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Toxicology

Chronic exposure of *Oreochromis niloticus* to sub-lethal copper concentrations: Effects on growth, antioxidant, non-enzymatic antioxidant, oxidative stress and non-specific immune responses

Narayanan Gopi[a], Sekar Vijayakumar[a], Rajagopalan Thaya[b], Marimuthu Govindarajan[c,d], Naiyf S. Alharbi[e], Shine Kadaikunnan[e], Jamal M. Khaled[e], Mohammed N. Al-Anbr[e], Baskaralingam Vaseeharan[a,*]

[a] Biomaterials and Biotechnology in Animal Health Lab, Department of Animal Health and Management, Alagappa University, Karaikudi, 630004, Tamil Nadu, India
[b] Foundation for Innovative Research in Science and Technology, Kelavannanvilai, NGO Colony Road, Nagercoil, Tamil Nadu, India
[c] Unit of Vector Control, Phytochemistry and Nanotechnology, Department of Zoology, Annamalai University, Annamalainagar, 608 002, Tamil Nadu, India
[d] Department of Zoology, Government College for Women, Kumbakonam 612 001, Tamil Nadu, India
[e] Department of Botany and Microbiology, College of Science, King Saud University, Riyadh 11451, Saudi Arabia

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ABSTRACT

Background: Trace elements of copper (Cu) are one of the main forms of ecological noxious waste in freshwater systems that affect the survival and development of organisms. The objective of the current study was to investigate the effects of chronic exposure to Cu on the growth, oxidative stress, immune and biochemical response in the Nile tilapia, *Oreochromis niloticus*.

Methods: Three groups of *O. niloticus* were tested as follows; the first group was used as the control (not treated with Cu in water), while the 2nd and 3rd groups were exposed to (low) 40 μg L^{-1} and (high) 400 μg L^{-1} concentrations of Cu added to water, respectively. The duration of the experiment, which was conducted in triplicate, was 60 d. End points were evaluated on days 30 and 60. Following 30 d and 60 d of exposure to Cu, the fish were removed from experimental tanks to determine growth. Consequently, blood samples were collected from caudal veins at the end of the trial period (30 d and 60 d) and serum was separated to evaluate different immunological parameters, such as lysozymes (LYZ), respiratory burst activity (RBA) and myeloperoxidase (MPO). Gill and liver tissues were collected for evaluation of Cu and certain biochemical parameters as follows: antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase (GST); non-enzymatic antioxidants such as glutathione (GSH) and metallothionein (MT), and oxidative stress indicators such as malondialdehyde (MDA) and protein carbonyl (PCO). The results pertaining to treatments and the control were compared using two-way ANOVA and Tukey's HSD test. The level of significance was set at $P \leq 0.05$. Data were expressed as mean ± SD.

Results: Chronic exposure to Cu did not induce any mortality in fish during the test period. However, following exposure to Cu, growth of fish in the exposed groups was affected more than that in the control group (unexposed to Cu). In addition, accumulation of Cu in the liver tissue was higher than that in the gill tissues of fish exposed to Cu, compared to that in the control. Gill and liver tissues of Cu-exposed fish showed a significant ($P \leq 0.05$) reduction in the activities of the antioxidant enzymes, SOD, CAT, GPx, and GST, compared to those of unexposed fish. Non-enzymatic antioxidants, GSH and MT, in gill and liver tissues were significantly increased ($P \leq 0.05$) in fish exposed to both concentrations of Cu, compared to those in unexposed fish. Oxidative stress indicators, MDA and PCO in gills and liver of Cu-exposed fish was significantly ($P \leq 0.05$) at both tested concentrations, when compared to control group. Non-specific immune response of LYZ, RBA, and MPO activity in serum decreased significantly ($P \leq 0.05$) in Cu-exposed fish, compared with that of unexposed fish.

Conclusion: Overall, the present results highlighted that chronic exposure to Cu ions may exert a strong effect on the antioxidant and immune responses of *O. niloticus*. Changes in antioxidant enzymes, oxidative stress effects and immune parameters during post-chronic metal exposure may indicate the potential of these parameters as biomarkers of metal toxicity in aquatic ecosystems.

* Corresponding author.
E-mail address: vaseeharanb@gmail.com (B. Vaseeharan).

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1. Introduction

In recent years, environmental contaminants have increased considerably due to industrial and agricultural activities. Environmental contamination due to metals is a major environmental health concern [1]. Currently, an increasing number of studies are being conducted on copper (Cu) contamination in freshwater organisms [2]. Cu, a trace element acting as a cofactor for many enzymes and proteins, is essential for the regulation of bio-cellular functions [3–5]. Modification of Cu metabolism may interrupt regular cellular functions, leading to disorders in antioxidant based immune defense systems and enhancement of other pathological issues in aquatic animals and humans [6–8].

Cu salts are used in aquatic ecosystems to manage undesirable organisms, including microbial pathogens [9,10]. Cu may be used as a disinfectant against protozoan ecto-parasites, such as *Cryptocarion* and *Amyloodinium*, as well as against bacteria [11]. Therefore, it is important to evaluate Cu toxicity in fish. Cu at high concentrations shows a tendency to accumulate in fish tissue. Between 0.2 to 30 mg L^{-1} of Cu are found naturally in unpolluted freshwater [12]. However, Cu may become toxic when its concentration rises above a certain level. Earlier reports indicated that dissolved Cu concentrations ranging from 50 to 560 mg L^{-1} were lethal to several aquatic species [13,14]. High levels of Cu can cause deleterious effects as well as biochemical and physiological disorders in freshwater organisms [15–17].

Deleterious effects of xenobiotics are difficult to detect in living systems due to long-term mechanisms at play. Hence, early-warning signals, or environmental biomarkers are used to detect biochemical responses triggered by contaminants, including toxic metals, in freshwater organisms [18].

Oreochromis niloticus, a freshwater teleost fish cultivated worldwide, is commonly used in toxicological investigations and is frequently used as pollution bioindicator. It is easy to maintain under controlled conditions, and suitable for use as a model for assessing metal toxicity. The aquaculture industry accounts for 75% of the world's tilapia production and 5% of the total finfish aquaculture [19,20]. Currently research on effects of Cu in aquatic organisms appears to focus on acute Cu exposure rather than on long term exposure to Cu. Therefore, the current study evaluated the toxic effect of Cu accumulation on the growth of *O. niloticus*. The effect of chronic Cu exposure on non-specific immune parameters and biochemical responses of enzymatic and non-enzymatic antioxidants, and oxidative stress in *O. niloticus*, were also evaluated.

2. Material and methods

2.1. Experimental animal

A sample of 90 *O. niloticus* (45 males and 45 females), with a mean body length of 8 ± 0.3 cm and a mean weight of 31 ± 0.4 g, were obtained from a home fish culturing area in Karaikudi, India. The fish were acclimatized for 2 weeks in cement tanks (1000 L) and fed commercial diet (52% crude protein, 16.0% crude ash, 12% crude lipids, 12% water, 5% Ca, 3.0% crude fiber, 0.5% P, ≥ 2.3% lysine and ≤ 3.8% sodium chloride) twice a day at the rate of 5% of body weight (Tairoun Feed Company, Taipei, Taiwan). Feces and unconsumed feed were siphoned out frequently. Aeration was provided to prevent stress and physical as well as chemical parameters of the water were supervised during the acclimation period using a multiparameter water quality meter (HORIBA U-52, Japan) as follows: Temperature = 29.4 ± 1.1 °C; pH = 7.5 ± 0.3; salinity = 0.25 ± 0.05 ppt; total ammonia = 0.09 ± 0.01 mg $N\text{-}NH_4$ L^{-1}; dissolved oxygen = 6.9 ± 0.4 mg O_2 L^{-1}; conductivity = 342.6 ± 16.2 µs/cm; total hardness = 136.5 ± 9.6 mg $CaCO_3$/L, alkalinity = 42.7 ± 6.2 mg $CaCO_3$/L; and Cu concentration = 3.9 µg L^{-1} respectively. These physico-chemical parameters, with the exception of Cu concentrations, were not disturbed throughout the experimental period [21].

