm}^{-1}$).

206 **2.8. Lipid peroxidation (LPO)**

207 Oxidative damage was analysed in terms of LPO. Determination of
208 malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) concentration, both sub-
209 products of polyunsaturated fatty acid peroxidation, was realized using the method
210 described by Erdelmeier et al. (1998) and maximal absorbance at 586 nm.
211 Malondialdehyde bis-(dimethyl acetal) (Sigma-Aldrich) was used as a standard. The
212 LPO is expressed in nmol of MDA + 4-HNE mg^{-1} prot.

214 **2.9. Statistical analysis**

215 The non-parametric Kruskal-Wallis test was used to assess the significant
216 differences between exposure groups and tissues for each variable (biomarkers and
217 metals concentration). Results were significantly different when $p<0.05$. Principal
218 components analysis (PCA) was used in the ordination method to evaluate the
219 relationship between the different treatments and the analysed variables in the gills and
220 the hepatopancreas.

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223 3. Result and Discussion

224 3.1. Metal accumulation

225 The baseline metals concentrations in *R. exoculata* collected from TAG for the
226 different tissues are described in the Table 1. In all tissues the metal concentration
227 descending trend is the following Fe > Cu > Zn > Mn. In the total soft tissues, gills and
228 muscle Co is more abundant than Cd, and the opposite was noted in the hepatopancreas
229 (Table 1). The TAG black smoker presents hot fluids rich in metals (Fe 5590 μM , Cu
230 120-150 μM , Zn 46 μM , Mn 680 μM , Co <2 μM , Cd 66 nM) what is reflected by the
231 accumulation of these metals in *R. exoculata* tissues (Table 1). This is in agreement
232 with previous studies, with the same species but from another Mid-Atlantic Ridge vent
233 field - Rainbow (hot fluids metal contents: Fe 24000 μM , Cu 140 μM , Zn 160 μM , Mn
234 2250 μM , Co 13 μM , Cd 130 nM; Douville et al. 2002). At both sites, the concentration
235 of metals in different tissues is coherent with the variation of metals in the hot fluids.
236 Rainbow's hot fluid is much richer in iron than TAG's fluid and iron accumulation is
237 higher in shrimp's gills from the Rainbow vent site (90460 $\mu\text{g}\cdot\text{g}^{-1}$ d.w.; Kádár et al.
238 2006) when compared to those from TAG (6201 $\mu\text{g}\cdot\text{g}^{-1}$ d.w.; Table 1).

239 Vent shrimps do show higher natural Cu concentration in their body (379 $\mu\text{g}\cdot\text{g}^{-1}$
240 d.w.) than their coastal counterparts ($\sim 100\mu\text{g}\cdot\text{g}^{-1}$ d.w.; White and Rainbow 1985). In a
241 review by Pourang et al. (2004), the distribution of trace elements on coastal shrimps
242 were listed, and for Cu, gills always show higher Cu concentrations, followed by
243 hepatopancreas and then muscle. Nevertheless, in the present study, the hepatopancreas
244 showed higher Cu concentration, and this observation is different from other studies
245 where gills presented the highest concentration for the same species (Kádár et al. 2006,
246 Geret et al. 2002). However, the present results are consistent with other studies on vent
247 crustaceans, such as the accumulation of trace metals in the crab *Bythograea*
248 *thermydrion* where the highest Cu levels were found in the hepatopancreas (Cosson and
249 Vivier 1997). The high amount of Cu present in the hepatopancreas can be explained by
250 the presence of high amounts of haemocyanin, copper-sulphur containing granules and
251 MTs. Haemocyanin is one of the main protein synthesized in this organ (could represent
252 50% of total protein synthesized; Viarengo and Nott 1993).

253 For the copper exposure experiment, Cu concentrations in the tissues for the
254 different treatments are shown in the Figure 1 (see also Table A.1 in supplementary
255 material). The results do not show significant differences between groups ($p>0.05$),
256 except for the zinc with higher concentrations in the gills ($277 \mu\text{g.g}^{-1}$ d.w.) and in the
257 total soft tissues ($112 \mu\text{g.g}^{-1}$ d.w.) of the *in situ* group when compared to shrimps
258 exposed to $4 \mu\text{M}$ of Cu ($69 \mu\text{g.g}^{-1}$ d.w. and $49 \mu\text{g.g}^{-1}$ d.w., respectively; $p<0.05$). For the
259 *in situ* group, the concentration of Co and Mn is similar in the hepatopancreas and gills,
260 but in the groups incubated inside the IPOCAMP there is a higher concentration in the
261 gills. Cd is mainly accumulated in the hepatopancreas ($8.3 \mu\text{g.g}^{-1}$ d.w.), when compared
262 to the gills ($1.2 \mu\text{g.g}^{-1}$ d.w) and the muscle ($0.15 \mu\text{g.g}^{-1}$ d.w.; Fig. 1). As the *in situ*
263 group represents the baseline concentrations, these results suggest that after incubation
264 inside the IPOCAMP, where only Cu was added but not the other metals, there is a
265 redistribution of metals among the different tissues. As some of these are essential
266 metals, these results suggest their potential use in other biological functions in the gills,
267 or that their excess are further eliminated/regulated through the gills.

