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Effects of copper on larvae of the marbled crab *Pachygrapsus marmoratus* (Decapoda, Grapsidae): toxicity test and biochemical marker responses

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Abstract

The importance of trace elements in ecotoxicological investigations is a well-known issue when monitoring polluted areas such as commercial harbours. Copper represents one of the most common metal contaminants, often detected in these areas as it is widely employed in various fields and has many sources of inflow in the marine environment. *Pachygrapsus marmoratus* is a widespread intertidal crab species that has been extensively studied in ecology, ethology and population genetics. Ecotoxicological studies have also been performed, exclusively on the adult stage. In the present study we investigated the mortality and biochemical (oxidative stress and neurotoxicity) responses of *P. marmoratus* larvae exposure to environmental relevant concentration of copper. Results showed dose-dependent responses in terms of larval mortality, with a calculated LC_{50} value of 0.5 mg/L of Cu^{2+}. The LC_{50} concentration was used as the starting point for subsequent biochemical response evaluation. Results also demonstrated dose-dependent activation of antioxidant systems assuming a compensatory antioxidant activity to prevent higher cellular damage when larvae were exposed to the highest concentrations of copper. Moreover, a significant enhancement of neurotransmitter activities was observed, assuming a possible direct interaction of copper with the enzymes or an increase of free copper ion aliquot into the cells.

Keywords: oxidative stress; neurotoxicity; trace elements; ecotoxicity

1 - Introduction

Commercial harbors are known to be highly contaminated areas due to ordinary and non-ordinary activities, usually associated with water column and bottom sediments contamination (Denton et al., 2005). There are various sources of contamination including anthropic activities - such as traffic, dredging, effluent discharge - and natural events - such as river overflows and rainwater runoff (Bourg and Bertin, 1996; Schiff, 1995; Guerra-Garcia and Garcia-Gomez, 2005, Bretzel and Calderisi, 2006; Lye, 2009). Almost all main classes of contaminants are present in these areas, from Polycyclic Aromatic Hydrocarbons (PAH) to trace elements (Boyden, 1975; Denton et al., 2005; De Luca et al., 2005; Bretzel and Calderisi, 2006; Sprovieri et al., 2007; Chen et al., 2013). Copper is found in higher concentrations in harbors - both in the sediments and water - compared with other coastal areas, due to the presence of various sources of introduction, such as antifouling paints (Turner, 2010), as well as depending on physicochemical conditions (Förstner et al., 1986) and the concentration or degradation of the organic matter (Teasdale et al., 2003; Caplat et al., 2005).

Due to the high presence of contaminants in commercial harbors, chemical and ecotoxicological monitoring of water and sediments are usually mandatory and organized by environmental protection agencies or similar, especially during particular events, such as dredging. Despite of well-described and regulated procedures for chemical monitoring, ecotoxicology often focuses on mesocosms bioassays and usually occurs in laboratories. These assays, fundamental for acute and chronic description of bioavailable contaminant effects, have the limitation to be a pinpoint vision on what is happening in a specific environment. For this reason, ecotoxicological evaluations on organisms living in polluted areas, may increase the accuracy data collected from classical bioassays.

Pachygrapsus marmoratus (Fabricius, 1787) (Decapoda; Brachyura; Thoracotremata; Grapsidae) inhabits the rocky coasts of Mediterranean Sea, Black Sea and north-eastern Atlantic Ocean - from Brittany to Morocco including the Canary Islands, the Azores, and Madeira. (Zariquiey, 1968; Ingle, 1980 Flores and Paula, 2001). The marbled crab is highly prolific, with females producing thousands of fertilized eggs each reproductive season - between March and September. Dispersal - influenced by tidal and wind-driven currents - occurs through a series of planktonic larval stages lasting about a month, prior to settlement (Aydin et al., 2014). Adults are relatively sedentary (Cannicci et al., 1999). The complete zoeal development

of *P. marmoratus* underwent six stages and the mean duration in days have already been described by Cuesta and Rodríguez (2002): Zoea I: 3.5; Zoea II: 4; Zoea III: 4.5; Zoea IV: 4.5; Zoea V: 4; Zoea VI: till the end of the larval development.

