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Research Article

Interaction effect of salinity and ammonia on glutathione-dependent antioxidant defense system in the liver of Nile tilapia (*Oreochromis niloticus*)

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Abstract

The present study aimed to investigate the effect of salinity and ammonia interactions on oxidative stress response in the liver of Nile tilapia (*Oreochromis niloticus*). Nile tilapia were exposed to different ammonia and salinity concentrations including C (control group without ammonia and salinity), T1 (50% 96 h LC50 of ammonia and 1 ppt salinity level), T2 (50% 96 h LC50 of ammonia and 4 ppt salinity level), T3 (30% 96 h LC50 of ammonia and 1 ppt salinity level), and T4 (30% 96 h LC50 of ammonia and 4 ppt salinity level) for 96 h). Results showed significant increase for activities of superoxide dismutase (SOD), malondialdehyde (MDA), isocitrate dehydrogenase (ICDH), and malic enzyme (ME) in T1 and T2 groups compared to the control (p<0.05). As well, catalase (CAT) activity in accompany with reduced glutathione (GSH), and reduced/oxidized glutathione ratio (GSH/GSSG) showed remarkable reduction in T1 and T2groups Compared to the control (p<0.05). In T3 and T4 groups, only MDA content showed a significant decrease compared to the control (p<0.05). There were no differences in other parameters. In summary, results of this study showed that 96h exposure to 30% LC50 of ammonia plus 4 ppt saline increase antioxidant defense in Nile tilapia.

Keywords: Toxicity, Stressors, Superoxide dismutase, Catalase, Total antioxidant capacity

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Introduction

Ammonia is the most common pollutant in freshwater, which enters the aquatic environments through the destruction of nitrogen-containing organic matters, fertilizers, and industrial resources, as well as from the catabolism of proteins, purines, and pyrimidines in fish (Ip and Chew, 2010). Ammonia is one of the most toxic substances for aquatic animals, in a way that its high concentrations cause growth reduction, tissue damage, immune system suppression, and high mortality in aquatic organisms (Qi *et al.*, 2017). Two forms of ammonia are formed in water: non-ionized with chemical formula of NH3 (very toxic and easily absorbable through the gill epithelium to reach blood stream and organs such as liver) and ionized with a formula of NH4+ which has less toxicity compared to the former. The relative amount of NH3 and NH4+ in water depends on pH, temperature and salinity (Marcaggi and Coles, 2001). Exposure of fish to ammonia or other stressors can cause oxidative stress through increasing ROS production or decreasing antioxidant elements (Sinha *et al.* 2014; Cheng *et al.*, 2015). Accordingly, the most well-known mechanism of ammonia toxicity is induction of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), radical hydroxyl (HO), and radical superoxide (Murthy *et al.*, 2001; Hegazi *et al*., 2010). Overproduction of ROS in cells can lead to oxidation of proteins, DNA, and lipids, which leads in cell death (Martinez-Alvarez *et al*., 2005; Hegazi *et al*., 2010). As a defense mechanism, non-enzymatic and enzymatic antioxidant systems in aquatic organisms convert ROS into harmless metabolites (Sinha *et al.*, 2015; Ramirez-Duarte *et al.*, 2016). However, exposure to ammonia alters activities of antioxidant enzymes to prevent oxidative damage to the gills, muscles, liver, and brain of fish (Hegazi *et al.*, 2010; Qi *et al.*, 2017). Among these organs, the liver is a metabolically active organ that is proper for assessment of oxidative stress and antioxidant defense in living organisms. Liver glutathione oxidation and reduction systems including glutathione, glutathione peroxidase (GPx), glutathione S-transferase (GST), and glutathione reductase (GR) play an important role in maintaining cellular redox homeostasis and protecting cells against oxidative damage during environmental stress (Bainy *et al.*, 1996). Fish tolerance to ammonia exposure may increase in environments with greater salinity (Bianchini *et al.*, 1996; Sinha *et al.*, 2012). In fact, adding calculated amounts of chlorine to aquaculture water systems is a way to protect freshwater fish from ammonia toxicity. On the other hand, evidence shows that the adaptation of fish to different degrees of salinity by osmotic regulation is an energy-demanding process that is associated with metabolic activation and consequently, increased ROS formation. There is a direct relationship between the intensity of metabolism and the activation of oxidative enzymes (Shukry *et al*., 2021) Nevertheless, there is variability in

tolerance of fish species to salinity. Euryhaline fish (such as tilapia) can endure a wide range of salinity, which suggests that in case of exposure to salinity changes this species would alter its osmotic pressure and ions at a low cost of energy (Jumah *et al.*, 2016). By considering that lower energy demands would result in less oxidative stress, here, we aimed to investigate effects of salinity and ammonia levels on the glutathione-dependent redox system in Nile tilapia.

