

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

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### ABSTRACT

In light of deep-sea mining industry development, particularly interested in massive-sulphide deposits enriched in metals with high commercial value, efforts are increasing to better understand potential environmental impacts to local fauna. The aim of this study was to assess the natural background levels of biomarkers in the hydrothermal vent shrimp *Rimicaris exoculata* and their responses to copper exposure at *in situ* pressure (30 MPa) as well as the effects of depressurization and pressurization of the high-pressure aquarium IPOCAMP. *R. exoculata* were collected from the chimney walls of the hydrothermal vent site TAG (Mid Atlantic Ridge) at 3630 m depth during the BICOSE cruise in 2014. Tissue metal accumulation was quantified in different tissues (gills, hepatopancreas and muscle) and a battery of biomarkers was measured: metal exposure (metallothioneins), oxidative stress (catalase, superoxide dismutase, glutathione-S-transferase and glutathione peroxidase) and oxidative damage (lipid peroxidation). Data show a higher concentration of Cu in the hepatopancreas and a slight increase in the gills after incubations (for both exposed groups). Significant induction of metallothioneins was observed in the gills of shrimps exposed to 4 µM of Cu compared to the control group. Moreover, activities of enzymes were detected for the *in situ* group, showing a background protection against metal toxicity. Results suggest that the proposed method, including a physiologically critical step of pressurizing and depressurizing the test chamber to enable the seawater exchange during exposure to contaminants, is not affecting metal accumulation and biomarkers response and may prove a useful method to assess toxicity of contaminants in deep-sea species.

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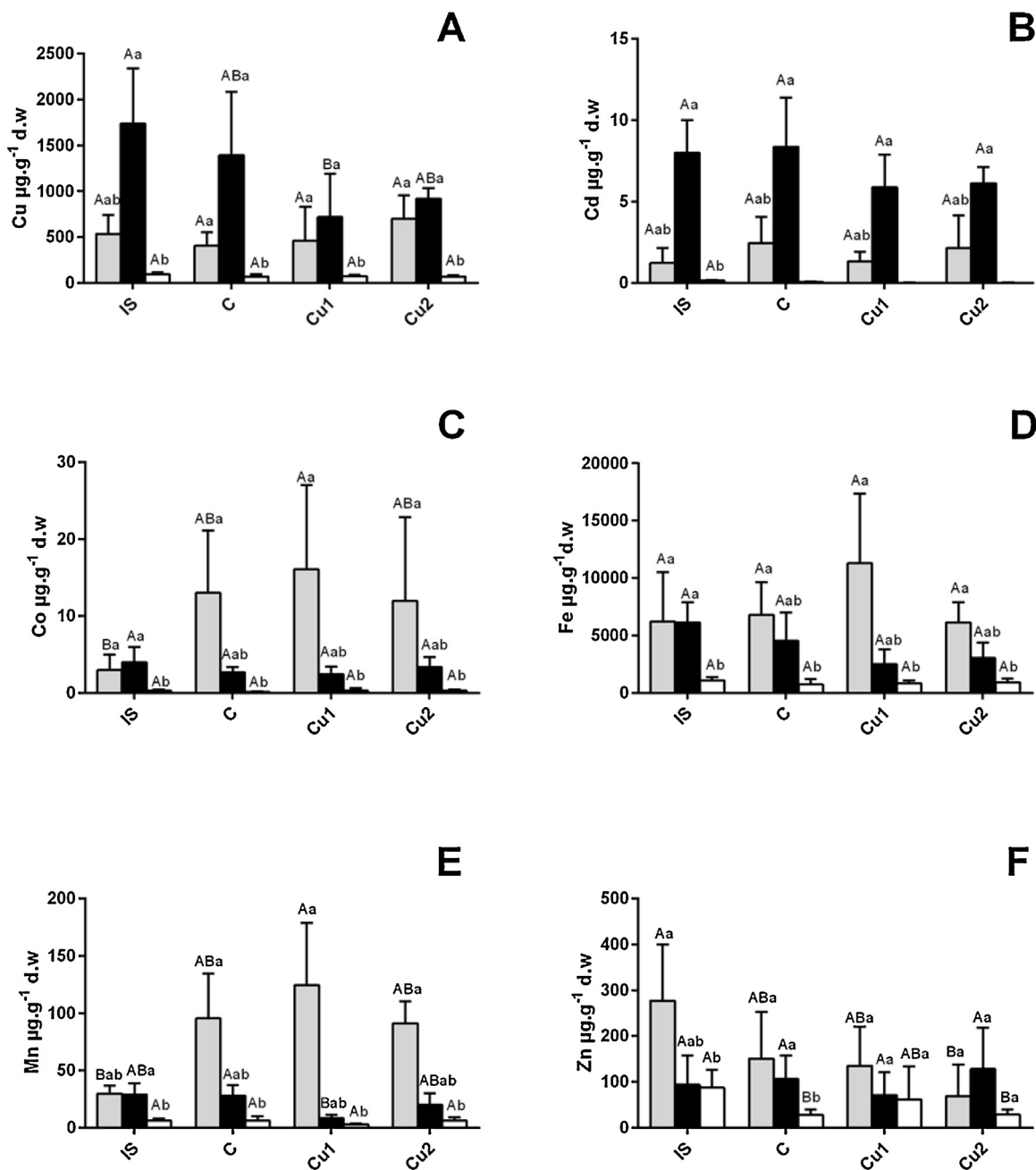
### 1. Introduction