Crabs are among the most important organisms in estuarine and coastal food-webs because they connect primary producers and organic detritus to secondary consumers, promoting nutrient cycling and water quality (Madeira et al., 2014). Moreover, intertidal organisms, such as *P. marmoratus,* are considered model species in studies focusing on temperature (see Vinagre et al., 2012) osmotic (Jayasundara et al., 2007) and trace elements stress (Fratini et al., 2008; Tejada et al., 2015; Rainbow et al., 2000). Adults of *P. marmoratus* exposed to different metal contaminates showed bioaccumulation in the hepatopancreas and gills (Fratini et al., 2008), oxidative stress responses (Tejada et al., 2015) and effect on the rate of uptake (Rainbow et al., 2000). Moreover, correlation between salinity acclimation and the effect of metal ion on different crab species has been shown. Vitale et al. (1999) found that at low salinity (2.5‰) cadmium has a median lethal effect (LC50) on *Neohelice granulata* (*Chasmagnatus granulatus*) more than 20 times lower than at high salinity (30‰). Bianchini et al. (2008) also reported numerous other implications of salinity related toxicity of copper, cadmium and other trace elements on *Neohelice granulate.* Concerning the larval stages, standard measures of toxicity such as mortality and LC_{50} after metal exposures (Ahsanullah et al., 1978; Mortimer et al., 1994; Botton, 2000; Greco et al., 2001; Ferrer et al., 2006; Neil et al., 2005) have already been investigated in other crab species. However, to our knowledge, there is no available data on biochemical responses of *P. marmoratus* larvae. Considering that studies using biochemical biomarkers can be helpful in establishing cause-effect relationships of specific contaminants and have been used to predict their effect on natural populations (Clements, 2000), The aim of this work is to investigate the effect of copper on larvae (zoea I) of the intertidal crab *P. marmoratus* after 48h acute copper exposure, evaluating survival (LC_{50}) and biochemical responses in terms of oxidative stress and neurotoxicity.

2 - Material and Methods

2.1 - Ovigerous female collection and maintaining

Several ovigerous females of *P. marmoratus* were collected in the commercial harbor of Livorno (Italy)

during spawning period (Mouneyrac et al., 2001) - from June to August 2018 (Note 1). The crabs were carefully transported to the laboratory and maintained in 20 L tanks (2-3 individuals per tank), filled with 5 L of filtered seawater (S=37‰, T=20±2 °C) collected from Quercianella (Livorno, Italy). Each aquarium was also provided with stones as hiding places and exposed areas where the females could aerate the eggs. Water was changed every two days. At the same time of each water renewal, fresh *Ulva lactuca* was added as food.

Note 1: According to the national law (Decree 26/14), that implements the Directive UE 2010/63 on the protection of animals used for scientific purposes, crabs are not under protection regimen.

2.2 - Larvae spawning, collection and maintenance

Larval spawning of *P. marmoratus* generally occurs synchronised with the new moon and full moon (Saigusa and Hidaka, 1978). For this reason, tanks were maintained at natural light and photoperiod. Shortly before the spawning, *P. marmoratus* females bearing fully mature embryos are easily recognizable, because of the size and colour of the eggs and due to their behaviour - spending more time outside the water and moving their pleiopods to aerate embryos. Females approaching spawning were isolated from the other females and transferred in 1 L beakers, with the same setup as the 20 L tanks. Spawning was checked daily. Once released, larvae were gently removed from beakers with a sterile 3 mL plastic pipette and put in flasks with fresh filtered seawater (FSW; S=37‰, T=20±2°C) and kept in the same conditions as adults. Larvae were fed daily with algal suspension (*Isochrysis galbana* + *Rhodomonas reticulata*) (10^5 cells/mL).

2.3 - Chemicals

Copper, as copper (II) sulfate pentahydrate ($CuSO_4*5H_2O$) (Sigma-Aldrich®) was used as reference toxicant for both 48-h acute toxicity assay and *in-vitro* exposure for biomarkers evaluation. All the concentrations presented in the graphics and tables are reported as Cu^{2+}. A 250 mg/L $CuSO_4*5H_2O$ stock solution was prepared dissolving 50 mg of the salt directly in ultrapure water. All dilutions were prepared in FSW. Nominal and measured concentrations for all testing dilutions are reported in Table 1.

