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ANTIOXIDANT RESPONSES IN *MYTILUS GALLOPROVINCIALIS* EXPOSED TO COPPER

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ABSTRACT

A laboratory experiment was conducted to investigate correlations between Cu concentrations in *Mytilus galloprovincialis* and antioxidant responses. The results of the experiment indicated that Cu accumulated in *M. galloprovincialis* during a 21-day exposure period. Mussels exposed to low dosages (40 µg/L) of Cu resulted in a 4-fold increase in Cu bioaccumulation, whereas mussels exposed to high dosages (100 µg/L) of Cu, resulted in a 10-fold increase. The potential of oxidative stress as a biomarker was investigated using a battery of antioxidant assays/methodologies. The total antioxidant capacity was measured using FRAP and ORAC, antioxidant enzyme activity was determined by measuring catalase, superoxide dismutase, while the oxidative stress status was assessed by measuring the glutathione (GSH) content and lipid peroxidation marker levels of conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS). The results showed that *M. galloprovincialis* exposed to high dosages of Cu had significantly ($P<0.05$) increased levels of TBARS, indicative of increased oxidative stress. Interestingly, for GSH levels and ORAC capacity, at day 21, both were significantly higher ($P<0.05$) than at the start of the experiment. The results suggested that oxidative stress responses in *M. galloprovincialis* could be considered as biomarkers of toxicity in southern Africa.

KEYWORDS:
Antioxidant responses, biomarkers, copper, *Mytilus galloprovincialis*, mussels, oxidative stress

INTRODUCTION

Excess oxidant by-products of normal metabolism are known to cause damage to DNA, proteins and lipids [1, 2, 3] and the effects of oxidants to the health of organisms are well documented [4, 5, 6, 7]. To counter the effects of oxidants, aerobic organisms have developed defence mechanisms against oxidative stress by using antioxidants [8, 9, 10]. Antioxidant defence systems include both enzymatic and non-enzymatic antioxidants [7, 11]. Endogenous antioxidant defence systems includes enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-PX) that act as detoxifying agents of reactive oxygen species (ROS) [7, 12, 13]. Glutathione reductase (GR) catalyses the reduction of oxidised glutathione (GSSG) to reduced glutathione (GSH) and thereby facilitating maintenance of the cellular redox status [1, 11, 13].

Antioxidants serve a protective function as the main and first line of defence by suppressing the formation of ROS [2, 9, 11]. This is done by reducing hydrogen peroxide and lipid hydroperoxides to water and lipid hydroxides, respectively, or by sequestering metal ions such as iron and copper. The scavenging antioxidants remove active species rapidly before the active species attack biologically essential molecules. These scavenging antioxidants act as the second line of defence *in vivo*. Also, various enzymes function in defence mechanisms by repairing damages, clearing the wastes, and reconstituting the lost function, act as the third line defence [14]).

In areas contaminated with chemicals, sentinel organisms such as mussels are not able to eliminate toxins and hence accumulate them. Cellular responses to contaminants include the development of mechanisms to protect themselves from toxic effects of increased ROS production [13]. The levels of antioxidants produced are therefore considered potential biomarkers of toxicity of chemicals [16, 13]. Since the use of an individual biomarker may not be able to reflect the status of an organism's health and the effects of each pollutant in a mixture [15, 16, 17], the use of a variety of biomarkers (biomarker battery) is considered better suited when determining the biological impact of pollutants (including metals) [18].

Mussels are widely used as sentinel organisms in marine pollution monitoring and are therefore considered ideal monitors of pollution in environments due to their ability to bioaccumulate pollutants [19]. Bivalves such as *M. galloprovincialis* are commonly used as bioindicators as these organisms are known to accumulate high levels of trace metals and organic compounds in their tissue [20]. The resultant

responses to metal exposure have resulted in oxidative stress through the formation of ROS and lipid peroxidation [21, 22, 23, 24, 25, 26].

Cu is an essential metal for many biological systems and is present in small concentrations in various cells and tissues. Numerous enzymes require Cu as a cofactor for structural and catalytic properties, including cytochrome c oxidase [27]. Several mechanisms have been proposed to explain Cu induced cellular toxicity, including the capacity of free Cu ions to participate in the formation of reactive oxygen species (ROS) [28]. The presence of Cu can play an important role in the formation of ROS in biological systems [2, 7]. Hence, the presence of Cu influences the formation of ROS and when the ROS production exceeds the rates of its decomposition by antioxidant defences and repair systems, it leads to oxidative stress [7].

Copper (Cu) is known to display a high affinity for thiol groups [29]. According to [28], *in vitro* oxidation of the cystein thiol group of GSH exposed to Cu resulted in the release of superoxide anions and hydrogen peroxides. Exposure to Cu in oysters (*Crassostrea virginica*) resulted in increased lipid peroxidation (LPO) [30] but at the same time, when exposed to Cu and a GSH inhibitor (buthionone sulfoximine), a lower LPO was reported, suggesting a protective role of GSH to Cu toxicity. According to [31], Cu is a potent oxidant agent in *M. galloprovincialis* as metallothioneins and LPO levels increased after exposure to Cu. These authors suggested that the antioxidant responses to Cu exposure are complex and that the contaminant mixtures interact differently based on target specific tissue and that this may lead to an imbalance in the health status of the mussel.

Cape Town is a major urban centre and has a large reservoir of Cu. [32] evaluated the uses of Cu in Cape Town and found that the city had approximately 110 million kg of in-use Cu. However, the effects of Cu on the antioxidant system in *M. galloprovincialis* in southern Africa have not been investigated as yet. The aim of this study was to apply a multi-biomarker approach to investigate antioxidant response of *M. galloprovincialis* to Cu exposure. To this end, several biochemical responses of the antioxidant system were assessed in whole soft tissue. Total antioxidant capacity was determined using the ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) assays. Antioxidant enzyme activity was determined for catalase (CAT), superoxide dismutase (SOD) while reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were also measured. Lipid peroxidation was determined using conjugated dienes (CDs) and thiobarbituric acid reactive substances (TBARS).

MATERIALS AND METHODS

Experimental design. Mussels *M. galloprovincialis* of similar size (6.62 ± 0.8 cm shell length) were collected from Bloubergstrand, Western Cape, South Africa and mean metal concentrations in mussels sampled from this site were below the Canadian sediment quality guidelines for the marine environment [33]. Mussels sampled were transported, with site water, to a laboratory at the Cape Town campus of the Cape Peninsula University of Technology (CPUT) where they were allowed to depurate and acclimate for 3 days. The mussels were then separated into triplicate groups of 45 specimens, placed in aerated containers, containing 0.5 L of seawater per mussel. The sea water was acquired from the research aquarium in Sea Point (Department of Agriculture, Forestry and Fisheries, DAFF, South Africa), and passed through a closed filtration system containing 0.10 µm, 0.5 µm and 0.1 µm filters as well as a UV filter before being placed into the aerated containers. After 3 days of acclimation in a temperature controlled room (18 °C), the mussels were fed MicroVert® invertebrate food as per the directions, the water changed one hour later and then dosed with Cu.