268 A high variability in Cu concentration among the shrimp replicates was noted
269 (Fig. 1). Still, the Cu concentration in the hepatopancreas decreases in the groups
270 exposed to Cu (Cu1: $440 \mu\text{g.g}^{-1}$ d.w., Cu2: $810 \mu\text{g.g}^{-1}$ d.w.) when compared to the *in*
271 *situ* and control groups (IS: $1500 \mu\text{g.g}^{-1}$ d.w., C: $1376 \mu\text{g.g}^{-1}$ d.w.). On the other hand, a
272 slight increase in Cu concentration is observed in the gills for the Cu exposed groups
273 while a non-significant decrease in Cu present in the muscle is noticed ($p>0.05$; Fig. 1).
274 Although, there is no published copper concentration values for *R. exoculata* habitat at
275 TAG, the present results observed for the hepatopancreas suggest an elimination
276 process during the 72 h incubation. Crustaceans possess Cu sulphur granules in the
277 hepatopancreas and these may play a role as a detoxification mechanism. In shrimps,
278 copper and sulphur are accumulated in the epithelial cells of the hepatopancreas as
279 insoluble granules, thus decreasing the potential toxic effect of Cu present in the
280 cytoplasm of cells (Viarengo and Nott 1993, Ahearn et al. 2004). Moreover, the total Cu
281 amount for the two exposed groups do not show significant differences which is in
282 favour of a potential regulation mechanism of metal metabolism by the shrimps. White
283 and Rainbow (1982) found that *Palaemon elegans* are able to regulate body levels of
284 copper up to a threshold exposure level, beyond which the metal is accumulated in
285 direct proportion to external metal levels (up to $100 \mu\text{g.L}^{-1}$ for the coastal shrimp). This

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286 threshold would presumably be higher for vent shrimps as they are constantly bathed in
287 seawater rich in metals. This regulation can result from a balance between accumulation
288 and elimination rate.

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290 **3.2. Metallothioneins**

291 The MTs levels increased in the gills of the Cu exposed groups when compared
292 to control and IS, with significant differences between the control ($155 \mu\text{g.g}^{-1}$ w.w.) and
293 Cu2 ($332 \mu\text{g.g}^{-1}$ w.w.; $p < 0.05$; Fig. 2). Higher amounts of MTs ($400 \mu\text{g.g}^{-1}$ w.w.) were
294 observed in the hepatopancreas (Fig. 2) while the muscle had the lowest, but in both
295 tissues no significant differences were observed between the different treatments
296 ($p > 0.05$).

297 To our knowledge only two studies on MTs levels in *R. exoculata* were
298 previously reported. The study by Geret et al. (2002) presents data of MTs per tissues
299 for *R. exoculata* from the Rainbow hydrothermal vent field. The MT data obtained in
300 the present study (gills: $161 \mu\text{g.g}^{-1}$ w.w.; muscle: $27 \mu\text{g.g}^{-1}$ w.w.; Fig. 2) are in the same
301 order of magnitude as for *R. exoculata* from the Rainbow (gills: $255 \mu\text{g.g}^{-1}$ w.w.;
302 muscle: $72 \mu\text{g.g}^{-1}$ w.w.; Geret et al. 2002). However, TAG shrimps have lower MT
303 levels in the hepatopancreas ($399 \mu\text{g.g}^{-1}$ w.w.) than those from Rainbow ($3012 \mu\text{g.g}^{-1}$
304 w.w.; Geret et al. 2002). The other study that investigated MTs levels analysed the soft
305 tissues of *R. exoculata* and *Mirocaris fortunata* which do not allow a direct comparison
306 with the present, per tissue, results (Gonzalez-Rey et al. 2008).

307 MTs are induced in response to the Cu exposure. The significant induction of
308 MTs in the gills at higher Cu concentration ($4 \mu\text{M}$) when compared to the control group
309 seems to follow the slight increase of Cu (Figs. 1 and 2). This is probably linked the role
310 of MTs in the detoxification and/or storage of Cu. An antioxidant role for MTs was
311 suggested in previous studies with the mussel *Mytilus galloprovincialis*, through
312 oxyradical scavenging protecting both isolated cells and the entire organism from
313 oxidative stress (Viarengo et al. 1999). In the hepatopancreas, MTs levels do not change
314 between groups ($p > 0.05$). Different explanations may be proposed. First, Campana et al.
315 (2015) suggest that in the shrimp *Pandalus montagui*, most of the Cu is accumulated in
316 a metabolically available form and excretion occurs from this component, implying that
317 Cu could be eliminated in a metabolically active form and so would not induce MTs

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318 response. On the other hand, MTs can be linked to Cd, which is mainly accumulated in
319 the hepatopancreas (Fig. 1) and whose amount does not vary after 72h of incubation.
320 The affinity of MTs with metals defines their binding abilities (Cu>Cd>Zn; Engel and
321 Brouwer 1989). Cd is a non-essential metal, thus to avoid toxicity the organisms have to
322 limit their accumulation or to complex the accumulated metal to a non- or less-toxic
323 form (Depledge and Rainbow 1990).

324

325 **3.3. Antioxidant enzymatic response**

326 The antioxidant enzymes activities show a tissue specific response and the
327 separate analysis per tissue (not whole organism) is recommended for future studies, for
328 higher resolution of the results. SOD activity is higher in the hepatopancreas with IS
329 and C groups showing the highest values (IS: 95 U.mg⁻¹ prot, C: 123 U.mg⁻¹ prot; Fig.
330 3) and following the trend of Cu concentration (Table 1 and Fig. 1). This is in line with
331 previous suggestions that the increase in cytosolic SOD activity is stimulated by higher
332 Cu concentrations (Greco et al. 1990, Li et al. 2006). In the gills, no significant change
333 was noted between the different treatments ($p>0.05$). The highest GPx activity is
334 observed in the hepatopancreas of the *in situ* group (706 nmol.min⁻¹.mg⁻¹ prot; Fig. 3),
335 which is significantly higher than in control (366 nmol.min⁻¹.mg⁻¹ prot, $p<0.05$).