2.4 - Mortality assay

Six concentrations of copper (II) sulfate pentahydrate ($CuSO_4*5H_2O$) (0.25 - 0.50 - 1 - 2 - 4 - 8 mg/L Cu^{2+}) were used for the mortality assay. Day-1 larvae were used as model organisms for a 48-h mortality assay. Three replicates for each copper concentration and control were prepared in 100 mL glass beakers. Each beaker was filled with 50 mL of sample or control. 10 actively swimming larvae were carefully pipetted in each beaker. All containers were then covered with Parafilm® strips to prevent water evaporation. All beakers where maintained at 20±1 °C, under a photoperiod of 12:12 light: darkness, for 48 h, without food. Mortality in each beaker was registered at 24 and 48 h of exposure and dead organisms removed. The criterion for determining death was the absence of movement in 1-2 min of observation.

2.5 - Cellular fraction preparation and in vitro exposure to copper

About 1000 larvae were collected at 48 h post-spawning and homogenized in a potter Elvejem in 100 mM phosphate buffer (pH 8,0) (1:2 w/v) for biomarkers analysis. Homogenates were then sonicated for 15 s at 4°C and centrifuged for 10 min at 9000x*g* at 4°C to obtain the supernatant (S9 fraction). The samples were stored at -80°C until used. The protein content was determined according to the Lowry method (Lowry, 1951) using Bovine Serum Albumin (BSA) as standard. Copper, as copper (II) sulfate pentahydrate ($CuSO_4*5H_2O$) solutions, used at the concentrations of 0.5, 0.25 and 0.125 mg/L of Cu^{2+}, were incubated with S9 fraction for 1 hour for the *in vitro* exposure. Biochemical analyses were performed in triplicate for each larval S9 cellular fraction collected at different times for each metal concentration with a BioTek Synergy HT micro-plate Reader.

2.6 - Biochemical analysis

2.6.1 Superoxide dismutase (SOD) activity

SOD activity was determined following Magnani et al. (2000) method, based on the ability of the enzyme to inhibit the autoxidation of pyrogallol. The autoxidation of pyrogallol in the presence of EDTA at pH 8.2 is 50%. The principle of this method is based on the competition between the pyrogallol autoxidation by $O_2^{\bullet -}$ and the dismutation of this radical by SOD. Absorption was read at the wavelength of 420 nm against Tris-EDTA buffer at time zero and after 1 minute of the addition of pyrogallol. SOD activities are expressed as units (U)/mL where one unit (U) is defined as the amount of enzyme required to cause 50% inhibition of

pyrogallol autoxidation.

2.6.2 Catalase (CAT) activity

CAT activity was determined by measuring the decrease of hydrogen peroxide in absorbance at 240 nm (Aebi, 1983). The mixture was composed of 200 µL of supernatant and 500 µL of 50 mM phosphate buffer (pH 7) and the reaction was started by the addition of 300 µL of hydrogen peroxide (30mM). The decrease in absorbance was recorded every 15 seconds up to 3 min. Catalase activity was expressed as µmol H_2O_2/min/mg protein.

2.6.3 Glutathione reductase (GR) activity

GR activity was determined using the method described in Wheeler et al. (1990). GR catalyses the reduction of glutathione disulfide (GSSG) to the sulfhydryl form glutathione (GSH), functioning as dimeric disulfide oxidoreductase and utilizes a FAD prosthetic group and NADPH to reduce one molar equivalent of GSSG to two molar equivalents of GSH. The absorbance was measured at 340 nm, the enzymatic activity was determined using ε=6.22 mM^{-1} cm^{-1} and the results expressed in nmol/min/mg protein.

2.6.4 Cholinesterases (ChEs) activities

ChEs activity was measured according to Ellman et al. (1961) method in 100 mM phosphate buffer (pH 8.0), 10 mM DTNB, and different concentrations of substrates (AChE; BChE; PChE). Enzyme activity was recorded continuously for 5 min at 412 nm and the specific activity was corrected for the spontaneous hydrolysis of the substrate and expressed as nmol/min/mg protein, using a molar extinction coefficient of $13.6x10^3M^{-1}cm^{-1}$ (the yellow dianion of 5-thio-2-nitrobenzoic acid, TNB).