Hydrothermal vents are massive sulphide deposits with high concentration of various metals (e.g. Cu, Ni, Zn, Mn, Co, Ag, Au) with potential economic interest to mining industries (Hannington et al., 2011). Recently, the Government of Papua New Guinea has given exploration license to Nautilus Minerals Corporation to explore this

type of mineral deposits in the Manus basin (Gena, 2013). However, it is likely that the impact of human activities will somehow change the natural equilibrium of this particular environment (Van Dover, 2014). The living fauna will likely be exposed to variations in environmental metal composition, hence the need to further explore the toxicity of chemicals associated with deep-sea exploitation. For this it is considered essential to adapt the existing ecotoxicological protocols from shallow-water to the deep-sea conditions to enable a better understanding of potential anthropogenic impact on deep-sea fauna (Mestre et al., 2014).

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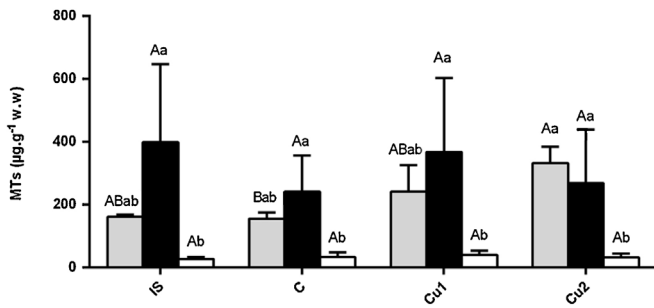


**Fig. 1.** Metals concentrations (mean  $\pm$  SD) in tissues of *Rimicaris exoculata* for the different treatments. Values followed by the same letter are not significantly different ( $p > 0.05$ ) with capital letters for comparison of one tissue between groups and lowercase letters for comparison among one group. Gills (grey), hepatopancreas (black), abdominal muscle (white), IS: *in situ* group, C: control, Cu1: exposed to 0.4  $\mu$ M, Cu2: exposed to 4  $\mu$ M.

The Trans-Atlantic Geotraverse (TAG) hydrothermal vent field is located on the Mid-Atlantic Ridge and its hot fluids are rich in various metals, including copper with concentrations ranging between 120 and 150  $\mu$ M (Edmonds et al., 1996). Copper is an essential trace element found in a variety of cells and tissues and used as cofactor of several enzymes. However, free Cu ions ( $\text{Cu}^+$  and  $\text{Cu}^{2+}$ ) can participate in the formation of reactive oxygen species (ROS) (Gaetke and Chow, 2003). As invertebrates possess the ability to concentrate certain metals from the surrounding medium, reflecting the variation in metal concentration in the environment (White and Rainbow, 1985), they are useful tools to monitor change of metal amounts as a result of anthropogenic activities.