2.2. Experimental design

Fish used in the present scientific study were cultured and sacrificed according to the terms of animal use stipulated and permitted by the Committee of Animal Care and Use, Alagappa University, India. Following acclimation, fish were randomly divided into three experimental groups in nine, 100 L capacity Fiberglass Reinforced Plastic (FRP) tanks containing 80 L of fresh water. The 1st, 2nd and 3rd groups were exposed to concentrations of 0 (control), 40 (low) and 400 (high) µg Cu L^{-1}, respectively. Each treatment was carried out in 3 tanks, containing 10 fish amounting to 30 fish per treatment group (n = 30). The control group was treated with tap water containing no addition of added Cu (3.9 µg L^{-1}), whereas fish from the remaining groups were exposed to 40 µg Cu L^{-1} and 400 µg Cu L^{-1} under laboratory conditions. The lowest Cu concentration (40 µg L^{-1}) was selected based on the ecologically significant concentrations and highest Cu concentration (400 µg Cu L^{-1}) was defined for toxicity induced on *O. mossambicus*, as reported earlier [13,22]. Cu solutions were prepared using copper sulfate ($CuSO_4$; Merck, Darmstadt, Germany). Cu concentrations were delivered daily in the morning following a change of water, with substitute water containing the same Cu concentration as that of the replaced water. The experiment was performed in triplicate and spanned 60 d. In order to minimize hunger during the waterborne Cu exposure period, the fish were hand fed with commercial diet (Tairoun Feed Company) twice a day at the rate of 5% of body weight. Aeration of water was maintained during the experimental period. Feces and unconsumed feed were siphoned out frequently. Temperature, pH, salinity, dissolved oxygen, total ammonia, conductivity, alkalinity, and total hardness were evaluated daily and maintained at values similar to those recorded during the acclimation period. Water samples were collected weekly to determine total and dissolved Cu concentrations in water samples, which were either filtered or not filtered through a 0.45 µm syringe filter (Millipore Millex HV/PVDF). For analyses, samples were acidified using HNO_3 and stored at 4 °C. Cu concentrations were determined using an atomic absorption spectrometer (AAS) from Perkin Elmer (PerkinElmer, Inc. Waltham, USA) with a detection limit of 0.0014 g L^{-1} [23]. The concentrations of metals are expressed as microgram per liter (µg L^{-1}) for water samples. Following 30 d and 60 d of Cu exposure, total lengths and body weights of 3 fish from each tank (n = 9 per group) were recorded, after which these fish were used for all biochemical and immunological parameter analyses.

At the end of the 30 d and 60 d exposure periods, 3 fish (n = 9 in each group) randomly selected from each tank were subjected to fasting for 24 h prior to sampling, in order to minimize contamination from metabolic waste and ensure a healthy environment. The fish were instantaneously anesthetized in 0.05% tricaine methane sulfonate (MS-222, Sigma Diagnostics INS, St. Louis, MO) [24]. Blood samples drawn from the caudal vein of each fish using a sterile syringe were stored at 4 °C in a heparinized tube and centrifuged at 3000 *g* for 10 min to separate serum from blood and stored at −80 °C. until needed for the analysis of immune parameters as described below. After the collection of blood, the gill and liver of the sampled fish were removed and preserved in an ultra-freezer at −80 °C until needed for analysis of biochemical parameters.

2.3. Measurements of Cu in tissues

Cu content in gill and liver tissues was measured after drying for 24 h at 60 °C. All samples were of a constant weight after drying. Dried tissue samples were completely digested in HNO_3 (5 N) (Suprapur, Merck, Darmstadt, Germany) using 10 mL of acid for 1 g of tissue at 60 °C. Fifty µl of the completely digested tissue solution was analyzed using an Atomic Absorption Spectrophotometer (AAS) from Perkin Elmer [25]. The wavelength for Cu measurements was 324.5 nm. Samples were analyzed in triplicate and Cu concentrations were expressed as µg g^{-1} dry weight (d.w.). Determination of Cu was

conducted using DOLT-2 Dogfish liver and DORG-2 Dogfish gill as standard reference material while simultaneously testing the control.

2.4. Antioxidant, non-enzymatic antioxidant and oxidative stress parameters

Gill and liver samples were weighed and homogenized (10 mL g^{-1} tissue) in phosphate buffer solution (0.1 M; pH 7.2). The homogenates were then centrifuged at 15,000 *g* for 30 min at 4 °C. The supernatant was used for analysis of oxidative stress as well as enzymatic and non-enzymatic antioxidant assays. All biochemical and immunological parameter related optical density values were determined using a microplate reader (FlexStation® 3, Molecular Devices, California, USA). Protein concentrations in gill and liver homogenates were measured using bovine serum albumin as the standard according to the method described by Lowry et al., [26].

Biochemical activities of gill and liver tissues were measured using kits existing in the market (SG Mitalia Co., USA and Stanbio LDH (UV-Rate) USA). SOD activity at 560 nm was determined according to the colorimetric assay described by Suzuki [27], where the superoxide detector used was water soluble tetrazolium salt. SOD activity was expressed as U mg^{-1} protein. CAT activity was measured via assessment of decomposition of H_2O_2 by measuring absorbance at 240 nm [28]. CAT activity was expressed as U mg^{-1} protein. Activity of GPx was measured as a function of GSH oxidation at 340 nm, according to a method previously described by Rotruck et al., [29]. GPx activity was revealed as nmol NADPH min^{-1}mg $protein^{-1}$. Bioactivity of GST was measured using the spectrophotometric method at 340 nm [30] with 1-chloro 2, 4-dinitrobenzene (CDNB) (Sigma-Aldrich, Inc., St. Louis, MO, USA) as the reducing substrate. GST activity was recorded as µmol CDNB min^{-1}mg $protein^{-1}$. GSH content was observed using the spectrometric method [31] where it's content was quantified for reaction with DTNB at 412 nm. GSH content was expressed as nmol L^{-1}. Metallothionein (MT) levels were observed at 412 nm as per Viarengo et al., [32]. MT content was expressed as µg MT g^{-1} tissue. LPO content was assessed by determining thiobarbituric acid responsive substances at 532 nm (TBARS) via the method described by Buege and Aust [33]. LPO content was expressed as nmol MDA mg^{-1} tissue. 1, and 1′, 3, 3′-tetramethoxy propane was used as the standard. Quantification of protein carbonyl content was measured at 360 nm as per Reznick and Packer [34]. PCO content was expressed as nmol mg^{-1} protein. Details of analyses pertaining to all biochemical parameters have been described in our previous publications [21].

2.5. Non-specific immune parameters

2.5.1. Lysozyme activity

Lysozyme activity was evaluated according to the assay of Ellis [35]. Briefly, 2 mL of *Micrococcus lysodeikticus* (Sigma, ATCC 4698) at a concentration of 0.02% (w/v) in biological phosphate buffer solution (0.05 M, pH 6.2) was mixed with 100 µL of serum. After 0.5 and 4.5 min of reaction time, change in the absorbance was recorded at 530 nm. Results were calculated as U mL^{-1}.

2.5.2. Respiratory burst activity

Respiratory burst activity was calculated based on the methods described by Secombes, [36]. Fifty microlitre of serum was loaded into a 96 well plate and incubated for 60 min at 30 ± 1 °C. The plate was washed thrice with PBS solution (pH 7.2). Next, the wells were treated with 50 µL of NBT (0.2%) and incubated for 60 min. The cells were fixed using absolute methanol for 3 min and washed thrice with methanol solution (30%, W/V). The plates were air-dried and 60 µL of potassium hydroxide (2 N) was added, followed by 70 µL of dimethyl sulphoxide (DMSO). Absorbance was read at 540 nm using a spectrophotometer. RBA activity was expressed as slope min^{-1}.

2.5.3. Myeloperoxidase (MPO) activity

MPO activity was quantified using an assay technique described by Kumari and Sahoo [37]. Dilution of serum to 10 µL was performed using Hank's Balanced Salt solution (HBSS) in 96-well plates. Samples were then treated with peroxidase substrate [35 µL of 20 mM 3, 3′, 5, 5′-tetramethylbenzidine hydrochloride (TMB) and 5 mM H_2O_2 (Hydrogen peroxide)] and incubated for 30 min at 30 °C. Next, 35 µL of sulphuric acid (H_2SO_4, 4 M) were added to stop the color reaction, and absorbance read at 450 nm using a spectrophotometer. A standard without serum was used as the blank. A unit of MPO activity was defined as µmol of H_2O_2 reduced by a liter of serum at 30 °C. MPO activity was expressed as U mL^{-1}.

2.6. Statistical analysis

The experiment was planned via an absolutely randomized design. The research was repeated thrice (n = 9 per treatment), and each measurement was done in triplicate. Prior to commencing statistical analyses, all data were tested for normality using the Shapiro–Wilk test and for homogeneity of variance using Levene's test. Two-way ANOVA followed by Tukey's HSD test was performed to test the effects of time and concentration of Cu concentration on biochemical and immunological parameters in each organ. The interaction effect between time and concentration of Cu treatment was also analyzed. The significance level was set at $P \leq 0.05$. Data were expressed as mean ± SD values. Statistical analysis was performed using STATISTICA version 12.0 for Windows (STATSOFT, Inc.).