The exposure experiment comprised triplicates of a control group (mussels in seawater but not exposed to any metal), a low dosage (40 µg/L of Cu from $CuSO_4$) and a high but sublethal dosage (100 µg/L of Cu from $CuSO_4$) exposure. These concentrations were selected as they represented the environmental condition for the Cape Peninsula [34] for the low dosage group and the mussels were considered to be stressed when exposed to 100 µg/L Cu [35]. The first 10 mussels were sampled at the start of the experiment (T_0) for both metal and antioxidant analysis. The water was changed every second day and 10 mussels sampled every seven days. Of the 10 mussels sampled, 5 were used for metal analysis and 5 for antioxidant analysis. Mussels to be analysed for antioxidant enzyme activity were immediately immersed in liquid nitrogen and stored at -80° until the analyses were done. The mussels destined for metal analysis were stored at -20°C until the analyses were done.

Metal analysis. Metal analysis was done according to the method of [36]. Frozen mussel samples (n=5) were defrosted and oven dried for 48 hrs at 60° C in a Memmert drying oven. The soft tissue and shells were weighed and separated before oven drying. The dried tissue samples were weighed and homogenized per individual mussel with a mortar and pestle. Aliquot samples of individual mussels (± 0.2 g) were digested using 10 mL of nitric acid (HNO_3). Samples were then heated to 40 °C in a Grant UBD heating block for one hour, thereafter to 120 °C for 3 hours. The digestates were allowed to cool and then filtered through a Whatman No. 6 filter

paper and then through a 0.45 µm membrane microfilter (Millipore) paper using a needle and syringe. Samples were then placed in plastic centrifuge tubes containing 5 mL digestate and 10 mL distilled water and stored in a refrigerator until further analysis was done. A blank accompanied all samples when analyses of samples were done. The concentrations Cu was analysed, with five replicates being done for each metal using the Inductively Coupled Plasma Mass Spectrophotometer (ICP-MS). Concentrations of metals are presented as µg/g dry weight.

Biochemical Analysis. Prior to biochemical analysis, soft tissues were removed from the shells and weighed. Mussels were freeze dried for 48 hours and thereafter soft tissues of five mussels were combined and split into three aliquots and stored at -20 °C until chemical analysis were done.

Samples were thawed on ice and 0.5 g freeze-dried tissue was added to 5 mL of homogenization buffer and prepared as described by [37], with slight modifications. The homogenization buffer contained 50 mM KPO_4, 0.1 M KCl, 0.1 mM EDTA, pH 7.4; with 20% glycerol to protect the enzymes. The samples were homogenized using a glass Potter Elvehjem homogenizer and kept on ice throughout the homogenization process until the homogenates were centrifuged at 12000 x *g* at 4 °C for 30 minutes. The supernatant was retained and kept at -80 °C for subsequent analysis.

Antioxidant enzyme activities. Catalase activity was determined according to [38]. The homogenates were thawed on ice and diluted to (1:5 v:v) homogenate to buffer. To a 96-well plate, an assay mixture containing 170 µL phosphate buffer (50 mM KPO_4 buffer, pH 7.0) and 5 µL of the homogenate sample, in triplicate, was added. Thereafter, 75 µL H_2O_2 stock solution (30% v/v) was added, the solution mixed well and the decrease in absorbance measured at 240 nm (ϵ = 0.00394 $mM^{-1}.cm^{-1}$) in a Multiskan spectrophotometer and the enzyme activity calculated. The results were expressed as mmol/mg protein. Superoxide dismutase (SOD) activity was determined according to [38]. The kinetics of the auto-oxidation of 6-HD was monitored at 490 nm and 25 °C for approximately 4 mins. The assay was done by adding, in triplicate, 170 µL DETAPAC solution (0.1 mM) in an SOD assay buffer (50 mM, pH 7.5), to a 96-well plate. Samples were diluted 1:10 (v:v), homogenate to buffer, and the SOD buffer was added to the wells to make up a final volume of 200 µL. A range of sample volumes were assayed (0, 6, 12, and 18 µL) beforehand and the 6 µL volume of sample was added to the wells. To the DETAPAC and sample solution, 15 µL of stock 6-HD (1.6 mM) was added to initiate the reaction, whereafter the combined solution was mixed and the amount of protein used that resulted in 50% inhibition of auto oxidation of the 6-HD was measured spectrophotometrically in a Multiskan reader at 490 nm. The results were expressed as U/mg protein. Reduced and oxidized glutathione (GSH:GSSG) levels were determined according to [39]. In this assay glutathione reductase is added and hence both GSH and GSSG measured, which indicates total glutathione presence. For the GSSG determination, the freeze dried mussel tissue were homogenised using 500 mM $NaPO_4$ with 1 mM EDTA (pH 7.5), containing M2VP and centrifuged at 15000 x g for 5 minutes at 4 °C. GSH determination was done on previously homogenised freeze dried mussels without M2VP. This enabled conjugation of GSH for the determination of GSSG. Samples of GSH or GSSG standards (50 µL) were prepared in triplicate and added to 96-microwell plates. To these wells, 50 µL (0.3 mM) DTNB and thereafter 50 µL of GR (1 u/50 µL) were added. The microwell plates were then mixed and incubated for 5 minutes at 25 ºC. To initiate the reaction, 50 µL of 1 mM NADPH was added to each well and the absorbance immediately measured at 412 nm in a Multiskan reader. The change in absorbance in either GSH or GSSG was determined using a linear function. Calibration curves for GSSG and GSH were determined separately and the GSH:GSSG ratios calculated by dividing the difference between GSH and GSSG concentrations by the concentrations of GSSG. The results were expressed as µmol/g.

The FRAP assay was carried out as described by [40]. The homogenized tissue was thawed on ice and thereafter mixed with 5% PCA (1:1). The PCA mixed sample was centrifuged at 4000 rpm for 5 minutes at 4 °C. Thereafter, 10 µL (in triplicate) of sample and standards were pipetted to microwell plates. The standards comprised various concentrations (0-1000 µM) of AA. Thereafter, 300 µL of FRAP reagent was added to the plates. The FRAP reagent was prepared as follows: 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution, 20 mM $FeCl_3$ solution and distilled water that made up a final volume of 300 µL. The final volume added to the plate was 310 µL. The plate was incubated at 37 ^oC for 30 min and read at 593 nm in a Multiskan reader. The results were expressed as µmol Ascorbic Acid Equivalents (AAE) /g. The ORAC method was performed using a fluorescence spectrophotometer until zero fluorescence occured. The method of [41] was used on samples that were homogenized as described previously. A 1:10 (5% PCA FRAP homogenate sample: ORAC buffer) diluted sample was used for the ORAC assay. Preparations of the samples were done on ice throughout the procedure. The PCA diluted sample was centrifuged at 4000 rpm for 5 minutes at 4 °C. Thereafter, 12 µL (in triplicate) of sample and standards were pipetted to black 96-microwell plates. The standards comprised various concentrations (0-417 µM) of Trolox solutions. Thereafter, 138 µL of fluorescein was added to the plates followed by 50 µL of AAPH, to initiate the

reaction, making up a final volume of 200 µL being added to the wells. The solution in the wells was read using a fluorescence plate reader. The fluorescence of fluorescein was recorded every 5 minutes for 2 hours after the addition of AAPH. The ORAC values were calculated using a regression equation ($Y = a + bx + cx^2$) between Trolox concentration (Y) (µmol) and the net area under the fluorescence decay curve (x). Data were expressed as micromoles of Trolox equivalents (TE) per gram of sample (µmol TE/g).