Material and methods

Fish and experimental design

Healthy Nile tilapia (*Oreochromis niloticus*) were provided from a commercial fish farm at Qom province and transferred to the aquatic animal health research laboratory of the faculty of veterinary medicine, Shahid Chamran University of Ahvaz, Iran. They temporarily reared in 300 L fiberglass tanks to adapt for two weeks. The chemical ammonium chloride (Merck, USA) was dissolved in double distilled water as an ammonia stock solution. The lethal concentration of ammonia for 96 h in Nile tilapia was calculated at 0.9 mg/L. The concentrations required for exposure were achieved by diluting the stock solution into the tank water. The actual experiment was conducted after selecting a total number of 150 Nile tilapias (50.35±1.80 g) with similar physical parameters. They were randomly distributed into five groups of 30 individuals each and held in 300 L tanks. In the experiment, we used 30%(Sub-acute toxicity) and 50% (Acute toxicity) 96 h LC50 of ammonia in 1 and 4 ppt salinity levels to stress the Nile tilapia for 96 h. The treatments of the present study were as follows: C (control group without ammonia and salinity), T1 (50% 96 h LC50 of ammonia and 1 ppt salinity level), T2 (50% 96 h LC50 of ammonia and 4 ppt salinity level), T3 (30% 96 h LC50 of ammonia and 1 ppt salinity level), and T4 (30% 96 h LC50 of ammonia and 4 ppt salinity level). Water stock was prepared for each treatment as the water was replaced every three days. During the trials, the fish were deprived of food and 100% medium was renewed every 24 h in a semi-static system. The natural photoperiod was maintained during the experiment. The water physicochemical parameters including temperature (25.2±0.6°C) and dissolved oxygen (7.3±0.1 mg), and pH (8.3±0.1) of water were checked daily.

Samples collection

The fishes were killed by the mechanical method after anesthetization (Rubino *et al.*, 2019). Then, liver samples were collected, ،washed with physiological serum and kept at at –70°C while waiting for analysis.

For antioxidant analysis, 500 mg of liver samples and 2.5 mL of 100 mM PBS buffer (Phosphate-buffered saline, pH: 7.4) were added in an electrical homogenizer (Heidolph Instruments, Germany) immersed in an ice water bath and squeezed (21900 g for 2 min). Then, the mixture was centrifuged at 18800 g for 10 min at 4°C and the

supernatant was separated and used for the analysis of oxidative stress and antioxidant parameters.

Oxidative stress indicators

The GSH measurement is based on the reduction of 5,5 dithiobis (2-nitrobenzoic acid) (DTNB) with reduced glutathione(GSH) to produce a yellow compound. The reduced chromogen is directly proportional to GSH concentration and its absorbance can be measured at 405 nm (Ellman, 1959). Briefly, 15 µL of the sample was added to 260 µL of phosphate buffer (pH: 8) containing one mmol of ethylenediaminetetraacetic acid (EDTA). Then, 5 µL of Elman's reagent was added to this solution and incubated for 15 min at room temperature. GSH activity was determined spectrophotometrically by measuring color intensity at 412 nm, expressed as µmol/ mg of protein. For the oxidized glutathione (GSSG) assay, in the first step, GSSG was reduced to GSH with sodium borohydride (NaBH4) and then the total level of reduced glutathione (GSSG+GSH) in the sample was determined. GSSG was calculated from the difference between total and reduced glutathione (Alisik *et al*., 2019). Liver glutathione peroxidase (GPx) activity was analyzed with commercially available assay kits (ZellBio, GmbH, Veltinerweg, Germany).

The activity of SOD enzyme in liver tissue was estimated by using the method of Kono (1978). The reaction mixture contained a sodium carbonate buffer (50 mM, pH 10.2), 0.1 mL (3 M) phenazine methosulfate (PMS), nitroblue tetrazolium (NBT, 24 µM), and EDTA (0.1 mM). The reaction was started by adding hydroxylamine (1 mM, pH: 6). This enzyme activity was determined spectrophotometrically by measuring color intensity at 560 nm, expressed as units/min/mg of protein.