336 Both CAT and GST activities follow a similar pattern (Fig. 3). An increasing
337 trend of these enzymes activities in the gills follow the increasing Cu concentration (IS:
338 1.3 μmol CAT.min⁻¹.mg⁻¹ prot, 116 μmol GST.min⁻¹.mg⁻¹ prot, Cu2: 2.8 μmol
339 CAT.min⁻¹.mg⁻¹ prot, 301 μmol GST.min⁻¹.mg⁻¹ prot) but are not significantly different
340 ($p>0.05$). Increasing CAT activity with increasing Cu concentration has been previously
341 observed (Li et al. 2006). Due to the low amount of tissue available, it was not possible
342 to determine the CAT activity for the hepatopancreas. The GST activity in the
343 hepatopancreas for the *in situ* group (225 μmol.min⁻¹.mg⁻¹ prot) was significantly lower
344 when compared to the control group (406 μmol.min⁻¹.mg⁻¹ prot, $p<0.05$). Moreover, for
345 the groups placed in the IPOCAMP, a slight downward trend in GST activity was
346 observed with increasing Cu concentration. CAT and GST activities in the muscle were
347 higher for the *in situ* and Cu1 groups, with Cu1 significantly higher than Cu2 for both
348 enzymes ($p<0.05$; Fig. 3). These enzyme activities detected in the tissues of shrimps
349 suggest a protective mechanism against the presence of ROS, as previously noted by

350 Gonzalez-Rey et al. (2008). In their study, they found that CAT in soft tissues of
351 shallow-water shrimps as lower activity than in vents shrimps, while higher values of
352 SOD and GPx activities were found in shallow-water shrimps (Gonzalez-Rey et al.
353 2008; Table 2). When comparing the enzyme activities in the hepatopancreas, shallow-
354 water shrimps show lower activity for GPx (8.8 and 4.71 nmol.min.mg⁻¹.prot for *P.*
355 *serratus*; Oliveira et al. 2012, Silva et al. 2013) and GST (6.4 and 3.1 nmol.min.mg⁻¹
356 ¹.prot for *P. serratus*; Oliveira et al. 2012, Silva et al. 2013) when compared to *R.*
357 *exoculata* (GPx: 804.2 nmol.min.mg⁻¹.prot, GST: 225.6 nmol.min.mg⁻¹.prot; Ttable 2).
358 Similarly, *R. exoculata* presents higher values of CAT activity in the muscle (0.55
359 μmol.min.mg⁻¹.prot) when compared to *P. elegans* (0.0053 μmol.min.mg⁻¹.prot for;
360 Vinagre et al. 2014; Table 2). These observations are suggesting a detoxification
361 strategy in this vent shrimp, adapted to an enriched metal extreme environment.

362

363 **3.4. Oxidative damage**

364 The LPO level in the gills of the *in situ* shrimps (1.3 nmol.mg⁻¹ prot) was lower
365 than in the other groups (Fig. 4). The control group shows higher damage (2.3 nmol.mg⁻¹
366 ¹ protein) in the gills followed by a smooth decreasing trend of LPO levels with
367 increasing Cu concentration (Cu1: 2 nmol.mg⁻¹ prot, Cu2: 1.7 nmol.mg⁻¹ prot), although
368 no significant difference was found ($p>0.05$). LPO in the muscle is similar for the two
369 groups exposed to Cu (0.50 and 0.54 nmol.mg⁻¹ prot for Cu1 and Cu2 respectively;
370 $p>0.05$) while in the *in situ* group it is more than 1.5-fold higher than in the exposed
371 groups (IS and Cu1 $p<0.05$). For the soft tissues of *R. exoculata*, from Rainbow vent,
372 LPO was of the same order of magnitude (0.3 ± 0.05 nmol.mg⁻¹ prot; Gonzalez-Rey et
373 al. 2008). These results are 3-fold higher than that obtained for the coastal shrimps *P.*
374 *elegans* and *P. varians*. (Gonzalez-Rey et al. 2008). Nevertheless, results suggest that
375 Cu, at the exposure concentrations used in this study, is not inducing lipid peroxidation
376 in *R. exoculata*.

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378

379 **3.5. Relationship between metal accumulation and biomarkers responses**

380 The present results reinforce the fact that vent fauna, especially *R. exoculata*,
381 have developed physiological adaptive strategies to face the metal-rich habitat they live

382 in. Moreover, the establishment of protection through the antioxidant enzymes activities
383 seems correlated with the decreasing trend of LPO levels in the gills. The activity of
384 enzymes seems to have a beneficial effect in decreasing the amount of ROS in cells and
385 consequently limit the attack of ROS on membrane lipids. This result is in favour of a
386 good detoxification process in the presence of copper. Nevertheless, the rather low
387 impact of 4 μ M of Cu exposure on the shrimps might be due to the fact that they may
388 have developed protective strategies as they are continuously bathed in a metal rich
389 medium. Several studies have suggested the possibility of detoxification through the
390 bacteria present in the gills chamber or in the gut (e.g. Jan et al. 2015). Part of the toxic
391 compounds could be taken up by the bacteria hosted in the gills chamber of the shrimps
392 and decreasing the amount of toxic compounds accumulated in shrimps tissues
393 (Compère et al. 2002, Zbinden and Cambon-Bonavita 2003).