Levels of lipid peroxidation were assessed by measurements of conjugated dienes (CDs) and thiobarbituric acid reactive substances (TBARS). The CDs were measured according to [42]. To the freeze-dried samples (50 mg), 1 mL of a chloroform:methanol solution (2:1) was added and kept on ice. Solutions were vortexed and then centrifuged at 10000 rpm for 10 minutes at 4 °C. The top layer was removed and to the bottom organic layer, 500 µL of HCl was added. This solution was then vortexed for 10 seconds and then centrifuged at 10 000 rpm for 3 minutes at 4ºC. From the bottom layer, 100 µL was removed and transferred to a new eppendorf tube and dried under nitrogen gas. To each dried residue eppendorf tube, 1 mL cyclohexane was added. The solution was then placed into micro well plates (in triplicate) and the absorbance measured at 234 nm using a Multiskan reader. The results were expressed as µmol/g.

The TBARS were measured according to [45] with slight modifications. The homogenized mussel tissue was thawed on ice and 100 µL added to eppendorf tubes. To the samples, 375 µL of H_3PO_4 (0.44 M) and 125 µL TBA (42 mM) was added and the mixture vortexed for 10 seconds and heated in a boiling-water bath for 60 min. The solution was allowed to cool on ice for 2 minutes and then left at room temperature for 5 minutes. To the cooled solution, 500 µL butanol and 50 µL saturated NaCl was added. The sample was vortexed for 10 seconds and then centrifuged at 12 000 rpm for 2 minutes at 4ºC. From this mixture, 150 µL of the supernatant was added to 96 well plates (in triplicate) and the absorbance read at 532 nm in a Multiskan plate reader. The results were expressed as TBARS µmol/L.

Total protein concentrations. Protein concentrations in homogenized tissue samples were determined using a commercially-available protein assay kit (Pierce® BCA Protein Assay Kit, Thermo Scientific). Bovine serum albumin (BSA) was used as the protein standard and quantified by measuring absorbance at 595 nm [62].

Statistical analysis. Data were reported as means (± SEM). All calculations and data analysis were done using Statistica v10 (Statsoft). One way ANOVA was used to determine whether there were differences in mean metal concentrations over time and between exposure groups in *M. galloprovincialis*. The data was tested for normality and homogeneity of variance using Kolmogorov-Smirnoff and Levene's tests respectively, prior to post hoc comparisons. When data did not follow these assumptions, they were logarithmically transformed. Post hoc ANOVA analysis were done using the Tukey Honest Significant Difference (HSD) Test to determine statistical significances between groups and over time ($p<0.05$). The use of the test resulted in the determination of significant differences ($p<0.05$) between control, low and high dosage groups as well as significant differences over time (7, 14 and 21 days). Further ANOVA analysis were done using the Dunnet Test to determine statistical significances between groups and time with control groups (no exposure group or at T=0) ($p<0.05$). Non-parametric Spearman rank correlations of assays in *M. galloprovincialis* were done to determine relationships between parameters.

RESULTS

Metal analysis. Exposure to copper (Cu) in the mussel, *Mytilus galloprovincialis* resulted in metal accumulation in the soft tissue of the organism over the 21 day exposure (Figure 1). Within the control group (no exposure to copper) the level of Cu in the tissue of mussels were relatively low and ranged between 1.43 and 2.21 µg/g dry weight (dw).

Copper accumulated in mussels exposed to low dosages (40 µg/L) of Cu. At the start of the exposure, the level of Cu in the mussel tissue was 2.83 ± 0.34 µg/g dw and increased 4 fold at the end of the 21 day exposure period to 12.55 ± 1.73 µg/g dw. The mean concentration of Cu in the low dosage group was 6.81 ± 6.86 µg/g dw. The increase (2.8 fold) in accumulated Cu within mussels was significant ($p<0.05$) after 14 days of exposure from the start of the exposure, with further significant ($p<0.05$) increases at day 21 (4.4 fold). There was also a significant ($p<0.05$) 3.5 fold increase in Cu in mussels within the low dosage group when compared to the control group. By days 14 and 21 there were significant differences in Cu concentration in *M. galloprovincialis* between the low and high dosage groups.

Mussels exposed to the high dosage of Cu (100 µg/L) showed a 10 fold increase in Cu accumulated within the tissue. At the start of the exposure, the level of Cu was 2.86 ± 0.76 µg/g dw and by day 21 the level of Cu recorded in the tissue was 28.28 ± 7.3 µg/g dw. The mean concentration of Cu within the high dosage exposure was 15. 42 ± 4.11 µg/g dw. Within the high dosage group, Cu increased 2.66 fold on day 7 (2.86 to 7.64 µg/g dw), to 22.90 µg/g dw on day 14 (7 fold increase from the control) and then to 28.28 µg/g dw on day 21. The increases at day 14 and 21 were significant ($p<0.05$) when compared to the levels of Cu at the start of the exposure.

The high dosage exposure group had significantly higher Cu concentrations (8.1 fold) when compared to the control group as well as from the start of the experiment.

Biochemical analyses. Catalase activity did not differ significantly in either group (control, low dosage and high dosage) or over time (Time = 0, 7, 14 and 21 days) ($p>0.05$) (Figure 2). At the start of the exposure, SOD activity was higher in the control and high dosage groups than the low dosage group (Figure 3). The differences however were not significant ($p> 0.05$). After 7 and 14 days, SOD activities in the control and high dosage groups were significantly ($p<0.05$) lower than at the start of the exposure. During the entire exposure period, there were no significant differences in SOD in the low and high dosage groups compared with the control groups on those respective days ($p>0.05$). The GSH level followed the same pattern throughout the exposure to Cu (Figure 4) where concentrations increased from the control, through the low, towards the high dosage groups. There were no significant differences between dosage groups at the start of the exposure. By day 7 there were significant ($p<0.05$) differences between low and high dosage groups, as well as between the high dosage and the control group. This situation was the same on days 14 and 21 of the exposure. Furthermore, on day 21 the GSH levels in the low and high dosage groups were significantly ($p<0.05$) higher when compared to that of day 0.

At the start of the exposure, FRAP was higher in the low dosage group than both control and high dosage groups (Figure 5), but the differences were not significant. For the remainder of the exposure period, the FRAP activity was constant in all 3 exposure groups. FRAP levels in the control and high dosages on day 14 as well as the high dosage group on day 21 however were significantly ($p<0.05$) higher than at the start of the exposure. The ORAC values were highly variable over the entire exposure period and displayed an increase from the control to high dosage group after the start of the exposure (Figure 6). At the start of the exposure, ORAC for the low dosage was significantly ($p<0.05$) higher than the control group. On day 7, ORAC for the high dosage group was significantly higher than the day 7 control group. By day 14, ORAC for both low and high dosage groups were significantly higher than the control group of day 14 ($p<0.05$). ORAC for the high dosage group of day 14 was significantly higher from what it was at the start of the exposure. The same pattern as day 14 was observed on day 21. In addition to this, ORAC for the low dosage group was also significantly higher than the high dosage group by day 21 ($p<0.05$).