3. Results

3.1. Growth parameters

Total and dissolved Cu concentrations in the experimental aquaria during the chronic exposure period were consistent with nominal concentrations (Table 1). Therefore, the current study exposed *O. niloticus* to 3 different ecologically relevant Cu concentrations of 0, 40, and 400 Cu L^{-1} for 60 d. Exposure of fish to these concentrations did not result in mortality rates any different than those of unexposed fish. Cu concentrations in the experimental water did not differ significantly from the nominal concentrations added to the water over the experimental exposure period (Table 1). Cu was not detected in the experimental water of the controls. Fish exposed to 400 µg Cu L^{-1} exhibited a mean weight that was significantly lower than that exhibited by control fish after 30 d and 60 d. The growth of fish exposed to 40 µg Cu L^{-1} was not significantly different ($P \leq 0.05$) compared to that of control fish after 30 d and 60 d. The growth of Cu exposed fish decreased significantly ($P \leq 0.05$) for both exposure time and tested dose over 60 d, compared to that of unexposed groups (Table 2).

3.2. Metal accumulation in tissues

Cu accumulation in the gills and liver of *O. niloticus* is shown (Table 3). When the time of exposure was lengthened to 60 d, a

Table 1
Total and dissolved Cu concentrations in tap water measured following exposure to varying Cu levels (0 (control), 40 (low) and 400 (high) µg Cu L^{-1}) in *Oreochromis niloticus* for 60 d.

	Nominal Cu concentration (µg Cu L^{-1})		
	Control	20 µg Cu L^{-1}	100 µg Cu L^{-1}
Total measured	ND	19.5 ± 0.3	98.6 ± 0.8
Dissolved	ND	20.4 ± 0.1	99.8 ± 0.5

ND: not detected.
Data are expressed as mean ± SE.

corresponding increase in Cu accumulation was noticed in the tissues of *O. niloticus*. The accumulation of Cu in the liver was higher than that in the gills. Cu accumulation in the liver after 60 d was 29.40 and 42.6 μg g^{-1} at 40 μg Cu L^{-1} and 400 μg Cu L^{-1}, respectively, while Cu accumulation in the gills after 60 d was 27.50 and 38.60 μg g^{-1} at 40 and 400 μg Cu L^{-1}, respectively.

3.3. Enzymatic and non-enzymatic antioxidant activities

Interestingly, a significant ($P \leq 0.05$) difference in SOD activity was observed between Cu exposed fish and Cu unexposed fish. The liver of Cu exposed groups showed lower SOD activity compared to the gills. SOD activity in the gills following 30 d of exposure to Cu was 0.233 U mg^{-1} protein at 40 μg Cu L^{-1}. This decreased to 0.168 U mg^{-1} protein when exposed to 40 μg Cu L^{-1} for 60 d. A significant ($P \leq 0.05$) decrease in SOD activity (0.166 and 0.098 U mg^{-1} protein) was observed in the gills at 400 μg Cu L^{-1} after 30 d and 60 d, compared to that in the control group. SOD activity in the liver was 0.186 and 0.135 U mg^{-1} protein at 40 μg Cu L^{-1} for 30 d and 60 d, respectively. SOD activity in the liver was 0.135 and 0.084 U mg^{-1} protein at 400 μg Cu L^{-1} after 30 d and 60 d, respectively (Fig. 1a).

CAT activity in the gill and liver tissue of *O. niloticus* was significantly ($P \leq 0.05$) decreased after 60 d in the Cu exposed groups, compared to that in the unexposed group. Interestingly, CAT activity in the liver was comparatively lower than that in the gills at both concentrations of Cu. Catalase activity in the gills was 0.245 and 0.159 U mg^{-1} protein at 40 μg Cu L^{-1}, after 30 d and 60 d, respectively (Fig. 1b). Following 30 d and 60 d, a significant ($P \leq 0.05$) reduction in catalase activity (0.153 and 0.092 U mg^{-1} protein respectively) was observed in the gills at 400 μg Cu L^{-1}. CAT activity in the liver at 40 μg Cu L^{-1} was 0.175 and 0.152 U mg^{-1} protein after 30 d and 60 d, respectively. At 400 μg Cu L^{-1}, CAT activity in the liver was reduced from 0.147 U mg^{-1} protein (30 d) to 0.083 U mg^{-1} protein (60 d).

Both GPx and GST activities were significantly ($P \leq 0.05$) reduced in the gill and liver tissues of Cu exposed fish compared to those of unexposed fish. Greater inhibition of GPx and GST activities were observed after 60 d in the groups exposed to both concentrations of Cu (40 and 400 μg Cu L^{-1}) compared to those in the control group (Fig. 1c and d). After 60 d, GPx activities in the gills were 0.308 and 0.155 nmol NADPH min^{-1} mg protein $^{-1}$ at 40 and 400 μg Cu L^{-1}, respectively, whereas in the liver, after 60 d, they were 0.246 and 0.135 nmol NADPH min^{-1} mg protein $^{-1}$, at 40 and 400 μg Cu L^{-1} respectively. After 60 d, GST activity in the gills were 0.327 and 0.238 μmol min^{-1} mg protein $^{-1}$ at 40 and 400 μg Cu L^{-1} respectively. However, after 60 d the activity in the liver was 0.324 and 0.186 μmol min^{-1} mg protein $^{-1}$ at 40 and 400 μg Cu L^{-1}, respectively.

GSH concentrations in gill and liver tissue of Cu exposed *O. niloticus* groups increased significantly ($P \leq 0.05$) in comparison with those of the control group (Fig. 2a). Notably GSH concentrations in Cu exposed groups were higher at 60 d compared to 30 d. After 30 d, gill and liver GSH concentrations were 0.272 and 0.329 nmol L^{-1} at 400 μg Cu L^{-1}, respectively. However, after 60 d, gill and liver GSH concentrations were markedly increased to 0.655 and 0.975 nmol L^{-1} at 400 μg Cu L^{-1}, respectively.

No significant differences were observed between MT concentrations in the gill and liver tissues of Cu exposed groups and those of the control group, after 30 d. By contrast, significant ($P \leq 0.05$) differences were observed in the MT concentrations of gill and liver tissues after 60 d. Interestingly, liver tissue showed higher MT concentrations than those of gills. After 30 d, MT concentrations in gill and liver tissues were 0.216 and 0.242 μg MT g^{-1} tissue, respectively, at 400 μg Cu L^{-1}. MT concentrations in gill and liver tissue were significantly increased (0.535 and 0.668 μg MT g^{-1} tissue at 400 μg Cu L^{-1}, respectively) after 60 d (Fig. 2b).

3.4. Oxidative stress

MDA concentrations in the groups exposed to Cu were increased significantly ($P \leq 0.05$) compared to those of the control group (Fig. 3a). MDA concentrations in the liver were higher than that in the gills. The livers of fish exposed to 40 and 400 μg L^{-1} Cu showed 0.235 and 0.538 nmol MDA mg^{-1} tissue after 30 d, while MDA concentrations increased to 0.543 and 0.992 nmol MDA mg^{-1} tissue, respectively, after 60 d. However, MDA levels in the gills were lower than those in the liver at 40 and 400 μg L^{-1} after 30 d and 60 d, respectively. After 30 d, the gills showed 0.185 and 0.402 nmol MDA mg^{-1} tissue at 40 and 400 μg L^{-1} respectively. In the gills, this was increased to 0.484 and 0.787 nmol MDA mg^{-1} tissue at 40 and 400 μg L^{-1} respectively after 60 d.

The control showed no significant differences in PCO after 30 d and 60 d. However, PCO concentrations in the Cu exposed groups were significantly increased ($P \leq 0.05$); (Fig. 3b). After 30 d, PCO level in the liver was 0.196 nmol mg^{-1}protein at 40 μg Cu L^{-1}. It was increased to 0.483 nmol mg^{-1} protein after 60 d. A significant increase ($P \leq 0.05$) in liver PCO was observed at 400 μg Cu L^{-1}, after 30 d (0.361 nmol mg^{-1} protein) and 60 d (0.755 nmol mg^{-1} protein). PCO levels in the gills were 0.152 and 0.391 nmol mg^{-1} protein at 40 μg Cu L^{-1} after 30 d and 60 d, respectively. PCO in the gills were 0.332 and 0.696 nmol mg^{-1} protein at 400 μg Cu L^{-1} after 30 d and 60 d, respectively.

3.5. Non-specific immune response

The non-specific immune parameters, LYZ, RBA and MPO, of *O. niloticus* were significantly affected in the groups exposed to Cu, compared to those in the control group.

There was no significant difference in LYZ activity of *O. niloticus* between the control and 40 μg Cu L^{-1} (0.208 and 0.218 U mL^{-1} respectively) groups after 30 d. Interestingly, it was reduced to 0.171 U mL^{-1} at 400 μg Cu L^{-1} after 30 d (Fig. 4a). However, after 60 d, LYZ activity was significantly ($P \leq 0.05$) decreased to 0.176 and 0.113 U mL^{-1} at 40 and 400 μg Cu L^{-1}, respectively, in groups exposed to CU, compared to the control group.