394 Given the small differences observed between the *in situ* and control groups for
395 the variables assessed, the results suggest that the shrimps are apparently tolerant to the
396 change of pressure (occurring during the water renewal) for at least the period of 72 h.
397 The proposed protocol, including the pressurization and depressurization steps of the
398 high-pressure aquarium for seawater renewal, seems to be an adequate method to assess
399 toxicity to contaminants in deep-sea species. This may enable future longer term assays
400 under high-pressure and low-temperature conditions, which will be more realistic from
401 the perspective of potential deep-sea mining impacts. Nevertheless, future studies
402 should attempt to increase the samples size and also look at the effects of other
403 contaminants likely to be released during mining activities.

404

405 **4. Conclusions**

406 This study gives a first appraisal of the amounts of metals, MTs, antioxidant
407 enzymes and LPO in different tissues of the vent shrimp *Rimicaris exoculata* from the
408 TAG hydrothermal vent field. *R. exoculata* shows a background protection against
409 metal toxicity as the activities of enzymes were detected for the *in situ* group. A tissue
410 specific response was observed for the biomarkers analysed. Shrimps appear to develop
411 protection against exposure to Cu, with increasing activity of antioxidant enzymes
412 (CAT and GST) and induction of MT in the higher Cu concentration exposure (4 μ M).
413 The ubiquity of the shrimp *R. exoculata* along the Mid-Atlantic Ridge and the similar

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414 responses observed in other vent sites (e.g. Rainbow) with those obtained in this study
415 render this species as a good model for a possible monitoring of mining effects in
416 hydrothermal vent fauna. Moreover, results suggest that the use of the pressurized
417 aquarium do not affect the shrimps in terms of biomarkers responses to metal exposure.
418 This method looks promising for further biomarkers responses studies with other deep-
419 sea fauna.

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421

422 **Acknowledgements**

423 This work was developed under the MIDAS project (Managing impacts of deep-sea
424 resource exploitation), funded by the European Commission 7th Framework
425 Programme under the theme “Sustainable management of Europe’s deep sea and sub-
426 seafloor resources” (Grant Agreement 603418). We thank the captain and the crew of
427 the *Pourquoi pas?* Vessel and ROV Victor 6000 and the chief scientist of the BICOSE
428 cruise. MA was supported by a MER MSc grant and the French mobility grants Idex
429 and Aquimob.

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643 **Figure captions**

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2 644 **Figure 1.** Metals concentrations (mean \pm SD) in tissues of *Rimicaris exoculata* for the
3
4 645 different treatments. Values followed by the same letter are not significantly different (p
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6 646 > 0.05) with capital letters for comparison of one tissue between groups and lowercase
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8 647 letters for comparison among one group. Gills (grey), Hepatopancreas (black),
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10 648 Abdominal muscle (white), IS: In situ group, C: Control, Cu1: exposed to 0.4 μ M, Cu2:
11 649 exposed to 4 μ M.

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16 651 **Figure 2.** Metallothioneins (MTs, mean \pm SD) in tissues of *Rimicaris exoculata* for the
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18 652 different treatments. Values followed by the same letter are not significantly different (p
19
20 653 > 0.05) with capital letters for comparison of one tissue between groups and lowercase
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22 654 letters for comparison among one group. Gills (grey), Hepatopancreas (black),
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24 655 Abdominal muscle (white), IS: In situ group, C: Control, Cu1: exposed to 0.4 μ M, Cu2:
25 656 exposed to 4 μ M.

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30 658 **Figure 3.** Superoxide dismutase (SOD), catalase (CAT), total glutathione peroxidase
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32 659 (GPx) and glutathione-S-transferase (GST) activities (mean \pm SD) in tissues of
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34 660 *Rimicaris exoculata* for the different treatments. Values followed by the same letter are
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36 661 not significantly different ($p > 0.05$) with capital letters for comparison of one tissue
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38 662 between groups and lowercase letters for comparison among one group. Gills (grey),
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40 663 Hepatopancreas (black), Abdominal muscle (white), IS: In situ group, C: Control, Cu1:
41 664 exposed to 0.4 μ M, Cu2: exposed to 4 μ M. Given the small amount of tissue available
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43 665 for HP, CAT was not analysed. GPx activity was not detected in the abdominal muscle.

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47 667 **Figure 4.** Lipid peroxidation (LPO, mean \pm SD) in tissues of *Rimicaris exoculata* for
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49 668 the different treatments. Values followed by the same letter are not significantly
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51 669 different ($p>0.05$) with capital letters for comparison of one tissue between groups and
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53 670 lowercase letters for comparison among one group. Gills (grey), Hepatopancreas
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55 671 (black), Abdominal muscle (white), IS: In situ group, C: Control, Cu1: exposed to
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57 672 0.4 μ M, Cu2: exposed to 4 μ M.

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674 **Table captions**

675 **Table 1.** Metal concentration (mean \pm SD) in tissues of *Rimicaris exoculata* and
676 *Mirocaris fortunata*. Data expressed in $\mu\text{g.g}^{-1}$ d.w. or $\mu\text{mol.g}^{-1}$ w.w.. G: Gills; H:
677 Hepatopancreas; M: Abdominal muscle; S.T.: Soft tissue.

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679 **Table 2.** Antioxidant enzymes activities (SOD, CAT, GPx, GST) (mean \pm SD) in
680 tissues of the vent shrimp *Rimicaris exoculata* and coastal shrimps *Palaemon elegans*,
681 *Palaemon serratus*, *Palaemon varians*. G: Gills; H: Hepatopancreas; M: Abdominal
682 muscle; S.T.: Soft tissue.