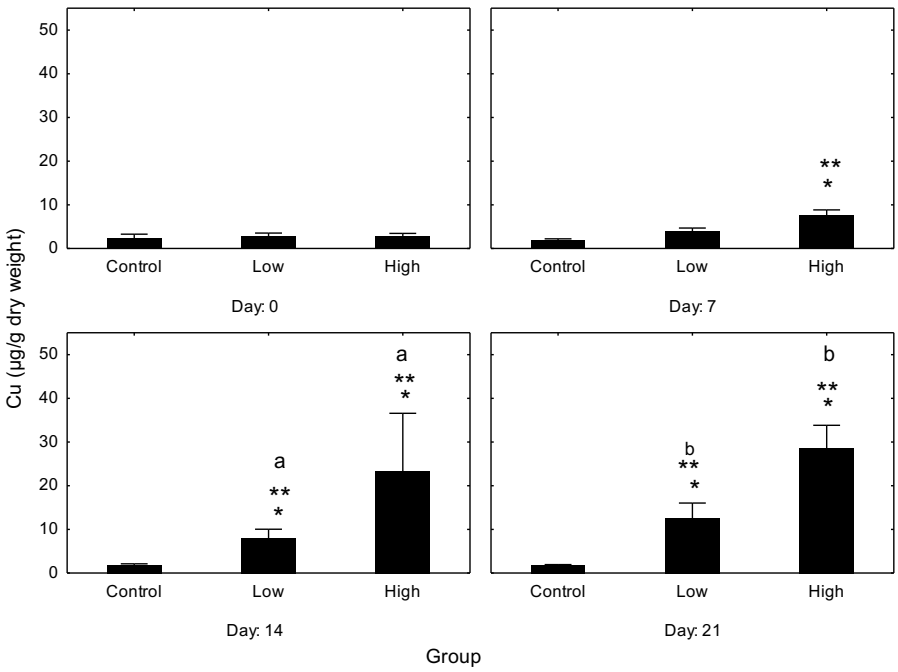

FIGURE 1

Mean (± SE.) copper (Cu) concentrations (µg/g dry weight) in the whole tissue of Mytilus galloprovincialis exposed for 7, 14 and 21 days to 40 µg/L (low dosage) and 100 µg/L (high dosage) of copper. * indicates significant difference of exposure to the control of that group, indicated by $p<0.05$ (one-way ANOVA, Dunnett post hoc test). Similar letters indicate significant difference between exposure groups indicated by $p<0.005$ (one-way ANOVA, Tukey HSD post hoc test). ** indicates significant difference to that of the start of the exposure, indicated by $p<0.05$ (one-way ANOVA, Dunnett post hoc test).

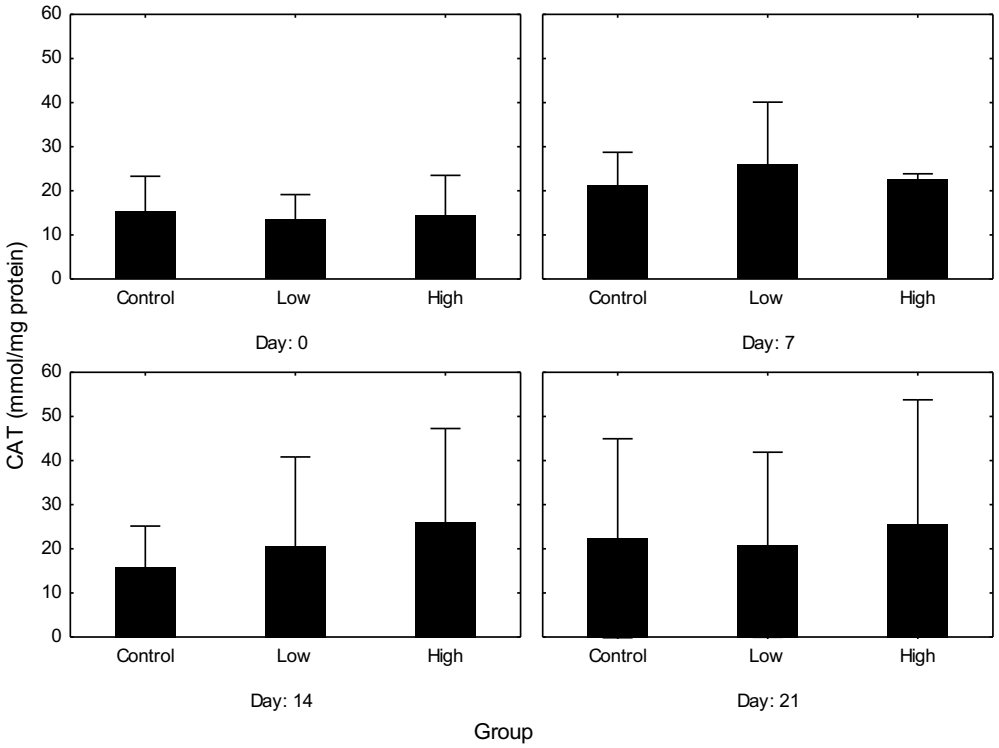

FIGURE 2

Mean (± SE.) catalase (CAT) activity in the whole tissue of *Mytilus galloprovincialis* exposed for 7, 14 and 21 days to 0 µg/L (control), 40 µg/L (low dosage) and 100 µg/L (high dosage) of copper.

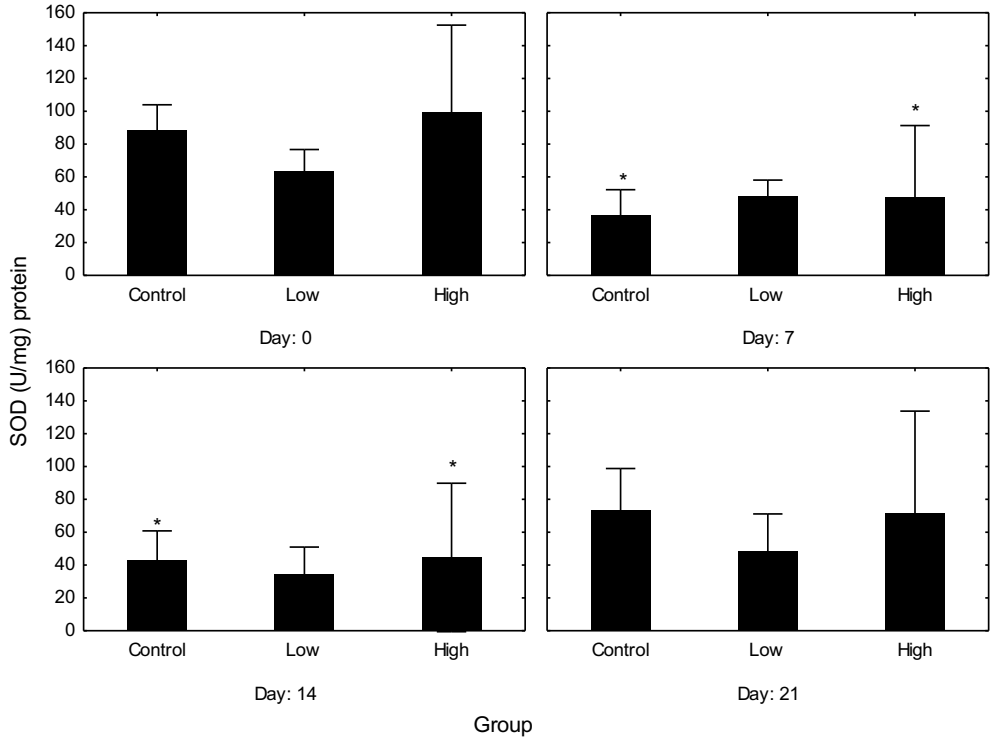

FIGURE 3

Mean (± SE.) SOD activity in the whole tissue of *Mytilus galloprovincialis* exposed for 7, 14 and 21 days to 0 µg/L (control), 40 µg/L (low dosage) and 100 µg/L (high dosage) of copper.
*** indicates significant difference to that of the start of the exposure, indicated by $p<0.05$ (one-way ANOVA, Dunnett post hoc test).**