RBA activity of tilapia was significantly ($P \leq 0.05$) increased (0.284 slope min^{-1}) at 40 μg Cu L^{-1} after 30 d, compared to that of 400 μg Cu L^{-1} (0.158 slope min^{-1}); (Fig. 4b). After 60 d, RBA activity was significantly ($P \leq 0.05$) inhibited at 40 and 400 μg Cu L^{-1} (0.174 and 0.079 slope min^{-1}) respectively.

There was no significant difference between MPO activities of *O. niloticus* at 40 and 400 μg Cu L^{-1} (0.221 and 0.202 U mL^{-1} respectively) after 30 d (Fig. 4c). However, after 60 d, MPO activity was significantly decreased ($P \leq 0.05$) at both 40 and 400 μg of Cu L^{-1} (0.152 and 0.098 U mL^{-1}) respectively.

Table 2
Growth parameters of *Oreochromis niloticus* exposed to varying Cu levels (0 (control), 40 (low) and 400 (high) μg Cu L^{-1}) in tap water for different exposure periods (30 d and 60 d).

Parameter	Control	40 μg Cu L^{-1} as $CuSO_4$	400 μg Cu L^{-1} as $CuSO_4$
Survival	100 [a]	100 [a]	100 [a]
Initial weight (g)	31.8 ± 0.5 [a]	31.4 ± 0.5 [a]	31.6 ± 0.5 [a]
Final weight (g) (After 30 d)	33.5 ± 2.3 [b]	28.7 ± 2.1[c]	24.3 ± 2.4[d]
Final weight (g) (After 60 d)	37.5 ± 2.3 [b]	25.7 ± 2.1[c]	20.3 ± 2.4[d]

Values are presented as mean ± SE of three replicates (n = 9 fish in each group). Within a row, significant differences ($P \leq 0.05$) between Cu exposed groups and the control group are indicated by different superscript letters (two way ANOVA followed by Tukey's HSD test).

Table 3
Accumulation of Cu in the gills and liver tissues of *Oreochromis niloticus* exposed to varying Cu levels (0 (control), 40 (low) and 400 (high) μg Cu L^{-1}) in tap water during different exposure periods (30 d and 60 d).

Cu in tissues (μg/g d.w.)	Duration (d)	40 μg Cu L^{-1}	400 μg Cu L^{-1}
Gill	0	7.43 ± 0.01[a]	7.43 ± 0.03[a]
	30	14.87 ± 0.45[b]	23.31 ± 0.21[c]
	60	27.50 ± 0.20[d]	38.60 ± 0.32[e]
Liver	0	8.70 ± 0.40[a]	8.70 ± 0.06[a]
	30	18.20 ± 0.32[b]	30.1 ± 0.25[c]
	60	29.40 ± 0.52[d]	42.6 ± 0.62[e]

Values are presented as mean ± SE of three replicates (n = 9 fish in each group). Significant differences ($P \leq 0.05$) between Cu exposed and control groups are indicated by different superscript letters (two way ANOVA followed by Tukey's HSD test).

4. Discussion

Analysis of chronic toxicity is useful for evaluating the continuous effect of pollutants on aquatic systems, as also for defining tolerable limits of chemicals used to protect aquatic biota [38]. In this study, Cu-exposed *O. niloticus* groups survived completely. Atli and Canli [39] reported similar findings, where no mortality was observed in *O. niloticus* following chronic exposure to selected metal ions (Cd, Cu, Cr, Zn, and Fe). Furthermore, in this study, significant reduction in the growth of *O. niloticus* was recorded at 400 μg Cu L^{-1} after 60 d, which may have been due to over accumulation of Cu in the exposed groups. A similar reduction in growth was observed by Kim and Kang [40], who reported that, even at low concentrations, increased accumulation of Cu was associated with increased malformation of growth, compared to that in the control. Reduced growth response is reportedly due to metabolic activity being increased to enable the removal of accumulated Cu and maintain homeostasis [41].

The present study showed that increased Cu^{2+} concentration and longer exposure time directly increased the accumulation of Cu in gill and liver tissues, indicating that the rate of Cu absorption was greater than the elimination of Cu in the fish. Gill tissues are the primary target organs for metal pollution, which disturbs ion regulatory mechanisms in the gills. This is due to Cu being deposited first in the gills, before moving to other vital organs such as the liver [42]. In the current study, at a concentration of 400 μg Cu L^{-1}, Cu deposition in the liver was significantly higher compared to that in the gills, after 60 d. Similarly, Cogun et al., reported that the liver acts as the prime organ for deposition of trace metals [43].

Antioxidant enzymes play an important role in the defense mechanism against oxidative stress [44]. Cellular enzymatic and non-enzymatic antioxidant defenses are activated in aquatic animals exposed to toxicants, leading to the production of free radicals. SOD and CAT enzymes act as a first line of defense against ROS, thus protecting organisms from oxidative stress [45]. The present study showed that SOD and CAT enzymes were significantly reduced in gill and liver tissues of *O. niloticus* after 60 d of Cu exposure. Lengthy exposure to metals leads to the accumulation of metals in organisms. Such accumulation of metals enhances the generation of high superoxide radicals ($O_2^{\bullet -}$), or their derivatives such as hydrogen peroxide (H_2O_2), resulting in oxidation of cysteine in SOD, which deactivates SOD. Hence, decreased SOD activity may be caused by the overproduction of ROS which damage the SOD enzyme. These results were supported by the observations of Vutukuru et al., [46], indicating that exposure to Cu reduced the activity of SOD in the liver of *Esomus danricus*. Our results were also substantiated by those of Ruijuan et al., (2014) who reported that SOD activity was reduced in *Carassius auratus* exposed to zinc [47]. Pandey et al., noted that CAT activity was suppressed in the liver of *Channa punctatus* [48]. Generally, CAT activity inhibition is related to binding of metal ions to –SH groups of the enzyme, which increases $O_2^{\bullet -}$ or H_2O_2 [49,50]. In the current study, reduction in SOD and CAT activity was due to Cu promoting excessive generation of ROS via the Fenton reaction [51].

The activity of glutathione related enzymes such as GPx and GST in gill and liver tissues of *O. niloticus* was reduced following exposure to Cu. The current study indicates that GPx activity in *O. niloticus* was potentially inhibited by exposure to waterborne Cu at 40 and 400 μg Cu L^{-1} after 60 d. GPx is a peroxidase enzyme which is important for detoxifying hydroperoxide into water (H_2O) and hydroxyl compounds [52]. The present study observed that the activity of CAT and GPx was reduced indicating that they had failed to protect tissues from H_2O_2 production [53]. Moreover, previous studies reported that GPx activity was inhibited in fish exposed to pollutants [54,55]. Glutathione-S-transferase (GST) plays a major role in the detoxification of harmful electrophilic xenobiotics such as environmental toxicants [56]. The current study showed that GST activity of *O. niloticus* was potentially inhibited by exposure to waterborne Cu at 40 and 400 μg Cu L^{-1} after 60 d. This was supported by the work of Atli and Canli [39] who reported that the GST activity was decreased after chronic exposures to metals in *O. mossambicus*.

Non-enzymatic antioxidant response of GSH plays a role in reducing xenobiotic pollutants [57]. At the end of the experimental period (60

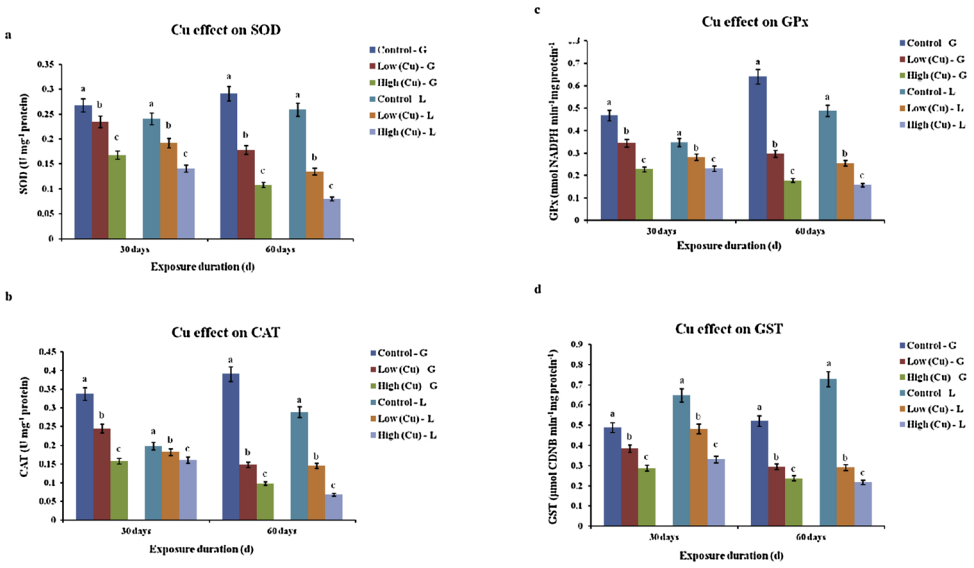

Fig. 1. Antioxidant parameters of Superoxide dismutase (a), Catalase (b), Glutathione peroxidase (c) and Glutathione-S-transferase (d) in *Oreochromis niloticus* exposed to varying Cu levels (0 (control), 40 (low) and 400 (high) μg Cu L^{-1}) in tap water, for different exposure periods (30 d and 60 d). G denotes gills and L denotes liver. Data are presented as mean ± S.E of three replicates (n = 9). Within treatment groups, values with different letters are significantly different ($P \leq 0.05$) as per two way ANOVA followed by Tukey's HSD test.