The activity of CAT was measured based on the decomposition of H_2O_2 according to the method of Góth (1991). A 100 µL of the sample was added to 1mL of 4% ammonium molybdate and 2 mL of 0.03% H_2O_2. The reaction was initiated by adding 20 µL of enzyme extract. The CAT activity was estimated spectrophotometrically in terms of Units/min/mg of the protein by measuring the rate of decomposition of H_2O_2 at 410 nm for 10 s intervals for 60 s using the extinction coefficient of H_2O_2.

The level of lipid peroxidation in the liver was measured by estimating malondialdehyde (MDA) using the thiobarbituric acid test according to the method of Placer *et al*. (1996). The reaction mixture contained 0.25 mL of distilled water, and 5.37% TBA in 0.25% hydrochloric acid and 15% trichloroacetic acid (TCA). 0.1 mL of the sample was added to the mixture and kept in a hot water bath for 15 min. Then the measurement was based on the absorbance measurement of the pink complex at 535 nm. TBA concentration was expressed in nmol MDA mg protein-1 following the calibration curve for MDA.

Malic enzyme and isocitrate dehydrogenase were measured spectrophotometrically at 340 nm based on the changes in OD caused by the reduction of $NADP^+$ to NADPH.

Optimum conditions for Malic enzyme, were 40 mM L- malate, 2 mM $NADP^+$, 1mM Mncl2 and 50 mM Tris–HCl buffer, pH 8.0 in a total volume of 200 µL with 10 µL of liver extract used per assay. The activity was measured as U/mg (Zelewski and Swierczyński 1991).

The optimal conditions for isocitrate dehydrogenase were 40 mM L-isocitrate, 2 mM NADP+, 1 mM Mncl2, and 50 mM Tris-HCl buffer, pH 8.0 in a total volume of 200 µL with 10 µL of liver extract used in each assay. The activity was measured as U/mg (Sinha *et al*., 2012)

Data Analysis

All the analyses were performed by SPSS software (SPSS, Release 16.0, SPSS, Inc.; Chicago, IL, USA). After confirmation of normality by the Kolmogorov-Smirnov test, all oxidative stress indicators were analyzed using one-way analysis of variance (ANOVA). Duncan's test was used to find significant differences among the treatments. Significant differences were considered when $p<0.05$. All results are presented as Mean±SEM.

Results

Alteration in activities of hepatic antioxidant enzymes and proteins could be a measure for functional effect of ammonia: saline ratio (ASR) on oxidative stress. Here, we have shown that different ratios of ammonia: saline would result in a variations at levels of hepatic enzymes and proteins affiliated to oxidative stress.

In our research, While different ASR in T1, T2 and T3 groups suppressed CAT activities compared to the control ($p<0.05$) following 96 h exposure to ammonia and saline. T4 group did not show any significant changes (Fig. 1).

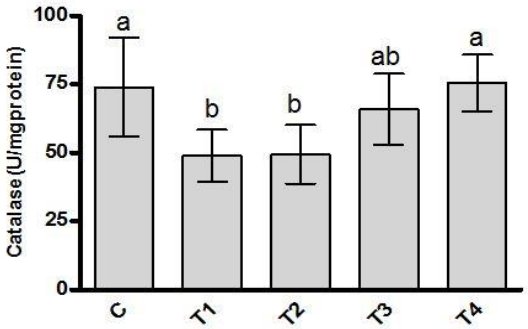

Figure 1: Liver CAT value in *Oreochromis niloticus* (mean±SD, n=9) exposed to different ammonia and salinity concentrations. Non-similar letters show significant differences at $p<0.05$.

Figure 2 Shows, different ASR in T1, T2 and T3 groups increased SOD activities compared to the control ($p<0.05$) following 96 h exposure to ammonia and saline. T4 group did not show any significant changes.

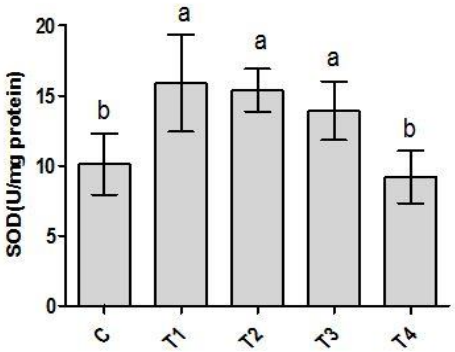

Figure 2: Liver SOD value in *Oreochromis niloticus* (mean±SD, n=9) exposed to different ammonia and salinity concentrations. Non-similar letters show significant differences at $p<0.05$.