Increasing total dissolved metal concentration will lead to a higher metal uptake rate (Rainbow, 1998). To prevent the increase

of metal accumulation in cells, organisms possess defence mechanisms such as specific proteins which play a role in binding metals, as for example the metallothioneins (MTs). These cysteine-rich non-enzymatic proteins are vital for various biological functions like storage, transport, compartmentalization of essential and non-essential metals (detoxification mechanisms) and can also act as oxyradicals scavengers protecting cells against ROS (Roesijadi, 1992). In the presence of certain metals and/or high-metals concentrations, ROS formation will induce an antioxidant defence mechanism. This mechanism consists in the action of specific enzymatic proteins, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase (GST). These enzymes will limit the ROS levels and prevent oxidative damage (Di Giulio et al., 1995). Nevertheless, if the action of these



**Fig. 2.** Metallothioneins (MTs, mean  $\pm$  SD) in tissues of *Rimicaris exoculata* for the different treatments. Values followed by the same letter are not significantly different ( $p > 0.05$ ) with capital letters for comparison of one tissue between groups and lowercase letters for comparison among one group. Gills (grey), hepatopancreas (black), abdominal muscle (white), IS: *in situ* group, C: control, Cu1: exposed to 0.4  $\mu\text{M}$ , Cu2: exposed to 4  $\mu\text{M}$ .

enzymes is not sufficient, the free radicals will reach the cellular membranes and cause oxidative damage, such as lipid peroxidation (LPO), which is the oxidation of polyunsaturated fatty acids (PUFAs; Halliwell and Gutteridge, 1984).

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

The chimney walls at TAG are covered by dense swarms (1200–3000 indiv  $\text{m}^{-2}$ ) of the vent shrimp *R. exoculata*, also known to be one of the most abundant invertebrate species in the Mid-Atlantic Ridge (Desbruyères et al., 2000; Schmidt et al., 2008). *R. exoculata* is also one of the most studied deep-sea species, making it a good model for investigating deep-sea environmental changes (e.g. Van Dover et al., 1988; Gebruk et al., 1993; Vereshchaka, 1997; Rieley et al., 1999; Allen et al., 2001; Geret et al., 2002; Ravaux et al., 2003; Zbinden et al., 2004, 2008; Copley et al., 2007; Gonzalez-Rey et al., 2007, 2008; Anderson et al., 2008; Corbari et al., 2008; Schmidt et al., 2008, 2009; Cottin et al., 2010; Ponsard et al., 2013; Jan et al., 2014).

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

This study assesses the biomarkers (MTs, antioxidant enzymes and LPO) response in different tissues (gills, hepatopancreas and abdominal muscle) in *R. exoculata* exposed to 0.4  $\mu\text{M}$  (25  $\mu\text{g L}^{-1}$ ) and 4  $\mu\text{M}$  (254  $\mu\text{g L}^{-1}$ ) of copper at *in situ* pressure and temperature (30 MPa, 10 °C). It also compares the metal accumulation (Cu, Zn, Fe, Mn, Co, Cd) and biomarkers baseline levels under natural conditions (isobaric sampling) and after maintenance in a high-pressure aquarium IPOCAMP (no metal exposure). Finally, this study also compares the effects of depressurization and pressurization of the

test chamber (conditions necessary for the Cu enriched seawater and oxygen renewal during the test).

## 2. Materials and methods

### 2.1. Sample collection

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

### 2.2. Cu exposure experiments

A total of 24 shrimps were used in this study, divided into 4 groups. The first group ( $n=6$ ) called *in situ* (IS) was dissected under sterile conditions and flash frozen in liquid nitrogen straight after collection and stored at  $-80^{\circ}\text{C}$  until further use. The other shrimps were directly transferred into the pressurized aquarium IPOCAMP for a Cu exposure experiment at 10 °C. A copper solution was prepared using  $\text{CuSO}_4$  (analytical grade, Sigma). Shrimps ( $n=6$  per condition) were isolated in 3.8 L boxes for: control (C; only seawater); 0.4  $\mu\text{M}$  of Cu (Cu1); and 4  $\mu\text{M}$  of Cu (Cu2). The experiment was carried out at 30 MPa and 10 °C for 72 h. For seawater and oxygen renewal, the IPOCAMP was depressurized every 12 h, and the viability of the animals was checked. At the end of the exposure period (72 h), survival of the shrimp was 100%, although one of the animals of the Cu2 group appeared less responsive to stimulation. The tissues were dissected (gills, hepatopancreas and muscle; without gut or exoskeleton) and immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further analyses.