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684 **Supplementary Material**

685 **Table A.1.** Metal concentration per tissues (gills, hepatopancreas, muscle) and weighted
686 mean (soft tissue) for *Rimicaris exoculata* in the different treatments (mean \pm SD),
687 expressed in $\mu\text{g.g}^{-1}$ d.w.. Values followed by the same letter are not significantly
688 different ($p>0.05$) with capital letters for comparison of one tissue between groups and
689 lowercase letters for comparison among one group (for each metal). IS: In situ group; C:
690 Control; Cu1: exposed to $0.4\mu\text{M}$; Cu2: exposed to $4\mu\text{M}$.

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691 **Table 1.**

Species	Sites	Tissues	Cu	Cd	Co	Fe	Mn	Zn	References
<i>Rimicaris exoculata</i>	TAG ($\mu\text{g}\cdot\text{g}^{-1}$ d.w.)	G	535 ± 205	1.24 ± 0.91	3 ± 2	6201 ± 4299	26 ± 11	277 ± 123	this study
		H	1562 ± 592	8.32 ± 1.68	3.7 ± 1.7	6122 ± 1764	29 ± 10	94 ± 64	
		M	98 ± 23	0.15 ± 0.03	0.3 ± 0.1	1052 ± 307	6 ± 2	88 ± 39	
		S.T.	379 ± 146	1.3 ± 0.4	1.6 ± 0.3	2740 ± 1191	16 ± 3.5	112 ± 31	
	(μmol.g ⁻¹ w.w.)	G	0.75 ± 0.18	0.32 10 ⁻³ ± 0.1 10 ⁻³	3.1 10 ⁻³ ± 5 10 ⁻⁴	3.6 ± 1.1	0.022 ± 0.004	0.22 ± 0.06	this study
		H	2.51 ± 0.78	8.710 ⁻³ ± 2.1 10 ⁻³	5.6 10 ⁻³ ± 2 10 ⁻⁴	12.9 ± 2.6	0.06 ± 0.02	0.24 ± 0.09	
		M	0.11 ± 0.02	0.1 10 ⁻³ ± 0.03 10 ⁻³	0.5 10 ⁻³ ± 1 10 ⁻⁴	1.5 ± 0.4	0.008 ± 0.002	0.13 ± 0.02	
		S.T.	0.32 ± 0.18	0.0008 ± 0.0003	0.002 ± 0.0016	2.8 ± 1.5	0.02 ± 0.01	0.13 ± 0.04	
	Rainbow (μmol.g ⁻¹ w.w.)	G	1.2 ± 0.6	0.014 ± 4 10 ⁻³	-	25.5 ± 11.4	0.316 ± 0.18	17.9 ± 5.0	Geret et al. 2002
		H	0.61 ± 0.21	3.5 10 ⁻³ ± 9 10 ⁻⁴	-	24.6 ± 1.8	0.076 ± 0.009	5.7 ± 1.7	
		M	0.26 ± 0.08	1.1 10 ⁻³ ± 9 10 ⁻⁴	-	3.1 ± 1.86	0.039 ± 0.026	1.5 ± 1.1	
	Rainbow (μg.g ⁻¹ d.w.)	G	1060 ± 200	-	-	90460 ± 48600	-	2900 ± 400	Kádár et al. 2006
H		700 ± 200	-	-	6640 ± 2230	-	800 ± 700		
M		300 ± 160	-	-	610 ± 260	-	300 ± 100		
Rainbow (μg.g ⁻¹ d.w.)	S.T.	176.7 ± 74	0.49 ± 0.02	-	2122 ± 867	43.2 ± 10.4	209 ± 111	Gonzalez-Rey et al. 2008	
<i>Mirocaris fortunata</i>	Rainbow (μg.g ⁻¹ d.w.)	G	1700 ± 500	-	-	122970 ± 41600	-	4300 ± 800	Kádár et al. 2006
		H	400 ± 100	-	-	3010 ± 620	-	600 ± 100	
		M	200 ± 60	-	-	940 ± 240	-	200 ± 40	
	Lucky Strike (μg.g ⁻¹ d.w.)	G	1400 ± 500	-	-	44170 ± 15320	-	1900 ± 800	Kádár et al. 2006
		H	500 ± 200	-	-	1870 ± 250	-	900 ± 600	
		M	40 ± 10	-	-	190 ± 80	-	60 ± 30	