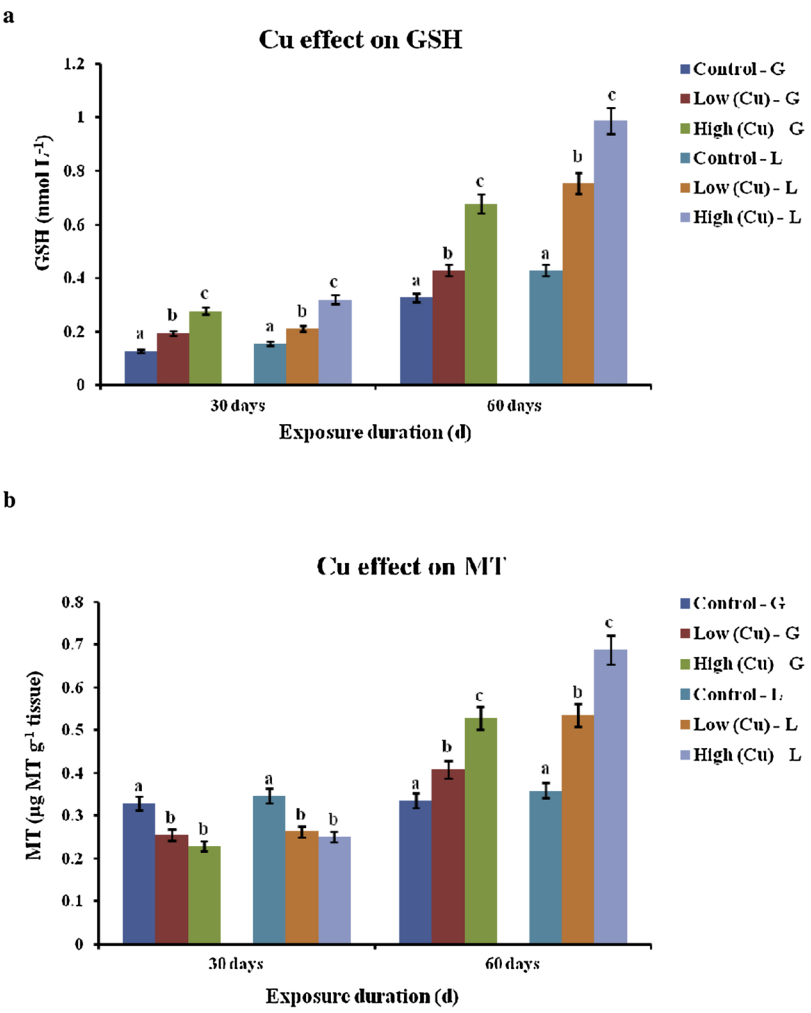

Fig. 2. Non-enzymatic antioxidant parameters of Glutathione (a) and Metallothionein (b) in *Oreochromis niloticus* exposed to varying Cu levels (0 (control), 40 (low) and 400 (high) µg Cu L^{-1}) in tap water during different exposure periods (30 d and 60 d). G denotes gills and L denotes liver. Data are presented as mean ± S.E of three replicates (n = 9). Within treatment groups, values with different letters are significantly different ($P \leq 0.05$) as per two way ANOVA followed by Tukey's HSD test.

d), GSH activity was increased in the gills and tissues of *O. niloticus* exposed to 40 and 400 µg Cu L^{-1} compared to that of the control. The present study revealed that the observed reduction in glutathione dependent enzymes, such as GST and GPx, following exposure to Cu, was directly proportional to the increase or decrease of GSH, which is an essential cofactor of GST and GPx. In addition, glutathione, which is a direct scavenger of oxyradicals, regulates the cellular detoxification mechanism [58].

Metallothionein (MT) is a protein which maintains homeostasis of essential metals, and also plays a role in the removal of heavy metals from the environment [59,60]. MT acts as a biomarker of metal exposure and is therefore studied and considered as a vital biochemical useful for understanding mechanisms underlying toxicity in aquatic animals [61]. At the end of the study, MT activity in the liver of *O. niloticus* exposed to 40 and 400 µg Cu L^{-1} was significantly increased compared to that in the gills. Amiard et al., and Filipovi and Raspor reported that increased activity of MT in organs like the gills and liver indicated its involvement in the ingestion and excretion of pollutants [61,62]. MT Synthesis is directly proportional to the increase in metal concentration mediated by regulatory factors. Our findings were positively correlated with the findings of Simonato et al. [63], which demonstrated that MT concentration in the liver was increased in juvenile *Prochilodus lineatus* following exposure to Cu at concentrations of 5, 9 and 20 g L^{-1}.

Malondialdeyde (MDA) is the final product of lipid peroxidation. Peroxidation of membrane polyunsaturated fatty acids during Cu exposure leads to elevated MDA levels [64]. In the current study, *O. niloticus* exposed to 40 and 400 µg Cu L^{-1} showed a substantial increase in MDA levels in the gills and liver after 30 d and 60 d. According to Halliwell and Gutteridge [65] reduced activity of CAT, SOD and GPx results in oxidative stress leading to the overproduction of free radicals, thereby stimulating MDA.

These highly oxidizable lipids attack nearby proteins to form protein carbonyls that are used as biomarkers of environmental impact [64]. Generally, elevated protein carbonyl levels indicate an alteration in normal protein metabolism due to over accumulation of metals [66]. In the present study, the protein carbonyl levels in *O. niloticus* increased, causing a significant increase in ROS which in turn leads to oxidative damage to proteins. Similar findings were reported by De Boeck et al., who observed that protein levels in the freshwater common carp were reduced following exposure to waterborne Cu [67].

Non-specific immune systems are sensitive to environmental contamination and therefore may serve as potential biomarkers [18]. Among non-specific immune responses, lysozyme activity in fish acts as

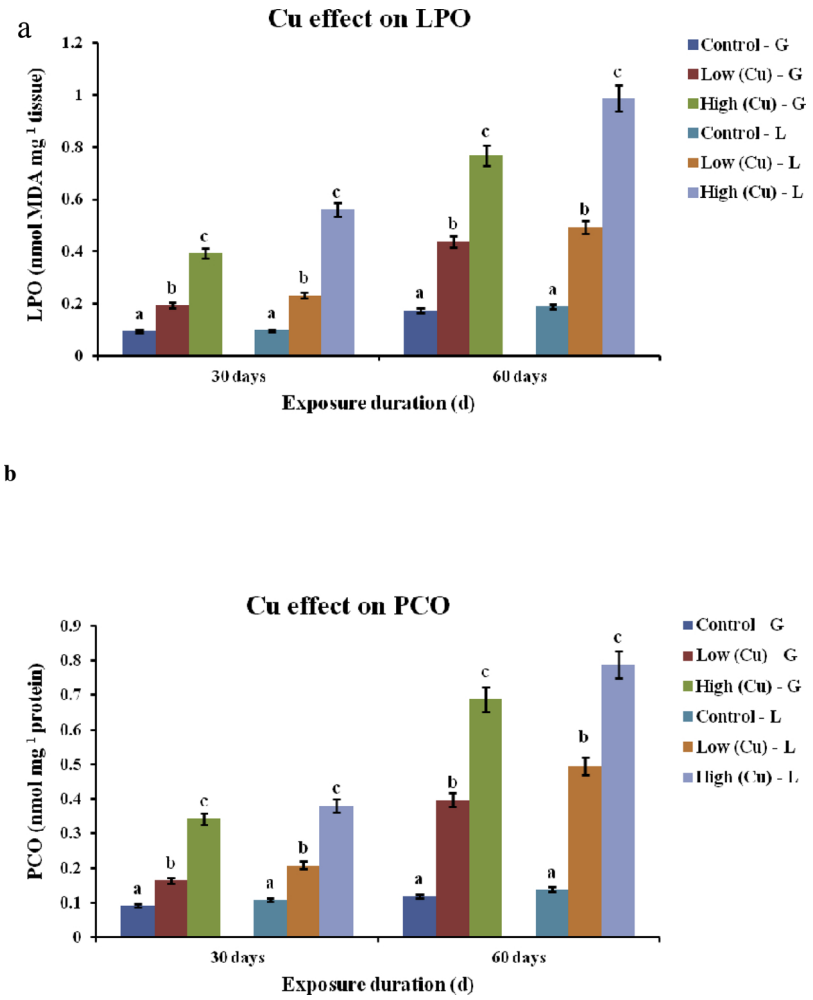

Fig. 3. Oxidative stress parameters of lipid peroxidation (a) and protein carbonyls (b) in *Oreochromis niloticus* exposed to varying Cu levels (0 (control), 40 (low) and 400 (high) μg Cu L^{-1}) in tap water during different exposure periods (30 d and 60 d). G denotes gills and L denotes liver. Data are presented as mean ± S.E of three replicates (n = 9). Within treatment group, values with different letters are significantly different ($P \leq 0.05$) as determined by two way ANOVA followed by Tukey's HSD test.