### 2.3. Tissue preparation

Tissues were individually homogenized at 4 °C in a Tris-HCl (0.02 M, 5  $\text{mL g}^{-1}$  soft tissue) buffer with butylated hydroxytoluene (BHT, 10  $\mu\text{L mL}^{-1}$ ), pH 8.6. The homogenate (3 mL) was separated in soluble and insoluble fractions by centrifugation (30,000g, 45 min, 4 °C) and the remaining homogenate was used for determination of metal concentrations. After centrifugation, a part of the supernatant was used for the measurement of LPO and total protein content. The other part of the supernatant was heat treated at 80 °C for 10 min followed by a centrifugation (30,000g, 45 min, 4 °C) to separate the low molecular weight proteins, and the supernatant was used for MT measurements.

For antioxidant enzyme analysis, tissues were individually homogenized in 50 mM Tris-HCl buffer, pH 7.6, containing sucrose (250 mM),  $\text{MgCl}_2$  (5 mM), DTT (1 mM). After 10 min of incubation, the homogenates were centrifuged at 1000g for 10 min at 4 °C and the cytosolic fraction was kept for analysis.

### 2.4. Metal analysis

For metal concentration determination, homogenates were weighed, dried (80 °C, 48 h) and submitted to wet acid digestion with 67% nitric acid in a hot plate (80 °C, 2 h). Six metals (Cu, Zn, Fe, Mn, Co, Cd) were analysed by graphite furnace absorption spectrometry (AAS, AAnalyst 800-PerkinElmer). Accuracy of the analytical method was checked by analysing certified reference material TORT-2 (NRC-CNRC) (lobster hepatopancreas), measured values (99  $\pm$  17  $\mu\text{g g}^{-1}$  Cu, 138  $\pm$  28 Fe, 124  $\pm$  52 Zn, 14  $\pm$  2 Mn, 0.49  $\pm$  0.08 Co, 33  $\pm$  3 Cd) were within the values of certified values in the reference material (106  $\pm$  10  $\mu\text{g g}^{-1}$  Cu, 105  $\pm$  13 Fe, 180  $\pm$  6

Zn,  $13.6 \pm 1.2$  Mn,  $0.51 \pm 0.09$  Co,  $26.7 \pm 0.6$  Cd). Data expressed as  $\mu\text{g g}^{-1}$  of dry tissue weight (d.w.). The values for total soft tissues correspond to the sum of gills, hepatopancreas and muscle, taking into the account the contribution of the weight of each tissue.

## 2.5. Metallothioneins (MTs)

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

## 2.6. Total proteins

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

## 2.7. Antioxidant enzymes activities

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

Catalase (CAT) activity was determined according to Greenwald (1985). The activity was determined as the decrease in absorbance during 1 min at 240 nm due to  $\text{H}_2\text{O}_2$  consumption in buffer of monobasic and dibasic potassium, pH 7.5. CAT activity is expressed as  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  prot.

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

Glutathione-S-transferase (GST) activity was determined by following the conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB; Habig et al., 1974). A buffer containing  $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$  (0.2 M), pH 7.9, was used. The change in absorbance was recorded at 340 nm for 1 min. The activity was calculated as  $\text{nmol of CDNB formed per min}^{-1} \text{mg}^{-1}$  prot (using a molar extinction coefficient of  $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$ ).

## 2.8. Lipid peroxidation (LPO)

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

## 2.9. Statistical analysis

The non-parametric Kruskal-Wallis test was used to assess the significant differences between exposure groups and tissues for each variable (biomarkers and metals concentration). Results were significantly different when  $p < 0.05$ . Principal components analysis