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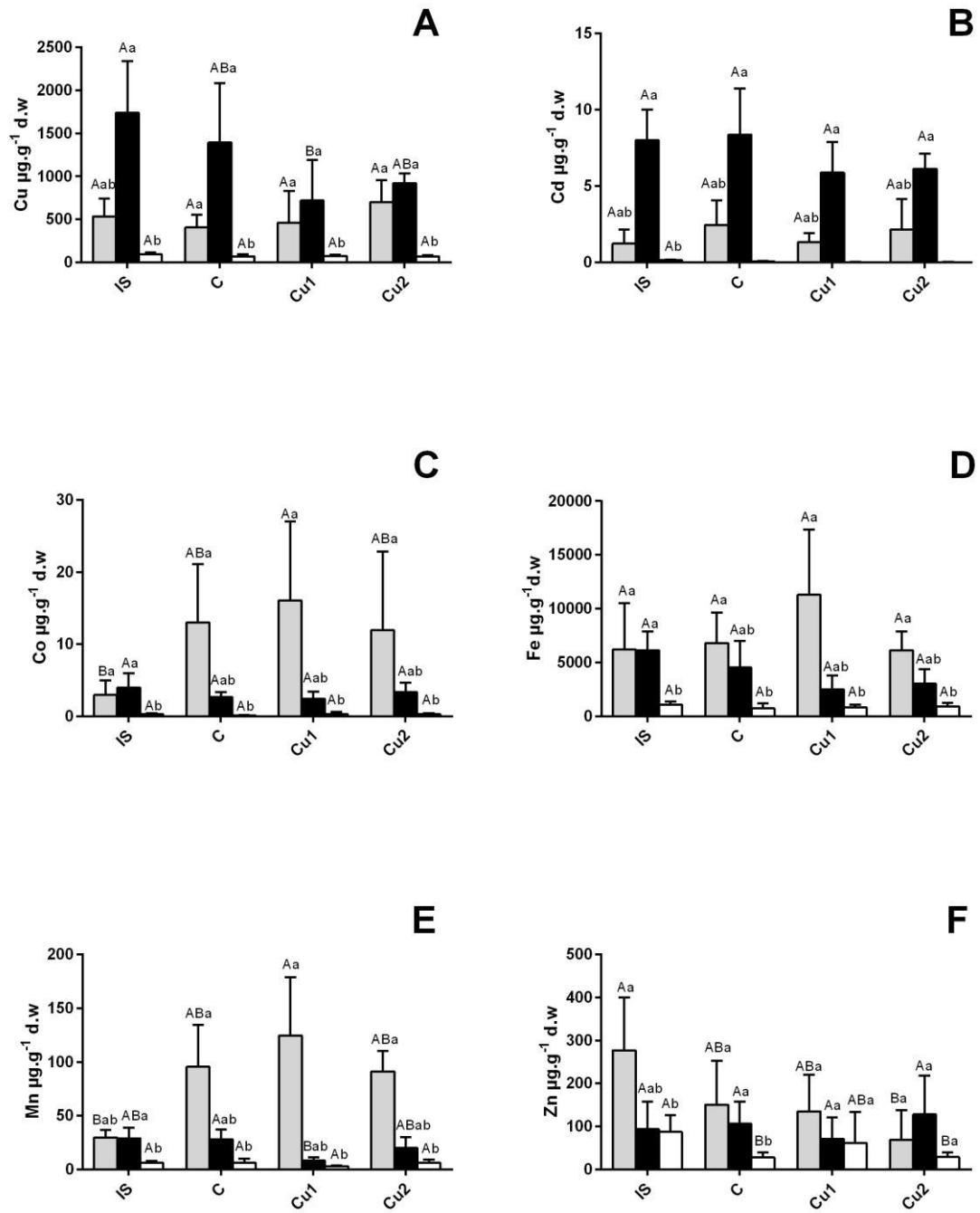
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693 **Table 2.**

Species		SOD (U.mg ⁻¹ .prot)	CAT (μmol.min.mg ⁻¹ .prot)	GPx (nmol.min.mg ⁻¹ .prot)	GST (nmol.min.mg ⁻¹ .prot)	References
<i>R. exoculata</i>	G	53.3 ± 8.8	1.3 ± 0.5	191.9 ± 68.8	116.3 ± 52.7	this study
	H	95.3 ± 26.2	-	804.2 ± 167.7	225.6 ± 40.8	
	M	19.2 ± 5.1	0.6 ± 0.2	-	12.5 ± 2.2	
	S.T	1.9 ± 0.4	4.2 ± 0.3	10 ± 1	-	
<i>P. elegans</i>	M	-	5.3 ± 0.3*	-	0.56 ± 0.3	Vinagre et.al 2014
	S.T	3.9 ± 1.3	1.2 ± 0.3	15 ± 3	-	Gonzalez-Rey et al. 2008
<i>P. serratus</i>	M	-	0.7 ± 0.3*	-	0.3 ± 0.1	Vinagre et.al 2014
	H	-	1.5 ± 0.1	8.8 ± 0.6	3.1 ± 0.3	Silva et al. 2013
	H	-	1.5 ± 0.3*	4.7 ± 0.3	6.4 ± 0.4	Oliveira et al.2012
<i>P. varians</i>	S.T	4.4 ± 1.1	2 ± 0.4	22 ± 6	-	Gonzalez-Rey et al. 2008