a valuable parameter of the impact of toxic metals on fish and their immune regulatory functions [68]. In the present study, exposure of *O. niloticus* to waterborne Cu at 40 and 400 μg Cu L^{-1} for 60 d reduced lysozyme activity. Similarly, Shariff et al., [69] reported decreased lysozyme activity in *Puntius gonionotus* following exposure to Cu.

In fish, phagocytes constitute the primary defense apparatus, which destroys invading pathogens by producing ROS, which can be quantified via method of respiratory burst activity [70]. Previously, respiratory burst activity was proposed as an indicator of fish health [71]. For example, exposure to Cu inhibited the respiratory burst activity in the rainbow trout, *Oncorhynchus mykiss* [72]. Findings of the current study indicated that Cu concentrations at 40 and 400 μg Cu L^{-1} reduced respiratory burst activity in *O. niloticus*.

Myeloperoxidase is a microbicidal enzyme. Reduced or elevated levels of peroxidase may indicate pathological situations induced by stressful conditions [73]. In this study, peroxidase activity in *O. niloticus* was reduced following exposure to waterborne Cu at 40 and 400 μg Cu L^{-1} after 60 d. Similarly, Kim and Kang [74] reported that *Pagrus major* exposed to waterborne Se at higher concentration showed a decrease in myeloperoxidase activity.

5. Conclusion

The present study concludes that, the growth of *O. niloticus* was significantly reduced following prolonged exposure to Cu at 40 and 400 μg Cu L^{-1} for 60 d. Activities of SOD, CAT, GPx, and GST in gill and liver tissues of *O. niloticus* declined following exposure to 40 and 400 μg Cu L^{-1} during the period between 30 d and 60 d. An increase in the levels of non-enzymatic antioxidants, GSH, and MT may overcome increased ROS production in gill and liver tissues of *O. niloticus*. Inhibition of antioxidant enzymes indicated a failure in protection against ROS production, which may have generated a large amount of free radicals. Non-enzymatic antioxidants were activated in order to cope with these free radicals. However, the non-enzymatic antioxidant mechanism was insufficient to prevent oxidative stress as evidenced by a significant increase in MDA and PCO levels in gill and liver tissues of *O. niloticus*. The results of the present study demonstrated that exposure to waterborne Cu markedly regulated the immune response of *O. niloticus*, including LYZ, MPO and RBA activity. Thus, fish under stress due to metal exposure may serve as important biomarkers of metal pollution in aquatic ecosystems. Therefore, discharging trace metal elements into aquatic bodies must be controlled and remedial techniques should be applied to remove metal pollution from freshwater ecosystems.

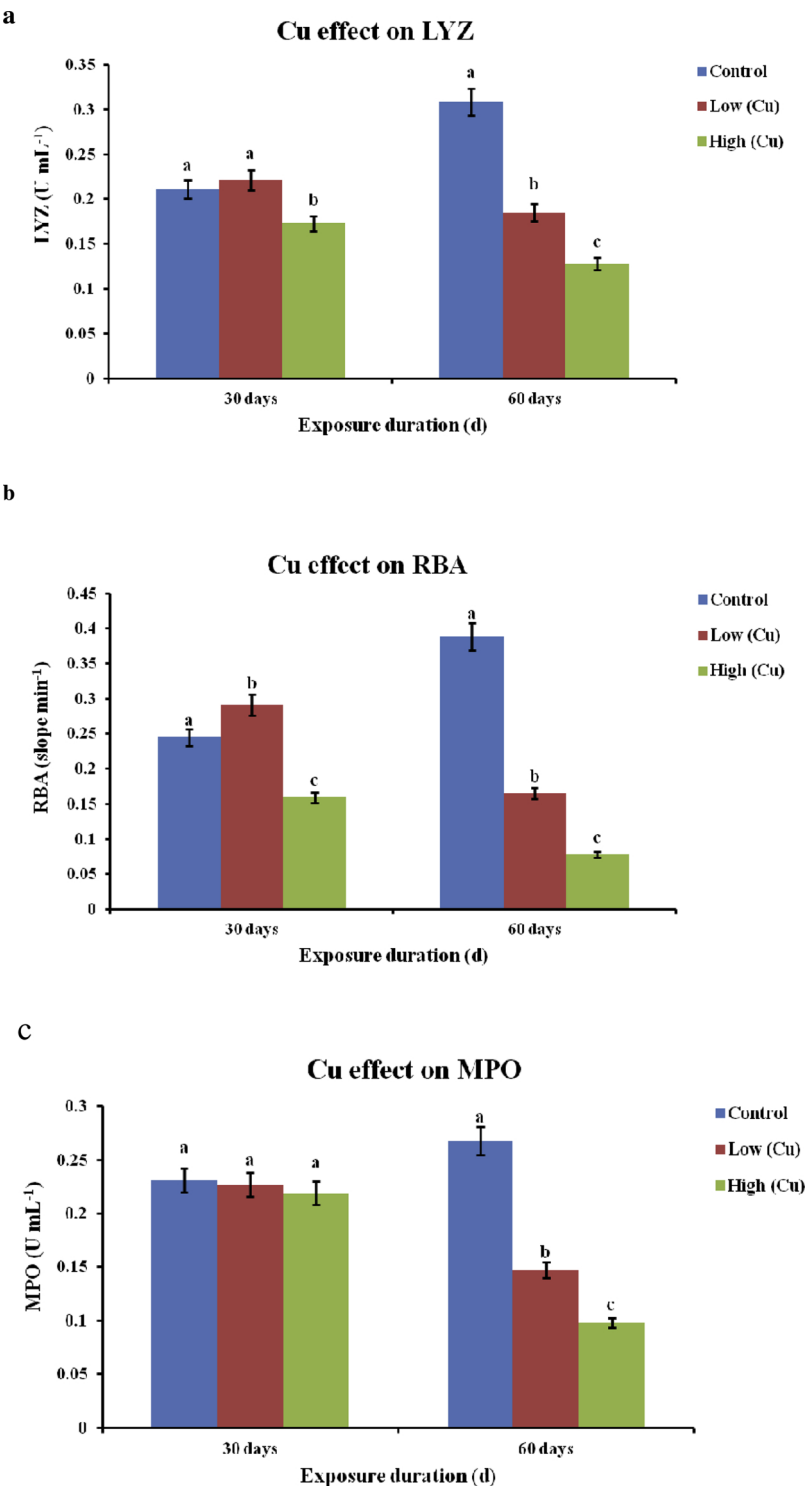

Fig. 4. Non-specific immune parameters of Lysozyme (a), Respiratory burst (b) and Myeloperoxidase (c) in *Oreochromis niloticus* exposed to varying Cu levels (0 (control), 40 (low) and 400 (high) μg Cu L^{-1}) in tap water during different exposure periods (30 d and 60 d). Data are presented as mean ± S.E of three replicates (n = 9). Within treatment group, values with different letters are significantly different ($P \leq 0.05$) as determined by two way ANOVA followed by Tukey's HSD test.