2.7 - Data analysis

All data were represented as mean ± standard deviation. One-way ANOVA were performed for data analyses (PRISM software, Graphpad Software). Magnitude values with $p<0.05$ were considered statistically significant. The mortality assay result, as LC_{50} value, was calculated using the PROBITS method (Finney, 1971).

3 – Results

3.1 Mortality assay

Results of 24-h and 48-h readings of larval mortality assay are reported in Table 2. No mortality was registered in controls after 24 h and 6.66% after 48 h. These results were used for a LC_{50} calculation using the method of PROBITS (Finney, 1971). LC_{50} values were calculated both for 24-h and 48-h of exposure, both as copper sulphate pentahydrate and as copper ion, and are reported in Table 3.

3.2 Biomarkers results

3.2.1 Superoxide dismutase (SOD) activity

SOD results for 1-h exposure of *P. marmoratus* to $CuSO_4*5H_2O$ (0.125; 0.25 and 0.5 mg/L Cu^{2+}) are showed in Fig. 1A. All the treatment groups, except the 0.125 mg/L group, were significantly different ($p < 0.05$) from control, while SOD activity was significantly higher in the specimens exposed to the two highest concentrations (0.25 and 0.50 mg/L Cu^{2+}) in comparison to larvae exposed to 0.125 mg/L Cu^{2+} ($p<0.05$).

3.2.2 Catalase (CAT) activity

A significant dose-dependent increase of CAT activity was detected in larvae exposed to all the contaminant concentrations (0.125; 0.25; 0.5 mg/L Cu^{2+}) in comparison to controls ($p < 0.05$). No significant differences were observed among exposure concentrations (Fig. 1B).

3.2.3 Glutathione reductase (GR) activity

Larvae exposed to 0.25 mg/L Cu^{2+} showed a GR activity significantly higher than the control group ($p < 0.05$) while no differences were observed when comparing the larvae exposed to 0.125 mg/L Cu^{2+} with the non-exposed larvae (Fig. 1C). The specimens exposed to the highest exposure concentration (0.50 mg/L $Cu2^{+}$) exhibited significant lower inhibition of the activity in comparison to control. Significant differences were observed between 0.125 and 0.5 mg/L Cu^{2+} ($p<0.05$), showing higher enzyme activity in larvae exposed to the lowest contaminant concentration.

3.2.4 Cholinesterases (ChEs) activities

In AChE activity results, no significant differences were observed among exposed and non-exposed larvae (data not shown).

BChE activity results - reported in Figure 2B - showed that 0.25 and 0.50 mg/L Cu^{2+} treated groups were significantly different from control and 0.125 mg/L Cu^{2+} ($p < 0.05$) showing higher BChE activity. No significant differences were observed among specimens contaminated with these two higher exposure concentrations. Regarding the PChE results, the group exposed at 0.50 mg/L Cu^{2+} was significantly different ($p < 0.05$) from the control (12.04 ± 9.06 nmol/min/mg protein) showing higher enzyme activity (50.15 ± 16.01 nmol/min/mg protein) compared to all the remaining concentrations (Fig. 2A).

4 – Discussion

4.1. Toxicity on larval stages

The evaluation of copper toxicity, as percentage of mortality on first larval stage (Zoea I) showed a dose-dependent response after both 24 and 48 hours of exposure. The calculated LC_{50} value decreased more than three times from 24-h to 48-h, underlining a putative time-dependent toxicity of copper, as reported in similar assays on other crab species, such as *Maja squinado* (Marino-Balsa et al., 2000), *Cancer anthonyi* (Macdonald et al., 1988), *Chasmagnatus granulata* (Lopez Greco et al., 2001; Ferrer et al., 2006). The authors reported a LC_{50} value of more than 1000 µg/L after 24 hours and 755.39 µg/L after 48 hours of exposure. Such concentrations of copper match with data obtained in the present work. *P. marmoratus* is generally known as a species with high resistance to different stress sources (Tejada et al., 2015), due to its peculiarity of inhabiting supralittoral crevices (Augusto and Flores, 2001). However, findings on acute copper toxicity showed that resistance present in adults is not present in larval stages. Contaminants, such as copper, can actively affect mortality percentages in the first hours of larval life, consequently affecting *P. marmoratus* dispersal.