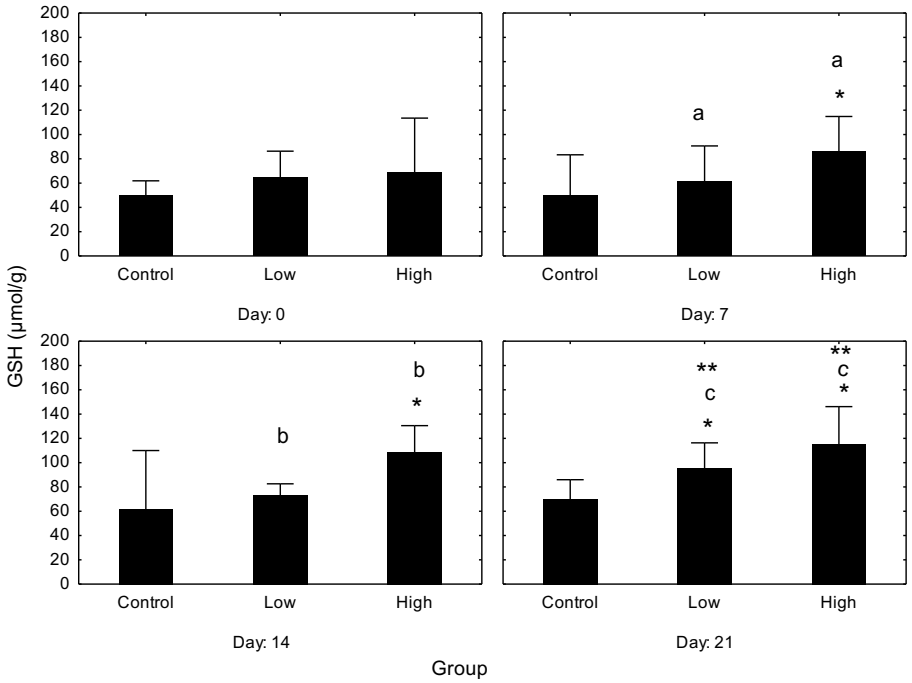

FIGURE 4

Mean (± SE.) GSH activity in the whole tissue of *Mytilus galloprovincialis* exposed for 7, 14 and 21 days to 0 µg/L (control), 40 µg/L (low dosage) and 100 µg/L (high dosage) of copper.
*** indicates significant difference of exposure to the control of that group, indicated by p<0.05 (one-way ANOVA, Dunnett post hoc test). Similar letters indicate significant difference between exposure groups indicated by p<0.005 (one-way ANOVA, Tukey HSD post hoc test). ** indicates significant difference to that of the start of the exposure, indicated by p<0.05 (one-way ANOVA, Dunnett post hoc test).**

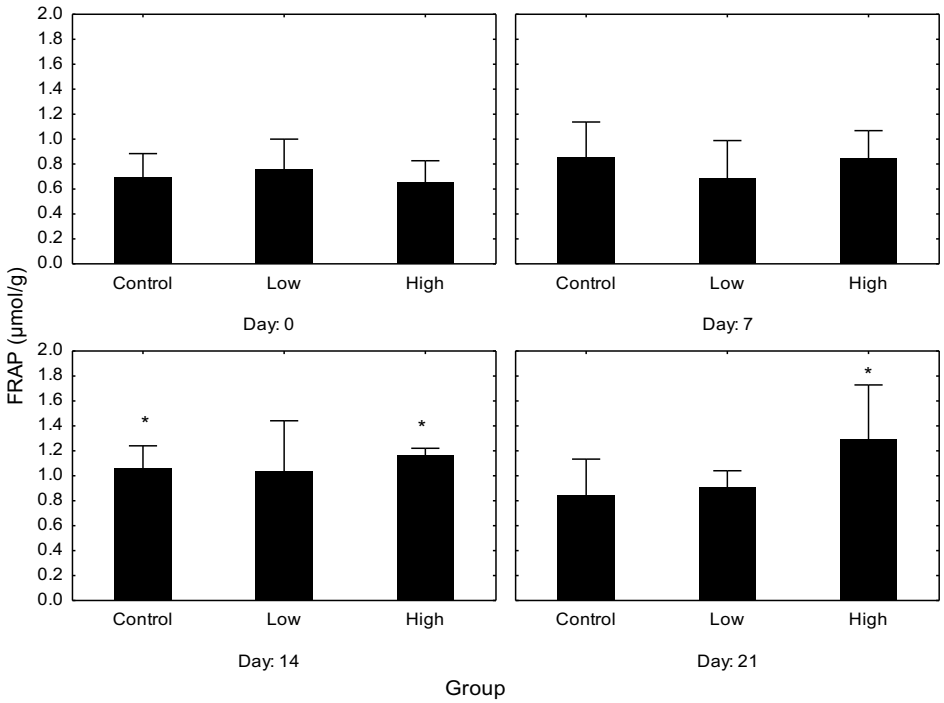

FIGURE 5

Mean (± SE.) FRAP activity in the whole tissue of *Mytilus galloprovincialis* exposed for 7, 14 and 21 days to 0 µg/L (control), 40 µg/L (low dosage) and 100 µg/L (high dosage) of copper.
*** indicates significant difference to that of the start of the exposure, indicated by p<0.05 (one-way ANOVA, Dunnett post hoc test).**