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

Species	Sites	Tissues	Cu	Cd	Co	Fe	Mn	Zn	References
<i>Rimicaris exoculata</i>	TAG ( $\mu\text{g g}^{-1}$ d.w.)	G	$535 \pm 205$	$1.24 \pm 0.91$	$3 \pm 2$	$6201 \pm 4299$	$26 \pm 11$	$277 \pm 123$	This study
		H	$1562 \pm 592$	$8.32 \pm 1.68$	$3.7 \pm 1.7$	$6122 \pm 1764$	$29 \pm 10$	$94 \pm 64$	
		M	$98 \pm 23$	$0.15 \pm 0.03$	$0.3 \pm 0.1$	$1052 \pm 307$	$6 \pm 2$	$88 \pm 39$	
		S.T.	$379 \pm 146$	$1.3 \pm 0.4$	$1.6 \pm 0.3$	$2740 \pm 1191$	$16 \pm 3.5$	$112 \pm 31$	
	TAG ( $\mu\text{mol g}^{-1}$ w.w.)	G	$0.75 \pm 0.18$	$0.32 \times 10^{-3} \pm 0.1 \times 10^{-3}$	$3.1 \times 10^{-3} \pm 5 \times 10^{-4}$	$3.6 \pm 1.1$	$0.022 \pm 0.004$	$0.22 \pm 0.06$	This study
		H	$2.51 \pm 0.78$	$8.7 \times 10^{-3} \pm 2.1 \times 10^{-3}$	$5.6 \times 10^{-3} \pm 2 \times 10^{-4}$	$12.9 \pm 2.6$	$0.06 \pm 0.02$	$0.24 \pm 0.09$	
		M	$0.11 \pm 0.02$	$0.1 \times 10^{-3} \pm 0.03 \times 10^{-3}$	$0.5 \times 10^{-3} \pm 1 \times 10^{-4}$	$1.5 \pm 0.4$	$0.008 \pm 0.002$	$0.13 \pm 0.02$	
		S.T.	$0.32 \pm 0.18$	$0.008 \pm 0.0003$	$0.002 \pm 0.0016$	$2.8 \pm 1.5$	$0.02 \pm 0.01$	$0.13 \pm 0.04$	
	Rainbow ( $\mu\text{mol g}^{-1}$ w.w.)	G	$1.2 \pm 0.6$	$0.014 \pm 4 \times 10^{-3}$	-	$25.5 \pm 11.4$	$0.316 \pm 0.18$	$17.9 \pm 5.0$	Geret et al., 2002
		H	$0.61 \pm 0.21$	$3.5 \times 10^{-3} \pm 9 \times 10^{-4}$	-	$24.6 \pm 1.8$	$0.076 \pm 0.009$	$5.7 \pm 1.7$	
<i>Mirocaris fortunata</i>	Rainbow ( $\mu\text{g g}^{-1}$ d.w.)	M	$0.26 \pm 0.08$	$1.1 \times 10^{-3} \pm 9 \times 10^{-4}$	-	$3.1 \pm 1.86$	$0.039 \pm 0.026$	$1.5 \pm 1.1$	Kádár et al., 2006
		G	$1060 \pm 200$	-	-	$90460 \pm 48600$	-	$2900 \pm 400$	
		H	$700 \pm 200$	-	-	$6640 \pm 2230$	-	$800 \pm 700$	
		M	$300 \pm 160$	-	-	$610 \pm 260$	-	$300 \pm 100$	
	Rainbow ( $\mu\text{g g}^{-1}$ d.w.)	S.T.	$176.7 \pm 74$	$0.49 \pm 0.02$	-	$2122 \pm 867$	$43.2 \pm 10.4$	$209 \pm 111$	Gonzalez-Rey et al., 2008
	Rainbow ( $\mu\text{g g}^{-1}$ d.w.)	G	$1700 \pm 500$	-	-	$122970 \pm 41600$	-	$4300 \pm 800$	Kádár et al., 2006
		H	$400 \pm 100$	-	-	$3010 \pm 620$	-	$600 \pm 100$	
		M	$200 \pm 60$	-	-	$940 \pm 240$	-	$200 \pm 40$	
	Lucky Strike ( $\mu\text{g g}^{-1}$ d.w.)	G	$1400 \pm 500$	-	-	$44170 \pm 15320$	-	$1900 \pm 800$	Kádár et al., 2006
		H	$500 \pm 200$	-	-	$1870 \pm 250$	-	$900 \pm 600$	
	M	$40 \pm 10$	-	-	$190 \pm 80$	-	$60 \pm 30$		



(PCA) was used in the ordination method to evaluate the relationship between the different treatments and the analysed variables in the gills and the hepatopancreas.