4.2. Biochemical responses

4.2.1 Oxidative stress related enzymes

Copper can induce cellular toxicity (Gaetke and Chow, 2003) with the formation of reactive oxygen species - ROS (Bremner, 1998; Kadiiska et al., 1993). ROS differ in terms of cellular reactivity and potentially cause

toxic insults to lipids, proteins and DNA (Regoli and Winston, 1999). In basal condition antioxidant systems - such as SOD, CAT, GPx, glutathione redox cycle - are involved in coping against cellular damage (Regoli and Giuliani, 2014). A concentration-dependent increase of SOD and CAT activities were observed, assuming a compensatory antioxidant activity to prevent higher cellular damage when larvae were exposed to the highest concentrations of copper. In a study conducted by Amin et al. (2010), the copper toxicity (40, 80 and 160 µg/L) on zoea I of *Lithodes santolla* (Decapoda: Anomura) was analysed showing that, after a 96-h exposure, lipid peroxidation values were higher in all treatments compared to controls. This confirmes impaired cellular function and alterations in physicochemical properties of cell membranes after contaminant exposure. These results could help justify the contribution of SOD and CAT observed in the present study as a behaviour to prevent possible higher cellular damages caused by metal exposure. The impacts of copper on the antioxidative enzymes have been investigated on adult crabs. In most studies, copper chelation and increased antioxidant defences were effective in limiting oxidative tissue damage when large amounts of copper accumulated in cells (Brouwer et al., 1998; Sabatini et al., 2009). However, when the ROS generation exceeds the scavenging ability of the defence system, excessive ROS can provoke oxidative stress. This could cause damage to biomolecules by free radical attachment to polyunsaturated fatty acid side chains in cells, leading to lipid peroxidation and consequent decrease of antioxidant system activities (Liu et al., 2014). Our results are in line with the present citations, showing a decrease of GR activity - ubiquitous enzyme essential for the glutathione redox cycle (Wu et al., 2011) - at 0.25 mg/L and a total inhibition at the highest exposure concentration. Similar variations of GR were reported for different crab species exposed to other trace elements. For example, Liu et al. (2014) evaluated the antioxidant defences induced by Pb in the freshwater crab *Sinopotamon henanense*, showing a dose-dependent suppression of GR activity. Using the crab *Sinopotamon yangtsekiense*, Liu et al. (2008) also observed a decrease in GR activity by 79% after exposition to Cd^{2+} for 96 h.

4.2.2 Neurotoxicity

Cholinesterases (ChEs) are carboxylic ester hydrolases that break down esters of choline. They include specific cholinesterase (AChE) and non-specific ones, or pseudo cholinesterase (PChE and BChE). The preferred substrate for AChE is acetylcholine, while non-specific cholinesterases prefer butyrylcholine and/or propionylcholine, depending on the species (Lionetto et al., 2011). Several studies demonstrated the

inhibition of ChE activities in non-model species - such as crabs - after contaminants exposure. Elumalai et al. (2007) found an inhibitory effect of zinc and mercury on ChE activity of the crab *Carcinus maenas*. Similar results were obtained by Narra (2015) for the freshwater crab *Barytelphusa guerini* after exposure to chlorpyrfos insecticide. However, in the present study, the activity trend of both examined ChEs seemed to be in contrast with results of cited literature, showing a dose-dependent increase of activity after *in vitro* copper exposure. Our findings can be explained with Khedher et al. (2017), which detected a significant enhancement of AChE activity in muscle tissues of the crab *C. maenas* after 2 days of time exposure, followed by a repression at 7 days. Similarly, Cunha et al. (2007) showed an increase of AChE activity in the gastropod *Nucella lapillus* exposed to 44000 µg/L Cu, which may be due to the direct interaction of this metal with the enzyme or an increase of free Cu aliquot into the cells, likely due to mechanisms of metal homeostasis (Romani et al., 2003). Moreover, the use of ChEs to account for responses in the antioxidant enzymes and lipid peroxidation levels is suggested to be a useful application to detect the possible exposure/effect induced by contaminants on living organisms (Lionetto et al., 2011). A significant correlation between ChEs and antioxidant enzyme activities was found in several species following contaminants exposure, as reported in mussels (Lionetto et al., 2003) and fish (Lionetto et al., 2003; Kavitha and Rao, 2008). For example, in mosquito fish, *Gambusia affinis*, Kavitha and Rao (2008) found the inhibition of brain AChE activity directly related to the inhibition of antioxidant enzymes, while the benthic fish *Mullus barbatus*, AChE activity showed a significant inverse correlation with glutathione reductase activity, but not with catalase activity (Lionetto et al., 2003). The results of the present study agree with the latter ones, underlining a similar trend in *P. marmoratus* as Lionetto et al. (2003) found in *M. barbatus*.