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References

[1] S.S. Vutukuru, N.A. Prabhath, M. Raghavender, A. Yerramilli, Effect of arsenic and chromium on the serum amino-transferases activity in Indian major carp, Labeo

rohita, Int. J. Environ. Res. Public Health 4 (2007) 224–227.
[2] J. Schjolden, J. Sorensen, G.E. Nilsson, A.B.S. Poleo, The toxicity of copper to crucian carp (*Carassius Carassius*) in soft water, Sci. Total Environ. 384 (2007) 239–251.
[3] S. Puig, D.J. Thiele, Molecular mechanism of copper uptake and distribution, Curr. Opin. Chem. Biol. 6 (2002) 171–180.
[4] D.G.N. Ellingsen Horn, J. Aaseth, Copper, in: G.F. Nordberg, B.A. Fowler, M. Nordberg, L. Friberg (Eds.), Handbook on the Toxicology of Metals, 3rd ed, Academic Press, California, 2007, pp. 529–546.
[5] M. Grosell, Copper. In. Homeostasis and toxicology of essential metals, in: C.M. Wood, A.P. Farrell, C.J. Brauner (Eds.), Fish Physiology, vol. 31A, Elsevier, California, 2011, pp. 53–133.
[6] D.N. Weber, S. Eisch, R.E. Spieler, D.H. Petering, Metal redistribution in largemouth bass (*Micropterus salmoides*) in response to restrainment stress and dietary cadmium: role of metallothionein and other metal-binding proteins, Comp. Biochem. Physiol. C. 101 (1992) 255–262.
[7] T.I. Moiseenko, L.P. Kudryavtseva, Trace metal accumulation and fish pathologies in areas affected by mining and metallurgical enterprises in the Kola Region, Russia, Environ. Pollut. 114 (2001) 285–297.
[8] K. Khan, Y. Lu, H. Khan, M. Ishtiaq, S. Khan, M. Waqas, L. Wei, T. Wang, Heavy metals in agricultural soils and crops and their health risks in Swat District, northern Pakistan, Food Chem. Toxicol. 58 (2013) 449–458.
[9] K. Wei, J. Yang, Oxidative damage induced by copper and beta-cypermethrin in gill of the freshwater crayfish Procambarus clarkia, Ecotoxicol. Environ. Safe 113 (2015) 446–453.
[10] H.J. Schlotfeldt, Current practices of chemotherapy in fish culture, in: C. Michel, D.J. Alderman (Eds.), Chemotherapy in Aquaculture: from Theory to Reality, Office International des Epizooties, Paris, 1992, pp. 25–38.
[11] M.F. Chen, J.A. Apperson, G.D. Marty, Y.W. Cheng, Copper sulfate treatment decreases hatchery mortality of larval white seabass Atractoscion nobilis, Aquaculture 254 (2006) 102–114.
[12] B.J. Shaw, G. Al-Bairuty, R.D. Handy, Effects of waterborne copper nanoparticles and copper sulphate on rainbow trout, (*Oncorhynchus mykiss*): physiology and accumulation, Aquat. Toxicol. 116-117 (2012) 90–101.
[13] USEPA, Aquatic Life Ambient Freshwater Quality Criteria-copper (CAS Registry Number 7440-50-8), U.S. Environmental Protection Agency Office of Water, Office of Science and Technology, Washington, DC, 2007.
[14] WHO, Copper, Environmental Health Criteria 200, World Health Organization, Geneva, 1996.
[15] B. Kulac, G. Atli, M. Canli, Response of ATPases in the osmoregulatory tissues of freshwater fish *Oreochromis niloticus* exposed to copper in increased salinity, Fish Physiol. Biochem. 39 (2013) 391–401.
[16] V.I. Lushchak, Environmentally induced oxidative stress in aquatic animals, Aquat. Toxicol. 101 (2011) 13–30.
[17] D.L. Madeira, H.N. Narciso, C. Cabral, M.S. Vinagre, Influence of temperature in thermal and oxidative stress responses in estuarine fish, Comp. Biochem. Phys. A 166 (2013) 237–243.
[18] A.C.D. Bainy, M.R.F. Marques, Global analysis of biomarker responses in aquatic organisms exposed to contaminants, Comments Toxicol. 9 (2003) 271–278.
[19] FAO, FAO Yearbook, Fishery and Aquaculture Statistics vol. 102, (2009), pp. 1–113 Nov http://wwwfaoorg/fishery/publications/yearbooks/en.
[20] A.F.M. El-Sayed, Tilapia Culture, CABI publishing CABI International, Oxfordshire, 2006.
[21] N. Gobi, B. Vaseeharan, R. Rekha, S. Vijayakumar, C. Faggio, Bioaccumulation, cytotoxicity and oxidative stress of the acute exposure selenium in Oreochromis mossambicus, Ecotoxicol. Environ. Saf. 162 (2018) 147–159.
[22] S.M. Monteiro, J.M. Mancera, A.F. Fernandes, M. Sousa, Copper induced alterations of biochemical parameters in the gill and plasma of Oreochromis niloticus, Comp. Biochem. Physiol. C. 141 (2005) 375–383.
[23] J.D. Simonato, M. Mela, H.B. Doria, I.C. Guiloski, M.A. Randi, P.S. Carvalho, P.C. Meletti, H.C. Silva de Assis, A. Bianchini, C.B. Martinez, Biomarkers of waterborne copper exposure in the Neotropical fish Prochilodus lineatus, Aquat. Toxicol. 170 (9) (2016) 31–41.
[24] Q.L. Chen, Y. Gong, Z. Luo, J.L. Zheng, Q.L. Zhu, Differential effect of waterborne cadmium exposure on lipid metabolism in liver and muscle of yellow catfish Pelteobagrus fulvidraco, Aquat. Toxicol. 142 (2013) 380–386.
[25] Perkin-Elmer Corporation, The Guide to Techniques and Applications of Atomic Spectroscopy, in: Atomic Spectroscopy Detection Limits, The Perkin-Elmer Corporation, Norwalk, 1995, pp. CO4–CO5.
[26] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurements with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[27] K. Suzuki, Measurement of Mn-SOD and Cu, Zn-SOD, in: N. Taniguchi, J. Gutteridge (Eds.), Experimental Protocols for Reactive Oxygen and Nitrogen Species, Oxford University Press, London, 2000, pp. 91–95.
[28] G. Cohen, D. Dembiec, J. Marcus, Measurement of catalase activity in tissue extracts, Anal. Biochem. 34 (1970) 30–38.
[29] J.T. Rotruck, A.L. Pope, H.E. Gasther, D.G. Hafeman, W.G. Hoekstra, Selenium biochemical role as a component of glutathione peroxidase, Science 179 (1973) 588–590.
[30] W.H. Habig, M.J. Pabst, W.B. Jakoby, Glutathione S-transferases. The first enzymatic step in mercapturic acid formation, J. Biol. Chem. 249 (1974) 7130–7139.
[31] D.J. Jollow, J.R. Mitchell, N. Zampaglione, J.R. Gillette, Bromobenzene induced liver necrosis. Protective role of glutathione and evidence for 3, 4-bromobenzene oxide as the hepato toxic metabolite, Pharmacol 11 (1974) 151–169.
[32] A. Viarengo, E. Ponzano, F. Dondero, R. Fabbri, A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic molluscs, Mar. Environ. Res. 44 (1997) 69–84.
[33] J.A. Buege, S.D. Aust, Microsomal lipid peroxidation, Methods Enzymol. 52 (1978) 302–310.
[34] A.Z. Reznick, L. Packer, Oxidative damage to proteins: spectrophotometric method for carbonyl assay, Methods Enzymol. 233 (1994) 357–363.
[35] A.E. Ellis, Lysozyme assays, in: J.S. Stolen, T.C. Fletcher, D.P. Anderson, B.S. Roberson, W.B. van Muiswinkel (Eds.), Techniques in Fish Immunology, Fish Immunology Technical Communications, SOS Publications, USA, 1990, pp. 101–103.
[36] C.J. Secombes, Isolation of salmonid macrophages and analysis of their killing activity, in: J.S. Stolen, T.C. Fletcher, D.P. Anderson, B.S. Robertsen, W.B. van Muiswinkel (Eds.), Techniques in Fish Immunology, Fish Immunology Technical Communications, SOS Publications, USA, 1990, pp. 137–154.
[37] J. Kumari, P.K. Sahoo, Dietary β-1, 3 glucan potentiates innate immunity and disease resistance of Asian catfish, *Clarias batrachus* (L.), J. Fish Dis. 29 (2006) 95–101.
[38] M.A. Aragão, R.P.A. Araújo, Métodos de ensaios de toxicidade com organismos aquáticos, in: P.A. Zagatto, E. Bertoletti (Eds.), Ecotoxicologia Aquática: Princípios E Aplicac¸ões, 1 ed., Rima, São Carlos, 2006, pp. 117–152.
[39] G. Atli, M. Canli, Response of antioxidant system of freshwater fish *Oreochromis niloticus* to acute and chronic metal (Cd Cu, Cr, Zn, Fe) exposures, Ecotoxicol. Environ. Saf. 73 (2010) 1884–1889.
[40] S.G. Kim, J.C. Kang, Effect of dietary copper exposure on accumulation, growth and hematological parameters of the juvenile rockfish, Sebastes schlegeli, Mar. Environ. Res. 58 (2004) 65–82.
[41] H. Campbell, R. Handy, D. Sims, Increased metabolic cost of swimming and consequent alterations to circadian activity in rainbow trout (*Oncorhynchus mykiss*) exposed to dietary copper, Can. J. Fish. Aquat. Sci. 59 (2002) 768–777.
[42] M. Grosell, C.M. Wood, Copper uptake across rainbow trout gills: mechanisms of apical entry, J. Exp. Biol. 205 (2002) 1179–1188.
[43] H.Y. Cogun, T.A. Yuzereroglu, O. Firat, G. Gok, F. Kargin, Metal concentrations in fish species from the Northeast Mediterranean sea, Environ. Monit. Assess. 121 (2006) 431–438.
[44] R. Van Der Oost, J. Beyer, N.P.E. Vermeulen, Fish bioaccumulation and biomarkers in environmental risk assessment: a review, Environ. Toxicol. Pharm. 13 (2003) 57–149.
[45] R.V. Zikic, A.S. Stajn, S.Z. Pavlovic, B.I. Ognjanovic, Z.S. Saicic, Activities of superoxide dismutase and catalase in erythrocytes and plasma transaminases of gold fish (*Carassius auratus gibelio* Bloch) exposed to cadmium, Physiol. Res. 50 (2001) 105–111.
[46] S.S. Vutukuru, S. Chintada, K.R. Madhavi, J. Venkateswara Rao, Y. Anjaneyulu, Acute effects of copper on superoxide dismutase, catalase and lipid peroxidation in the freshwater teleost fish, *Esomus danricus*, Fish Physiol. Biochem. 32 (2006) 221–229.
[47] Q. Ruijuan, F. Mingbao, W. Xinghao, Q. Li, W. Chao, W. Zunyao, W. Liansheng, Metal accumulation and oxidative stress biomarkers in liver of freshwater fish *Carassius auratus* following in vivo exposure to waterborne zinc under different pH values, Aquat. Toxicol. 150 (2014) 9–16.
[48] S. Pandey, I. Ahmad, S. Parvez, B. Bin-Hafeez, R. Haque, S. Raisuddin, Effect of endosulfan on antioxidants of freshwater fish *Channa punctatus* Bloch: 1. Protection against lipid peroxidation in liver by copper pre-exposure, Arch. Environ. Contam. Toxicol. 41 (2001) 345–352.
[49] N.S. Kono, I. Fridovich, Superoxide radical inhibits catalase, J. Biol. Chem. 257 (1982) 5751–5754.
[50] C.B.G. Ruas, C.D.S. Carvalho, H.S.S. de Araujo, E.L.G. Espindola, M.N. Fernandes, Oxidative stress biomarkers of exposure in the blood of cichlid species from a metal-contaminated river, Ecotoxicol. Environ. Saf. 71 (2008) 86–93.
[51] R.T. Di Giulio, J.N. Meyer, R.T. Di Giulio, Reactive oxygen species and oxidative stress, in: D.E. Hilton (Ed.), The Toxicology of Fishes, CRC Press, BocaRaton, 2008, pp. 273–326.
[52] E. Pinto, T.C.S. Sigaud-Kutner, M.A.S. Leitao, O.K. Okamoto, D. Morse, P. Colepicolo, Heavy metal-induced oxidative stress in algae, J. Phycol. 39 (2003) 1008–1018.
[53] H. Karadag, O. Firat, O. Firat, Use of oxidative stress biomarkers in *Cyprinus carpio* L. For the evaluation of water pollution in Ataturk Dam Lake (Adiyaman, Turkey), Bull. Environ. Contam. Toxicol. 92 (2014) 289–293.
[54] M. Fatima, I. Ahmad, I. Sayeed, M. Athar, S. Raissudin, Pollutant-induced over-activation of phagocytes is concomitantly associated with peroxidative damage in fish tissues, Aquat. Toxicol. 49 (2000) 243–250.
[55] Z.S. Talas, I. Orun, I. Ozdemir, K. Erdogan, A. Alkan, I. Yilmaz, Antioxidative role of selenium against the toxic effect of heavy metals (Cd^{+2}, Cr^{+3}) on liver of rainbow trout (*Oncorhynchus mykiss* Walbaum 1792), Fish Physiol. Biochem. 34 (2008) 217–222.
[56] J.H. Keen, W.H. Habig, W.B. Jakoby, Mechanism for the several activities of the glutathione S-transferase, J. Biol. Chem. 251 (1976) 6183–6188.
[57] E.G. Kanak, Z. Dogan, A. Eroglu, G. Atli, M. Canli, Effects of fish size on the response of antioxidant systems of *Oreochromis niloticus* following metal exposures, Fish Physiol. Biochem. 40 (2014) 1083–1091.
[58] G. Santovito, E. Piccinni, F. Boldrin, P. Irato, Comparative study on metal homeostasis and detoxification in two Antarctic teleosts, Comp. Biochem. Physiol. Part C. Toxicol. Pharmacol. 155 (2012) 580–586.
[59] S.M. Wu, C.F. Weng, M.J. Yu, C.C. Lin, S.T. Chen, J.C. Hwang, P.P. Hwang, Cadmium-inducible metallothionein in tilapia (*Oreochromis mossambicus*), Bull. Environ. Contam. Toxicol. 62 (1999) 758–768.
[60] G. Atli, M. Canli, Natural occurrence of metallothionein-like proteins in the liver of fish *Oreochromis niloticus* and effects of cadmium, lead, copper, zinc, and iron exposures on their profiles, Bull. Environ. Contam. Toxicol. 70 (2003) 619–627.