### 3. Result and discussion

#### 3.1. Metal accumulation

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

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

For the copper exposure experiment, Cu concentrations in the tissues for the different treatments are shown in Fig. 1 (see also Table A.1 in Supplementary material). The results do not show significant differences between groups ( $p > 0.05$ ), except for the zinc with higher concentrations in the gills (277  $\mu\text{g g}^{-1}$  d.w.) and in the total soft tissues (112  $\mu\text{g g}^{-1}$  d.w.) of the *in situ* group when compared to shrimps 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 *in situ* group, the concentration of Co and Mn is similar in the hepatopancreas and gills, but in the groups incubated inside the IPOCAMP there is a higher concentration in the gills. Cd is mainly accumulated in the hepatopancreas (8.3  $\mu\text{g g}^{-1}$  d.w.), when compared 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* group represents the baseline concentrations, these results suggest that after incubation inside the IPOCAMP, where only Cu was added but not the other metals, there is a redistribution of metals among the different tissues. As some of these are essential metals, these results suggest their potential use in other biological functions in the gills, or that their excess are further eliminated/regulated through the gills.

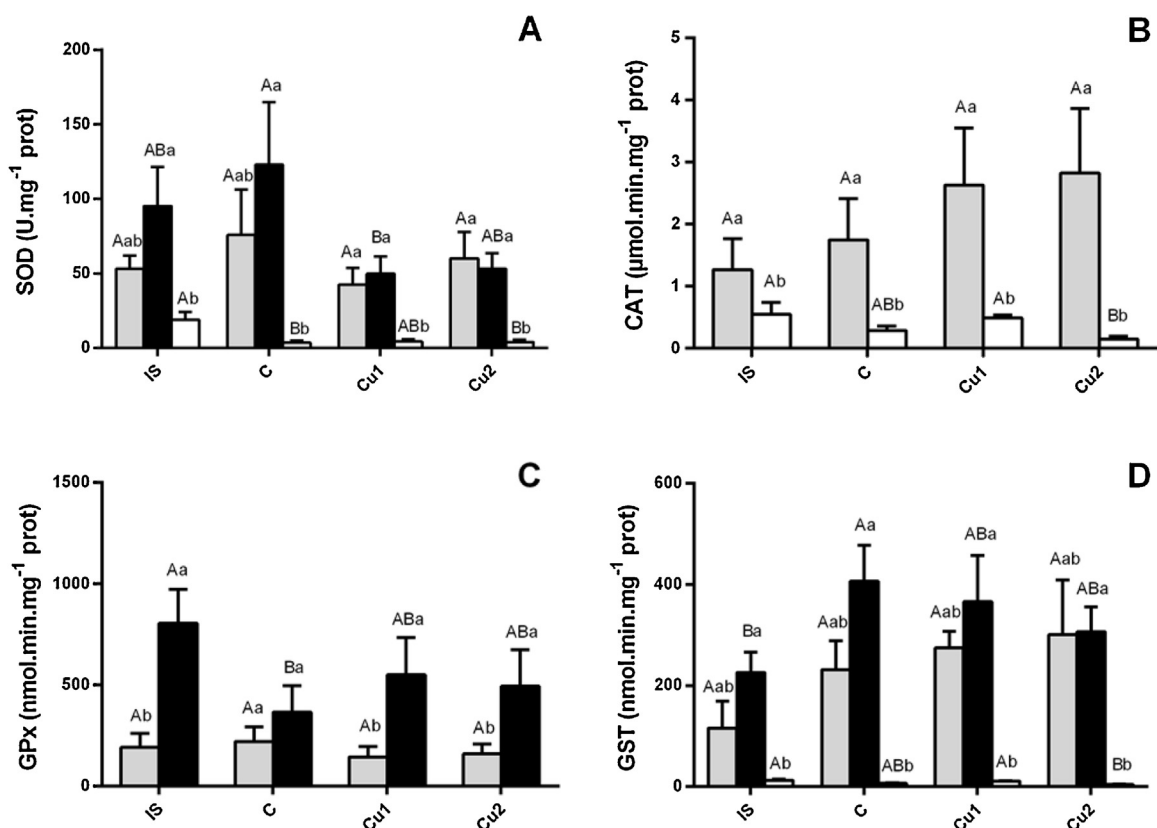
A high variability in Cu concentration among the shrimp replicates was noted (Fig. 1). Still, the Cu concentration in the hepatopancreas decreases in the groups exposed to Cu (Cu1: 440  $\mu\text{g g}^{-1}$  d.w., Cu2: 810  $\mu\text{g g}^{-1}$  d.w.) when compared to the *in situ* and control groups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