5 - Conclusions

A quantifiable and dose-dependent effect of Cu in *in vitro* exposure on *Pachygrapsus marmoratus* zoea I larval stage was observed. Results showed dose-dependent responses in terms of larval mortality as well as concentration-dependent activation of antioxidant systems assuming a compensatory antioxidant activity to prevent higher cellular damage when larvae were exposed to the highest concentrations of copper. Moreover, a significant enhancement of neurotransmitter activity was observed, hypothesizing a possible direct interaction of this metal with the enzyme or an increase of free Cu aliquot into the cells.

These results underlined the importance of investigating the effects of pollutants on different developmental stages of resistant species and to better understand their impact on dispersal behaviour of intertidal marine organisms. Further studies are necessary to consolidate these results at a different level between larval stages and adult stage to raise ecological significance from individual to population level.

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Figure captions

Figure 1. Oxidative stress enzyme (A - Superoxide dismutase (SOD); B - Catalase (CAT); C - Glutathione reductase (GR)) activities (mean ± standard deviation), in Pachygrapusus marmoratus exposed to different concentrations (0.125; 0.25 and 0.50 mg/L Cu2+) of CuSO4*5H2O. Significant differences ($p \leq 0.05$) are presented with bold asterisks (*).

Figure 2. Cholinesterase (A - Propionyl cholinesterase (PChE); B - Butyryl cholinesterase (BChE)) activities (mean ± standard deviation), in *Pachygrapusus marmoratus* exposed to different concentrations (0.125; 0.25 and 0.50 mg/L Cu^{2+}) of $CuSO_4*5H_2O$. Significant differences ($p \leq 0.05$) are presented with bold asterisks (*****).

Table 1: Nominal and measured concentration of Cu^{2+} ion, with relative percentage of recover, in all assessed dilutions used in the mortality bioassay; NC (nominal concentration), MC (measured concentration)

NC (Cu^{2+}) mg/L	MC (Cu^{2+}) mg/L	Recovery (%)
0.00 (control)	< LOQ	
0.254	0.208	82.03
0.509	0.415	81.47
1.018	0.785	77.12
2.036	1.730	84.95
4.072	3.313	81.36
8.144	6.941	85.23

Table 2: Mortality percentage of *Pachygrapsus marmoratus* zoea I at each assessed copper concentration, both at 24 h and 48 h of exposure. The toxicant (copper) is reported both as salt concentration used and relative ion concentration calculated.

Concentration (mg/L)		Mortality %	
$CuSO_4*5H_2O$	Cu^{2+}	24h	48h
0.00	0.00	0.00	6.66
1.00	0.25	6.67	13.33
2.00	0.50	25.00	70.00
4.00	1.01	33.33	80.00
8.00	2.03	56.67	86.66
16.00	4.07	71.33	90.00
32.00	8.14	84.66	96.66

Table 3: EC_{50} values calculated as both copper salt ($CuSO_4*5H_2O$) and the relative ion (Cu^{2+}) concentrations , at 24 h and 48 h of exposure

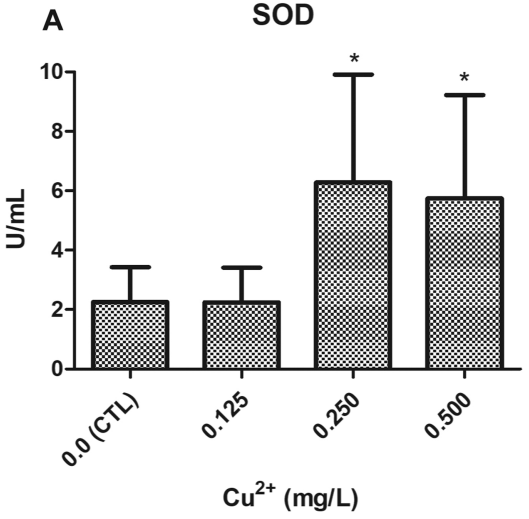
	LC 50 (95% CL) mg/L	
	24h	48h
$CuSO_4*5H_2O$	6.81 (5.78-8.08)	2.06 (0.13-4.70)
Cu^{2+}	1.73 (1.47-2.06)	0.52 (0.032-1.20)