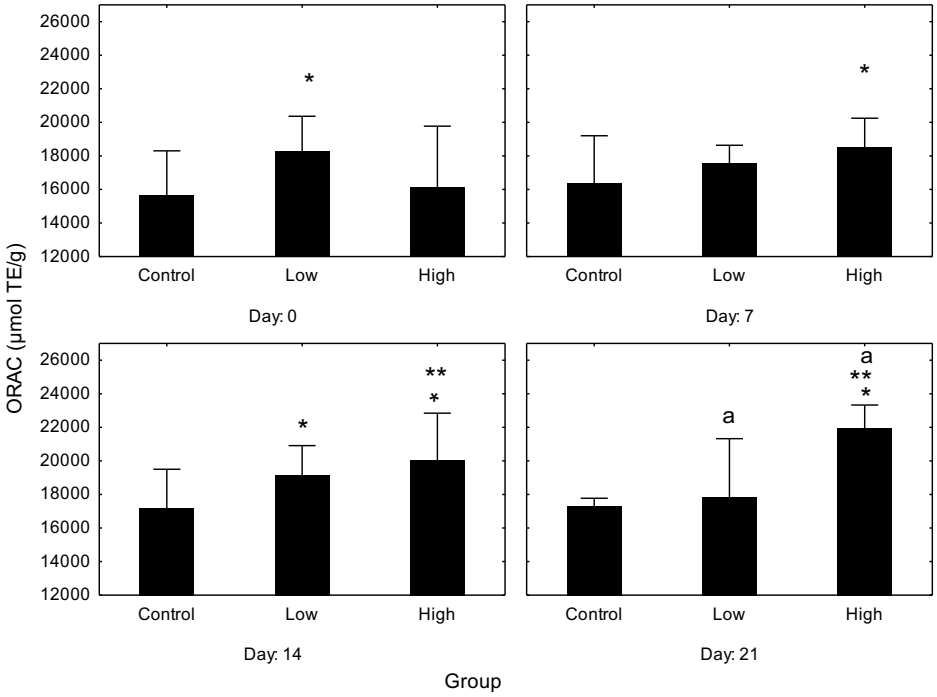

FIGURE 6

Mean (± SE.) ORAC activity in the whole tissue of *Mytilus galloprovincialis* exposed for 7, 14 and 21 days to 0 µg/L (control), 40 µg/L (low dosage) and 100 µg/L (high dosage) of copper. * indicates significant difference of exposure to the control of that group, indicated by p<0.05 (one-way ANOVA, Dunnett post hoc test). Similar letters indicate significant difference between exposure groups indicated by p<0.005 (one-way ANOVA, Tukey HSD post hoc test). ** indicates significant difference to that of the start of the exposure, indicated by p<0.05 (one-way ANOVA, Dunnett post hoc test).

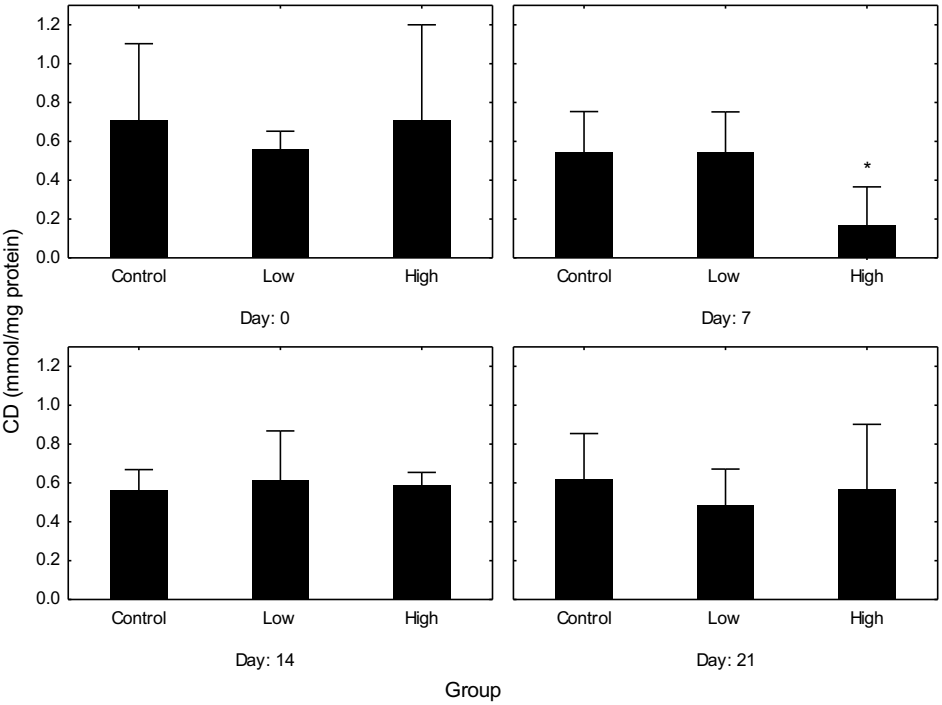

FIGURE 7

Mean (± SE.) CD activity in the whole tissue of *Mytilus galloprovincialis* exposed for 7, 14 and 21 days to 0 µg/L (control), 40 µg/L (low dosage) and 100 µg/L (high dosage) of copper. * indicates significant difference to that of the start of the exposure, indicated by p<0.05 (one-way ANOVA, Dunnett post hoc test).

The CD levels were similar for all groups and were consistently low for the duration of the exposure period (Fig 7). The high dosage group on day 7 had significantly ($p<0.05$) lower CD levels than day 0. The TBARS levels were similar throughout the entire exposure period indicating slight increases (not significant, $p>0.05$) from control to high dosage groups (Figure 5-8). The low dosage group on day 7 was significantly ($p<0.05$) higher than the start of the exposure and by day 21, TBARS levels were significantly ($p<0.05$) higher than at of the start of the exposure. The TBARS activity in the high dosage group on day 21 was significantly ($p<0.05$) higher than the control group of day 21.

There were significant positive correlations between Cu, GSH, ORAC, TBARS and both group and day ($p<0.05$) (Table 1). When considering exposure, significant positive correlations (provide r value) were recorded between group, Cu, CAT, GSH, ORAC and TBARS. Further significant positive correlations (provide r value) for antioxidants were recorded between GSH and ORAC, FRAP and ORAC, CAT and TBARS, respectively. There was only one significant negative correlation recorded, between SOD and FRAP.

DISCUSSION

Filter-feeding bivalves, such as mussels, are renowned for their ability to accumulate contaminants to high levels [43]. Hence, mussels that occur in areas contaminated with pollutants may suffer from oxidative stress due to the effects of oxyradicals [37]. It is for this reason that antioxidant responses to anthropogenic influences have been proposed to be used as biomarkers for field-based monitoring programmes [46, 38]. According to [44], laboratory-based experiments are able to facilitate interpretation of results from field studies and contribute to research of causative factors. The exposure of *M. galloprovincialis* prevalent in the Western Cape, to Cu, is a first attempt to ascertain if antioxidant/oxidative stress responses can be used as biomarkers of metal toxicity.

The literature on the effect of antioxidant enzyme activities in aquatic organisms shows highly variable results, mainly due to the lack of standard and calibrated methods [7, 47]. This is often due to the variety of methods of analyses used to measure antioxidant enzyme activities, ranging from measuring gills, digestive glands and whole organisms. The present study focused on antioxidant capacities of the whole organism as indications by [48] who found that CAT and GST activities in whole soft tissue in *Dressena polymorpha* were much higher than in the gills [47].

Mussel exposure to inorganic copper resulted in the changes to antioxidant capacity in this study. The results indicated that *M. galloprovincialis* exposed to high dosages of Cu, resulted in the accumulation of Cu in its tissue. By day 21 of the exposure, a 10 fold increase in Cu concentration in *M. galloprovincialis* was recorded. This result was higher than that reported in other studies [49] where 3-5 fold increases were indicated. [10] recorded a 2 to 3 fold increase in Cu accumulation in mussels. However, [50] reported a 5 fold increase in Cu in mussels dosed with 50 µg/L Cu. According to [3], sub-micromolar concentrations of Cu can affect glutathione and digestive glands in *M. galloprovincialis* after one day of exposure, but was followed by a tendency to recover at longer exposure times. Previous studies on mussels exposed to Cu indicated that exposure to high dosages with the resultant accumulation of Cu in the tissue, resulted in oxidative stress conditions being prevalent [21]. The effect of high concentrations of Cu was that oxidative stress became evident in impaired antioxidant defence systems [49] and an increase in the generation of ROS that leads to lipid peroxidation and DNA damage [10]. These effects were observed by day 21 in both the low and high Cu dosage groups of this study (Fig 4, 6 and 8) with respect to oxidative lipid damage (measured as TBARS).