#### 3.2. Metallothioneins

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

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

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



**Fig. 3.** Superoxide dismutase (SOD), catalase (CAT), total glutathione peroxidase (GPx) and glutathione-S-transferase (GST) activities (mean  $\pm$  SD) in tissues of *Rimicaris exoculata* for the different treatments. Values followed by the same letter are not significantly different ( $p > 0.05$ ) with capital letters for comparison of one tissue between groups and lowercase letters for comparison among one group. Gills (grey), hepatopancreas (black), abdominal muscle (white), IS: *in situ* group, C: control, Cu1: exposed to 0.4  $\mu$ M, Cu2: exposed to 4  $\mu$ M. Given the small amount of tissue available for HP, CAT was not analysed. GPx activity was not detected in the abdominal muscle.

(Fig. 1) and whose amount does not vary after 72 h of incubation. The affinity of MTs with metals defines their binding abilities ( $\text{Cu} > \text{Cd} > \text{Zn}$ ; Engel and Brouwer, 1989). Cd is a non-essential metal, thus to avoid toxicity the organisms have to limit their accumulation or to complex the accumulated metal to a non- or less-toxic form (Depledge and Rainbow, 1990).

### 3.3. Antioxidant enzymatic response

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

Both CAT and GST activities follow a similar pattern (Fig. 3). An increasing trend of these enzymes activities in the gills follow the increasing Cu concentration (IS: 1.3  $\mu\text{mol CAT min}^{-1} \text{mg}^{-1}$  prot, 116  $\mu\text{mol GST min}^{-1} \text{mg}^{-1}$  prot, Cu2: 2.8  $\mu\text{mol CAT min}^{-1} \text{mg}^{-1}$  prot, 301  $\mu\text{mol GST min}^{-1} \text{mg}^{-1}$  prot) but are not significantly different ( $p > 0.05$ ). Increasing CAT activity with increasing Cu con-

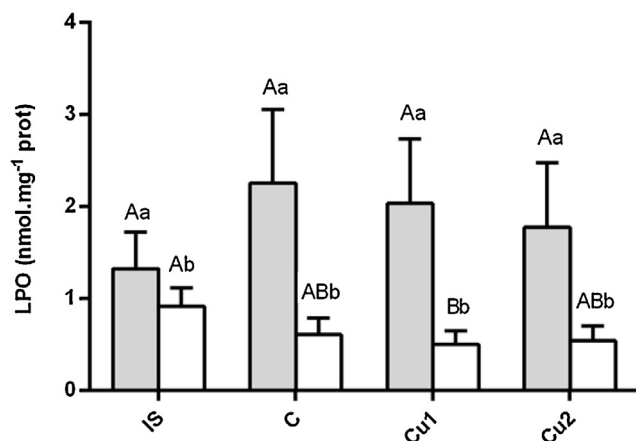
centration has been previously observed (Li et al., 2006). Due to the low amount of tissue available, it was not possible to determine the CAT activity for the hepatopancreas. The GST activity in the hepatopancreas for the *in situ* group (225  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  prot) was significantly lower when compared to the control group (406  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  prot,  $p < 0.05$ ). Moreover, for the groups placed in the IPOCAMP, a slight downward trend in GST activity was observed with increasing Cu concentration. CAT and GST activities in the muscle were higher for the *in situ* and Cu1 groups, with Cu1 significantly higher than Cu2 for both enzymes ( $p < 0.05$ ; Fig. 3). These enzyme activities detected in the tissues of shrimps suggest a protective mechanism against the presence of ROS, as previously noted by Gonzalez-Rey et al. (2008). In their study, they found that CAT in soft tissues of shallow-water shrimps as lower activity than in vent shrimps, while higher values of SOD and GPx activities were found in shallow-water shrimps (Gonzalez-Rey et al., 2008; Table 2). When comparing the enzyme activities in the hepatopancreas, shallow-water shrimps show lower activity for GPx (8.8 and 4.71  $\text{nmol min}^{-1} \text{mg}^{-1}$  prot for *Palaemon serratus*; Oliveira et al., 2012; Silva et al., 2013) and GST (6.4 and 3.1  $\text{nmol min}^{-1} \text{mg}^{-1}$  prot for *P. serratus*; Oliveira et al., 2012; Silva et al., 2013) when compared to *R. exoculata* (GPx: 804.2  $\text{nmol min}^{-1} \text{mg}^{-1}$  prot, GST: 225.6  $\text{nmol min}^{-1} \text{mg}^{-1}$  prot; Table 2). Similarly, *R. exoculata* presents higher values of CAT activity in the muscle (0.55  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  prot) when compared to *P. elegans* (0.0053  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  prot for; Vinagre et al., 2014; Table 2). These observations are suggesting a detoxification strategy in this vent shrimp, adapted to an enriched metal extreme environment.