Conflict of Interest

The Authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Highlights

- Dose dependent responses of larval mortality after both 24- and 48-hours copper exposure
- Dose dependent activation of antioxidant system of *P. marmoratus* larvae after copper exposure
- Dose dependent variation in cholinesterase activities after copper exposure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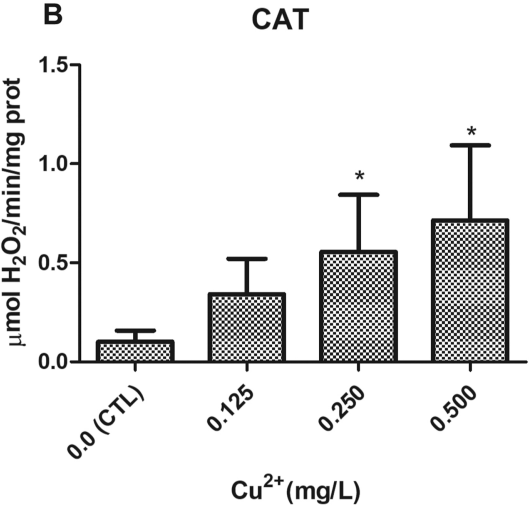
0.500
Cu2+ (mg/L)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Cu2+(mg/L)

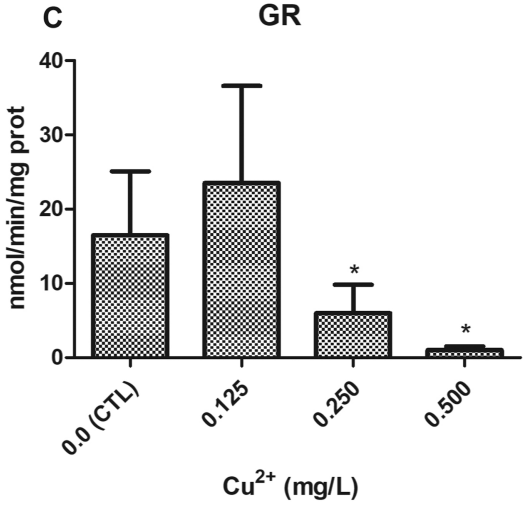
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Figure 1

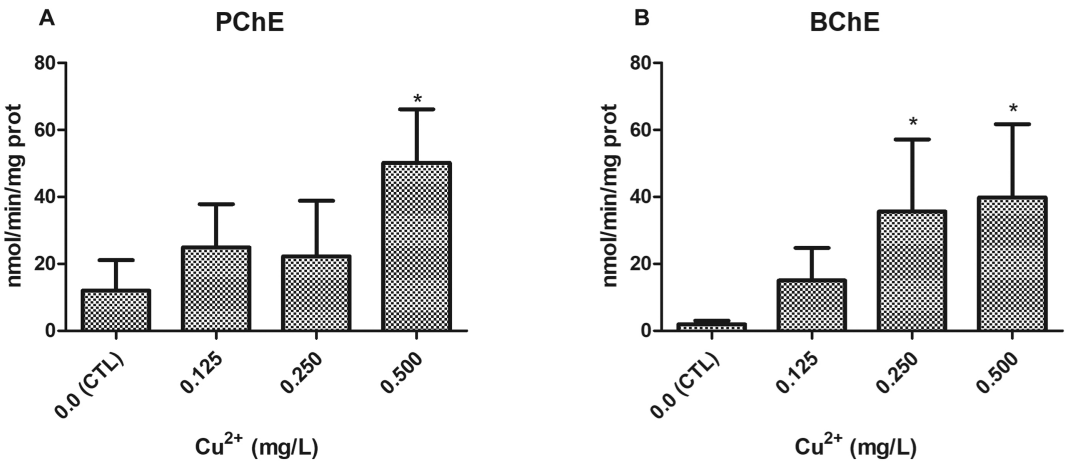
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Figure 2