The CAT enzyme catalyses the transformation of reactive oxygen compounds, *i.e.*, hydrogen peroxide to water [44]. Hence CAT is considered to be an important and sensitive biomarker of oxidative stress, even better than SOD, indicating biological effects on the redox status of marine organisms [51]. [47] exposed mussels to environmental concentrations of 4-nonylphenol (4-NP). The results of that study showed no significant differences between the higher dosage groups (1, 10 and 100 µg/L). The lack of significant results were also evident in a study by [52] who exposed mussels (*Tapes phippinarium*) to 25, 50, 100 and 200 µg/L of 4-NP, and reported no significant differences in CAT activity between the groups. It was hence argued by [47] that mussels might have a high tolerance to oxidative stress levels due to the presence of haemocytes rich in lysosomal hydrolases, a very efficient lysosomal system and/or an increased production of esterase enzymes after the exposure to toxic substances. [31] reported that CAT activity was only enhanced at lower Cu concentrations (5 µg/L). The results of that study are supported in the trends observed in present study, in particular the responses in the low dosage group on day 21. The higher CAT activity on day 14 in the low and high dosage groups could be evidence of enzyme activity due to the exposure to Cu. It is possible that the mussels initially responded to the stress, but thereafter was unable to reduce the effects of the high Cu exposure by using CAT as was suggested by [53].

TABLE 1
Summary of Spearman rank correlations for antioxidant response.

	Group	Day	Cu (µg/g dry weight)	CAT (mmole/mg protein)	SOD (U/mg)	GSH (µmol/g)	FRAP (µmol/g)	ORAC (µmol TE/g)	CD (mmol/mg protein)	TBARS (µmol/L)
Group	1.000									
Day	0.000	1.000								
Cu (µg/g dry weight)	**0.770**	**0.332**	1.000							
CAT(mmole/mg protein)	0.200	0.297	0.307	1.000						
SOD (U/mg)	0.056	-0.203	-0.085	-0.036	1.000					
GSH (µmol/g)	**0.648**	**0.507**	**0.704**	0.250	0.054	1.000				
FRAP (µmol/g)	0.098	**0.443**	0.246	0.036	**-0.388**	0.300	1.000			
ORAC (µmol TE/g)	**0.557**	**0.411**	**0.682**	0.122	-0.325	**0.637**	**0.576**	1.000		
CD (mmol/mg protein)	-0.164	-0.014	-0.096	0.299	0.315	-0.075	-0.015	-0.212	1.000	
TBARS (µmol/L)	**0.396**	0.249	**0.449**	**0.689**	0.037	**0.480**	-0.052	0.254	0.167	1.000

Figures in bold are statistically significant at $p < 0.05$ level.

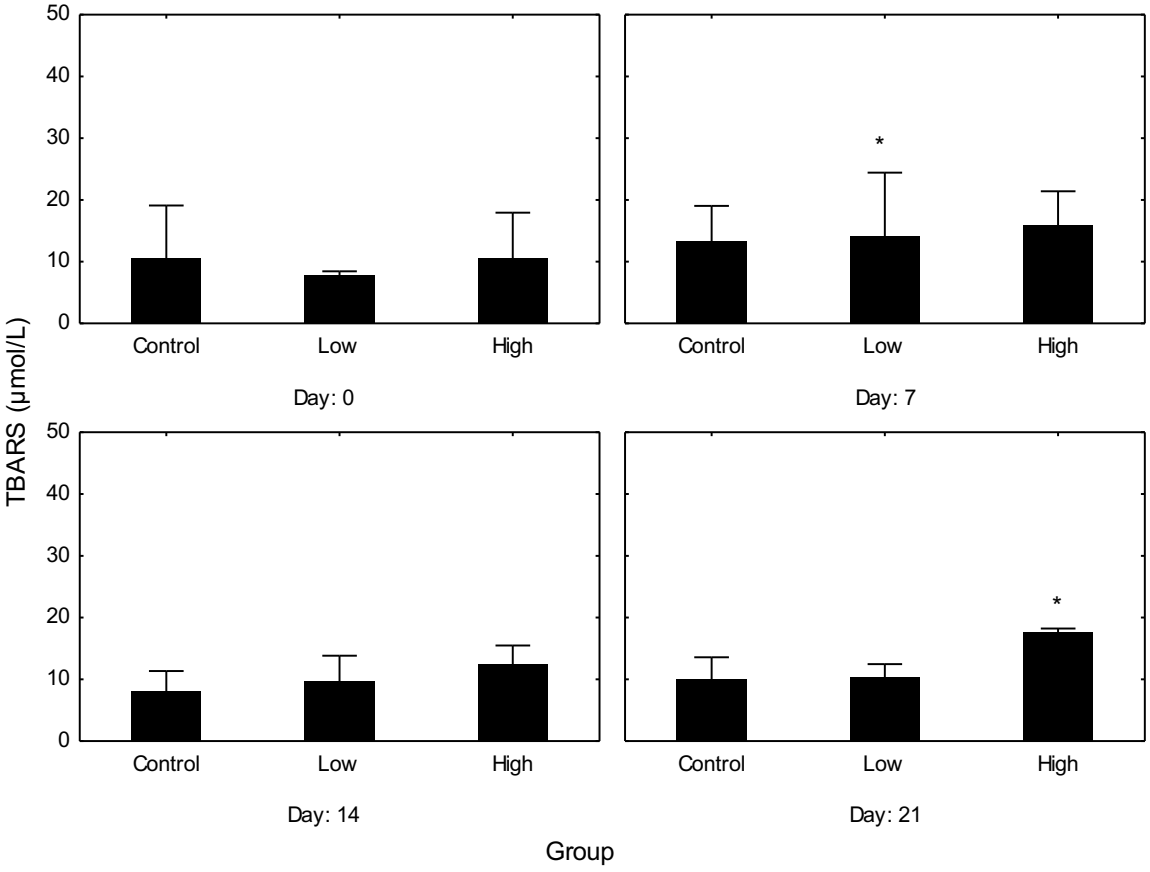

FIGURE 8
Mean (± SE.) TBARS activity in the whole tissue of *Mytilus galloprovincialis* exposed for 7, 14 and 21 days to 0 µg/L (control), 40 µg/L (low dosage) and 100 µg/L (high dosage) of copper.
*** indicates significant difference to that of the start of the exposure, indicated by $p<0.05$ (one-way ANOVA, Dunnett post hoc test).**

The SOD activity decreased after exposure to Cu, with significantly lower activities recorded in the control and high dosage groups on days 7 and 14. The lower SOD activity in the control group could be as a result low Cu concentrations in that group. Copper is an essential element and a co-factor for cytosolic SOD activity, and its deficiency will impair catalytic activity [31]. The results of this study are similar to others [31] who suggested that reduced SOD activity may be due to either increased ROS or

degenerating SOD activity, which in turn resulted in fewer active isoforms [54] or lower Cu presence [55]. Both these factors could account for the lower SOD activities in and high dosage and control groups on days 7 and 14, respectively, thus linking the lower Cu to the control group and the increased ROS to the high dosage group.