[61] J.C. Amiard, C. Amiard-Triquet, S. Barka, J. Pellerin, P.S. Rainbow, Metallothionein in aquatic invertebrates: their role in metal detoxification and their use as biomarkers, Aquat. Toxicol. 76 (2006) 160–202.
[62] V. Filipovi, B. Raspor, Metallothionein and metal levels in cytosol of liver, kidney and brain in relation to growth parameters of *Mullus surmuletus* and *Liza aurata* from the Eastern Adriatic Sea, Water Res. 37 (2003) 3253–3262.
[63] J.D. Simonato, M. Mela, B.D. Halina, C.G. Izonete, A.F.R. Marco, S.M.C. Paulo, C.M. Paulo, C.S. Helena, B. Adalto, B.R.M. Claudia, Biomarkers of waterborne copper exposure in the Neotropical fish Prochilodus lineatus, Aquat. Toxicol. 170 (2016) 31–41.
[64] I. Dalle-Donne, R. Rossi, D. Giustarini, A. Milzani, R. Colombo, Protein carbonyl groups as biomarkers of oxidative stress, Clin. Chim. Acta 329 (2003) 23–38.
[65] B. Halliwell, J.M.C. Gutteridge, Free Radicals in Biology and Medicine, second ed., Clarendon Press, Oxford, 1989.
[66] J.M. Fagan, B.G. Sleczka, I. Sohar, Quantization of oxidative damage to tissue proteins, Int. J. Biochem. Cell Biol. 31 (1999) 751–757.
[67] G. De Boeck, A. Vlaeminck, R. Blust, Effects of sublethal copper exposure on copper accumulation, food consumption, growth, energy stores, and nucleic acid content in common carp, Arch. Environ. Contam. Toxicol. 33 (1997) 415–422.
[68] N.C. Bols, J.L. Brubacher, R.C. Ganassin, L.E.J. Lee, Ecotoxicology and innate immunity in fish, Dev. Comp. Immunol. 25 (2001) 853–873.
[69] M. Shariff, P.A.H.L. Jayawardena, F.M. Yusoff, R. Subasinghe, Immunological parameters of Javanese carp *Puntius gonionotus* (Bleeker) exposed to copper and challenged with *Aeromonas hydrophila*, Fish Shellfish Immunol. 11 (2001) 281–291.
[70] B.G. Magor, K.E. Magor, Evolution of effectors and receptors of innate immunity, Dev. Comp. Immunol. 25 (2001) 651–682.
[71] C.D. Rice, D.H. Kergosien, S.M. Adams, Innate immune function as a bioindicator of pollution stress in fish, Ecotoxicol. Environ. Saf. 33 (1996) 186–192.
[72] M.S. Elsasser, B.S. Roberson, F.M. Hetrick, Effect of metals on chemiluminescent response of rainbow trout (*Salmo gairdneri*) phagocytes, Vet. Immunol. Immunopathol. 12 (1986) 243–250.
[73] A. Rodríguez, M.A. Esteban, J. Meseguer, Phagocytosis and peroxidase release by seabream (Sparus aurata L.) leucocytes in response to yeast cells, Anat. Rec. A. Discov. Mol. Cell. Evol. Biol. 272 (2003) 415–423.
[74] J.H. Kim, J.C. Kang, Oxidative stress, neurotoxicity, and non-specific immune responses in juvenile red sea bream, *Pagrus major*, exposed to different waterborne selenium concentrations, Chemosphere 135 (2015) 46–52.