**Table 2**

Antioxidant enzymes activities (SOD, CAT, GPx, GST) (mean  $\pm$  SD) in tissues of the vent shrimp *Rimicaris exoculata* and coastal shrimps *Palaemon elegans*, *Palaemon serratus*, *Palaemon varians*. G: Gills; H: hepatopancreas; M: abdominal muscle; S.T.: soft tissue.

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

<sup>a</sup> nmol min mg<sup>-1</sup> prot.



**Fig. 4.** Lipid peroxidation (LPO, mean  $\pm$  SD) in tissues of *Rimicaris exoculata* for the different treatments. Values followed by the same letter are not significantly different ( $p > 0.05$ ) with capital letters for comparison of one tissue between groups and lowercase letters for comparison among one group. Gills (grey), hepatopancreas (black), abdominal muscle (white), IS: *in situ* group, C: control, Cu1: exposed to 0.4  $\mu$ M, Cu2: exposed to 4  $\mu$ M.

### 3.4. Oxidative damage

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

### 3.5. Relationship between metal accumulation and biomarkers responses

The present results reinforce the fact that vent fauna, especially *R. exoculata*, have developed physiological adaptive strategies to face the metal-rich habitat they live in. Moreover, the establishment of protection through the antioxidant enzymes activities seems correlated with the decreasing trend of LPO levels in the gills. The activity of enzymes seems to have a beneficial effect in

decreasing the amount of ROS in cells and consequently limit the attack of ROS on membrane lipids. This result is in favour of a good detoxification process in the presence of copper. Nevertheless, the rather low impact of 4  $\mu$ M of Cu exposure on the shrimps might be due to the fact that they may have developed protective strategies as they are continuously bathed in a metal rich medium. Several studies have suggested the possibility of detoxification through the bacteria present in the gills chamber or in the gut (e.g. Jan et al., 2014). Part of the toxic compounds could be taken up by the bacteria hosted in the gills chamber of the shrimps and decreasing the amount of toxic compounds accumulated in shrimps tissues (Compère et al., 2002; Zbinden and Cambon-Bonavita, 2003).

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

## 4. Conclusions

This study gives a first appraisal of the amounts of metals, MTs, antioxidant enzymes and LPO in different tissues of the vent shrimp *R. exoculata* from the TAG hydrothermal vent field. *R. exoculata* shows a background protection against metal toxicity as the activities of enzymes were detected for the *in situ* group. A tissue specific response was observed for the biomarkers analysed. Shrimps appear to develop protection against exposure to Cu, with increasing activity of antioxidant enzymes (CAT and GST) and induction of MT in the higher Cu concentration exposure (4  $\mu$ M). The ubiquity of the shrimp *R. exoculata* along the Mid-Atlantic Ridge and the similar responses observed in other vent sites (e.g. Rainbow) with those obtained in this study render this species as a good model for a possible monitoring of mining effects in hydrothermal vent fauna. Moreover, results suggest that the use of the pressurized aquarium do not affect the shrimps in terms of biomarkers responses to metal exposure. This method looks promising for further biomarkers responses studies with other deep-sea fauna.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.aquatox.2016.03.024>.

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