According to [56], similar values between total glutathione and acid-soluble thiols indicated that GSH was the most abundant thiol in *M. galloprovincialis* and that the concentrations of GSSG were negligible in both field and laboratory conditions. Hence, the GSH values reported here are considered indicative of total glutathione concentrations. According to [3], GSH is considered to play a protective role against metal toxicity and seems that glutathione represents the first line of defence against metals. The effects should however be considered in conjunction with the different routes of metal accumulation, speciation within the cellular environment and sequestration in subcellular compartments which in turn results in different patterns of metal cation interaction with intracellular components [3]. In the current study the significantly higher GSH activities reported in the high dosage group suggests that Cu induces oxidative stress, resulting in higher GSH activity and hence supports the proposal about the protective nature of glutathione noted previously. [53] reported that GSH increased after exposure to a metal and suggested that the response to oxidative stress and consequent GSH responses was considered a good indicator of oxidative stress in mussels. The result of GSH increase in this study therefore supports the proposal that increased levels of GSH be a good indicator of oxidative stress.

Both FRAP and ORAC are considered ideal methods to measure total antioxidant capacity [11]. The methods however do not distinguish between reactivity and concentration and are considered semi-quantitative. The results of this study show a weak but significant relationship ($r=0.576$, $p<0.05$) between FRAP and ORAC. This result shows the importance of using more than one method to measure the antioxidant capacity of samples. These findings are similar to that of [57] who compared different analytical methods to measure *in vivo* total antioxidant capacity and found a weak but significant correlation between ORAC and FRAP serum ($r = 0.349$, $p<0.05$).

The FRAP assay did not display any significant variation during the first 7 days of exposure to Cu. By day 14, both the control and high dosage had significant differences from the start of the exposure. The significantly higher FRAP values for the control group suggests that factors other than Cu dosage could have been responsible for oxidative stress, including insufficient Cu concentration in the water of the control group and stress due to general handling [58]. The FRAP activity for the low dosage group remained constant throughout the study period and could be an indication that at that concentration, the Cu was beneficial for the mussels to facilitate biological system functioning. The significantly higher values for the high dosage groups suggest that Cu accumulation was responsible for the oxidative stress. [59] reported that mussels exposed to polluted sites recorded higher antioxidant activity. These results further support the proposal that the mussels were stressed by day 14 and it was possible that by day 21 no longer had the antioxidant capacity to deal with the Cu-induced stress.

According to [57], the ORAC assay measures the capacity of an antioxidant to directly quench free radicals. The result of the present investigation indicated that ORAC differed significantly between groups of respective stages of exposure to Cu, suggesting that mussels in those groups were suffering from oxidative stress. The higher ORAC values hence provide an indication of lower protection afforded in the cellular environment against the potential toxicity of Cu. The data show that, despite antioxidant activity taking place, the higher Cu exposure resulted in an inability to counteract the toxicity of Cu, making the organism more susceptible to oxidative stress [60].

Two methods were used to evaluate lipid peroxidation damage over the course of the 21 exposure period (Figures 7 and 8). Each of the methods measured damage at a different stage of the lipid peroxidation process. Conjugated dienes (CDs) represent the initial product of radical attack, a rearrangement of the double bonds in unsaturated fatty acids [61]. The results of CD levels in the present study showed that only the high dosage exposure group was significantly lower on day 7 when compared to the start of the exposure. This suggests that the lack of CD accumulation in both low and high dosage groups of mussels is indicative of mechanisms that exist to repair CDs by stabilizing the CD level. By doing so, the CDs were not able to react and produce peroxyl radicals [61]. However, by day 21, CD levels in the control and high dosage group decreased to that of the start of the exposure, suggesting that the mussels were no longer able to deal with the stress caused by high Cu exposure.

The TBARS assay measures one of the terminal products in the peroxidation consequence of breakdown of lipids, malondialdehyde [63]. [63] reported that TBARS levels in gastropods showed a dramatic increase when stressed, but that TBARS levels decreased to control levels when these stresses were removed. [10], however, reported that lipid peroxidation decreased significantly when exposed Cu. The results of the present study showed an increase in TBARS levels over the 21 day exposure period with only low (on day 7) and high (by day 21) dosage groups being significantly higher than the start of the exposure experiment. The significant increase was therefore indicative of adequate antioxidant defences to deal with the increase in oxygen radical generation

associated with the increase in stress from increased Cu body burdens and it was only toward the end of the exposure period that they were no longer able to respond to the stress. These results are supported by those of [61] who found that the formation of TBARS products accumulated under stressful conditions.

Antioxidant responses to pollutants are difficult to predict and a high degree of variability has been reported [60], mainly as a function of the category of chemicals, nature of the exposure, stage of biological and ecological cycle [46, 49, , 60, 61]. Although the intent of the present study was to detect mussel antioxidant responses to Cu, and correlate the responses to oxidative stress, interpretation of responses is difficult given the variability previously noted. Nevertheless, the results of the present study showed a clear dose-response to Cu by *M. galloprovincialis* and provided valuable information about the application of antioxidants as biomarkers in these mussels prevalent along the west coast of the Cape Peninsula in Cape Town. Although Cu bioaccumulated in the mussels, antioxidant responses were variable. For antioxidant enzyme activity, SOD and GSH are considered ideal biomarkers and for antioxidant capacity biomarkers (FRAP and ORAC), both are considered reliable responses, albeit that the FRAP responses were not significantly different. Lipid peroxidation assays indicated that TBARS are better suited as reliable responses to stress and it is suggested that other types of lipid peroxidation methods be considered. The ecological relevance of biomarkers is higher when, in addition to exposure to pollutants, they are indicative of adverse effects on organisms [50, 61]. The results of this study are indicative of this and can be considered for use as references for field based-studies.

CONCLUSION

To come to conclusive decisions about the use of antioxidant responses as biomarkers of toxicity is difficult. Only GSH and ORAC activities were dose-dependent during the experiment. Animals react to stress in different ways and these are situation specific. In response to exposure to toxins, antioxidant activity increases and in this way can be used as an indicator of toxicity. When the animals are stressed, the responses peak, then decrease, and this ultimately lead to death if the stress is too intense and/or for too long. It is therefore difficult to interpret at which stage of oxidative stress an organism is, when considering the data from antioxidant responses. Nevertheless, the antioxidant responses of *M. galloprovincialis* appear to be potentially useful biomarkers in the mentioned region, as evident from the responses reported in the present study. The reduced capability of the mussel to neutralise deleterious effects of Cu is evident in high dosage exposures and this would have resulted in an increased susceptibility to oxidative stress. Hence the measured parameters represent different aspects of antioxidant responses to Cu and are considered potentially useful biomarkers of metal contamination. This study forms an important basis for future studies to be launched in the region.

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