694 * nmol.min.mg⁻¹.prot

695 **Figure 1**



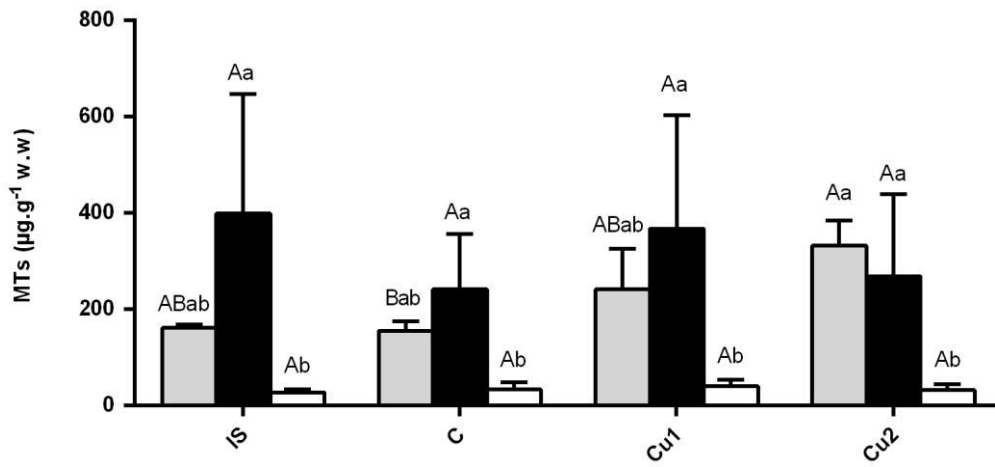
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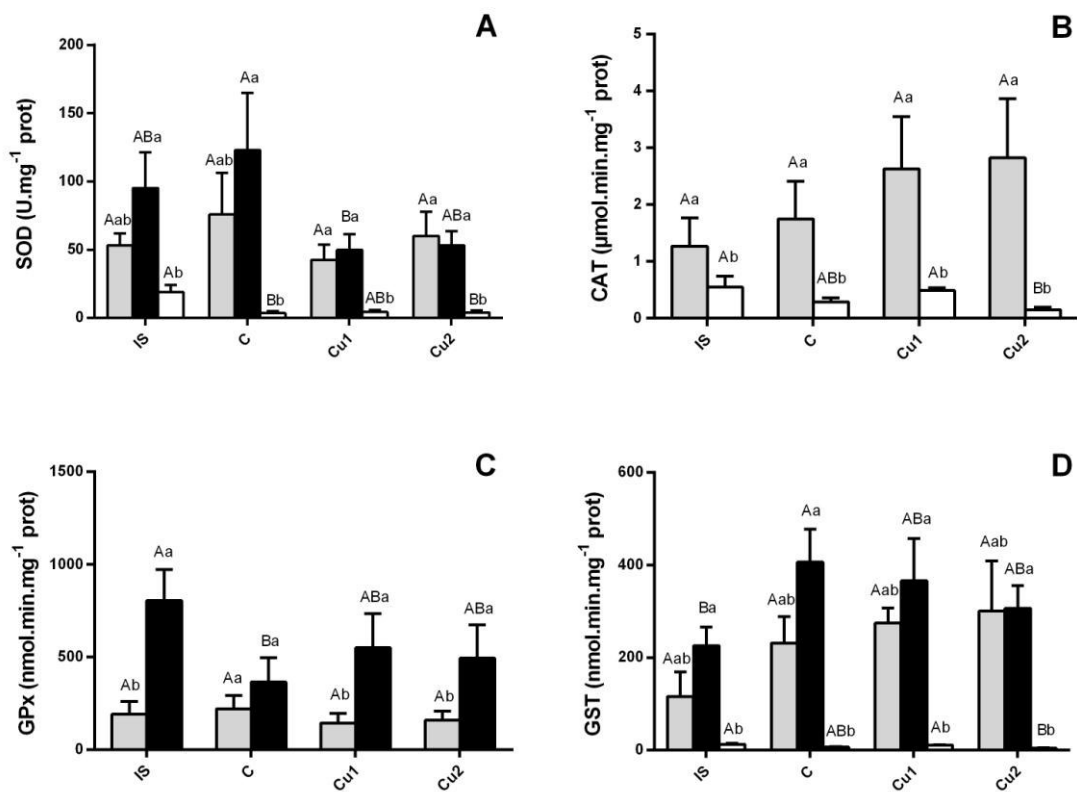
700 **Figure 2**



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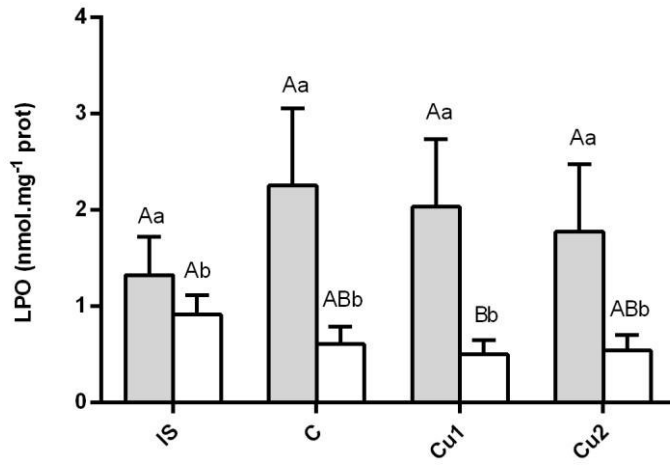
703 **Figure 3**



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706 **Figure 4**



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716 **Table A.1.**

		IS	C	Cu1	Cu2
Gills	Cu	535 ± 205 ^{Aab}	408 ± 145 ^{Aa}	461 ± 369 ^{Aa}	700 ± 258 ^{Aa}
	Cd	1 ± 0.9 ^{Aab}	2 ± 1.6 ^{Aab}	1 ± 0.6 ^{Aab}	2.2 ± 2 ^{Aab}
	Co	3 ± 2 ^{Ba}	13 ± 8 ^{ABa}	16 ± 11 ^{Aa}	12 ± 10.9 ^{ABa}
	Fe	6201 ± 4299 ^{Aa}	6791 ± 2851 ^{Aa}	11285 ± 6051 ^{Aa}	6129 ± 1751 ^{Aa}
	Mn	30 ± 7 ^{Bab}	96 ± 39 ^{ABa}	125 ± 54 ^{Aa}	91 ± 19 ^{ABa}
	Zn	277 ± 123 ^{Aa}	151 ± 102 ^{ABa}	135 ± 85 ^{ABa}	69 ± 69 ^{Ba}
	Hepato-pancreas	Cu	1740 ± 600 ^{Aa}	1395 ± 690 ^{ABa}	719 ± 474 ^{Ba}
Cd		8 ± 2 ^{Aa}	8 ± 3 ^{Aa}	6 ± 2 ^{Aa}	6 ± 1 ^{Aa}
Co		3.7 ± 2 ^{Aa}	2.7 ± 0.7 ^{Aab}	2 ± 0.9 ^{Aab}	3.4 ± 1.3 ^{Aab}
Fe		6122 ± 1764 ^{Aa}	4541 ± 2466 ^{Aab}	2488 ± 1297 ^{Aab}	3046 ± 1331 ^{Aab}
Mn		29 ± 10 ^{ABa}	28 ± 9 ^{Aab}	8 ± 3 ^{Bab}	20 ± 10 ^{ABab}
Zn		94 ± 64 ^{Aab}	107 ± 51 ^{Aa}	72 ± 50 ^{Aa}	128 ± 90 ^{Aa}
Muscle		Cu	94 ± 22 ^{Ab}	71 ± 24 ^{Ab}	75 ± 17 ^{Ab}
	Cd	0.15 ± 0.03 ^{Ab}	0.06 ± 0.05 ^{ABb}	0.02 ± 0.02 ^{Bb}	0.01 ± 0.03 ^{Bb}
	Co	0.34 ± 0.1 ^{Ab}	0.13 ± 0.09 ^{Ab}	0.34 ± 0.26 ^{Ab}	0.3 ± 0.1 ^{Ab}
	Fe	1052 ± 307 ^{Ab}	724 ± 490 ^{Ab}	813 ± 245 ^{Ab}	906 ± 349 ^{Ab}
	Mn	6.3 ± 1.6 ^{Ab}	6.2 ± 4 ^{Ab}	3 ± 0.8 ^{Ab}	6.5 ± 2.6 ^{Ab}
	Zn	88 ± 39 ^{Ab}	28 ± 12 ^{Bb}	62 ± 72 ^{ABa}	29 ± 11 ^{Ba}
	Soft tissue	Cu	379 ± 146 ^A	331 ± 154 ^A	265 ± 98 ^A
Cd		1.3 ± 0.4 ^A	1.4 ± 0.3 ^A	1.3 ± 0.45 ^A	1.5 ± 0.7 ^A
Co		1.1 ± 0.3 ^A	3.4 ± 2 ^A	3.5 ± 1.7 ^A	2.9 ± 1.5 ^A
Fe		2740 ± 1191 ^A	2342 ± 1041 ^A	3757 ± 1547 ^A	2340 ± 810 ^A
Mn		11 ± 3.4 ^A	30 ± 20 ^A	30 ± 22 ^A	32 ± 15 ^A
Zn		112 ± 31 ^A	63 ± 29 ^{AB}	85 ± 53 ^{AB}	49 ± 14 ^B

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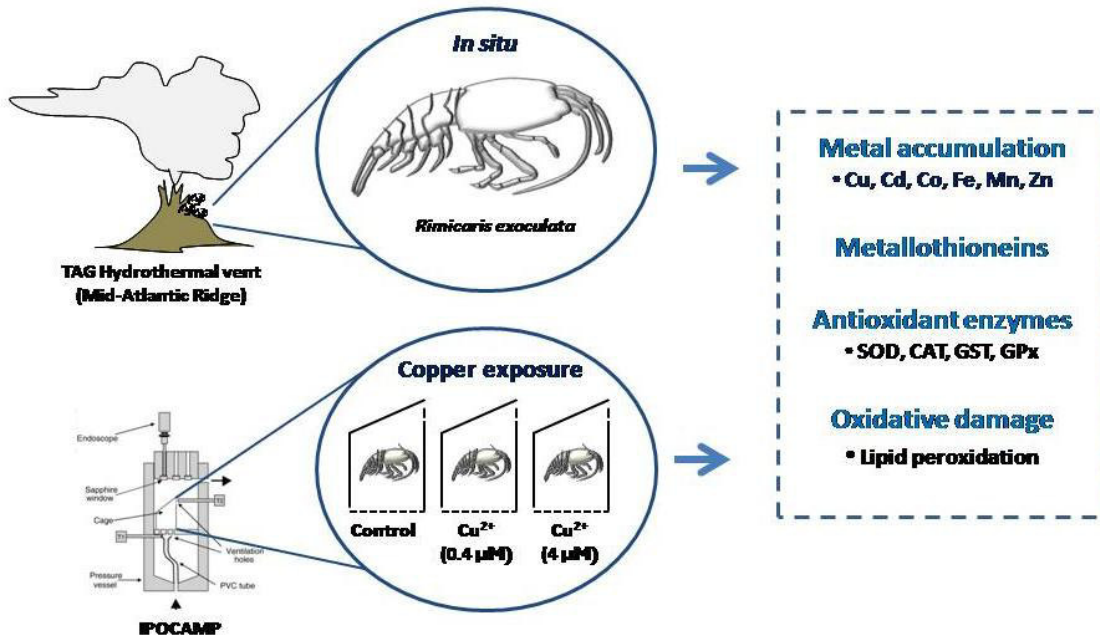
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728 Graphical Abstract



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1 **Highlights**

2 • *Rimicaris exoculata* have developed antioxidant defences to cope with a metal-
3 rich habitat.

4 • The depressurization of the test chamber every 12h did not affect the biomarkers
5 response.

6 • The proposed method seems useful to assess toxicity to contaminants in deep-
7 sea species.