As Figure 3 shows, MDA level was significantly (p<0.05) higher in T1 and T2 compared to the control group following 96 h exposure to ammonia and saline. T3 and T4 group did not show any significant difference.

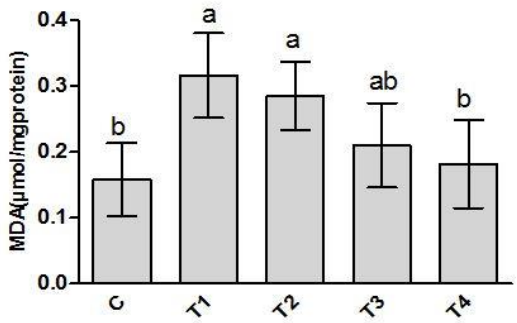

Figure 3: Liver MDA value in *Oreochromis niloticus* (mean±SD, n=9) exposed to different ammonia and salinity concentrations. Non-similar letters show significant differences at p<0.05.

Figure 4 Shows, different ASR in T1, T2 and groups significantly decreased GSH compared to the control (p<0.05) following 96 h exposure to ammonia and saline. T3 andT4 group did not show any significant changes.

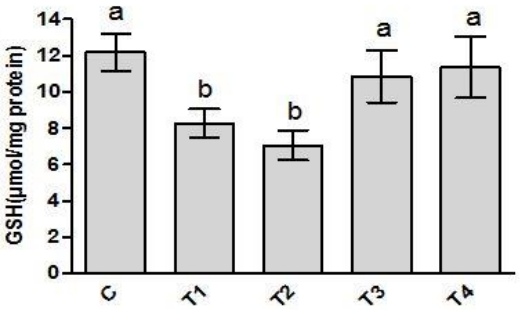

Figure 4: Liver GSH value in *Oreochromis niloticus* (mean±SD, n=9) exposed to different ammonia and salinity concentrations. Non-similar letters show significant differences at p<0.05.

As was shown in Figure 5, GSH/GSSG ratio in T4 and T2 groups shows a significant decrease and increase, compared to the control group, respectively (p<0.05).

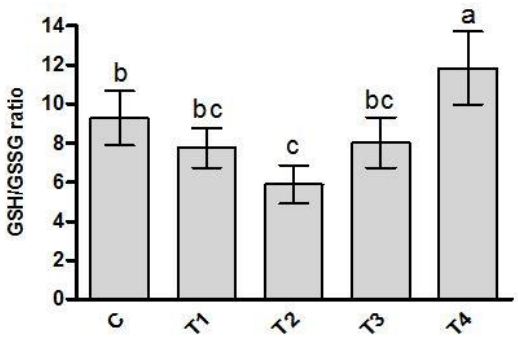

Figure 5: Liver GSH/GSSG ratio value in *Oreochromis niloticus* (mean±SD, n=9) exposed to different ammonia and salinity concentrations. Non-similar letters show significant differences at p<0.05.

As Figure 6 shows, GPX activity was significantly (p<0.05) decreased in T3 compared to the control group following 96 h exposure to ammonia and saline.T1, T2 and T4 group did not show any significant difference.

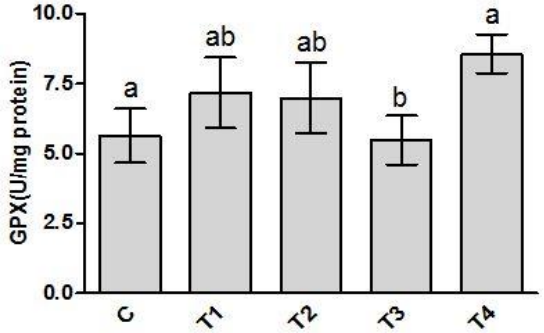

Figure 6: Liver GPX value in *Oreochromis niloticus* (mean±SD, n=9) exposed to different ammonia and salinity concentrations. Non-similar letters show significant differences at p<0.05.

Figure 7 show that there is a significant increase in GR activities in the T1, T2 groups compared to control ($p<0.05$), after 96 h exposed to ammonia and salinity.

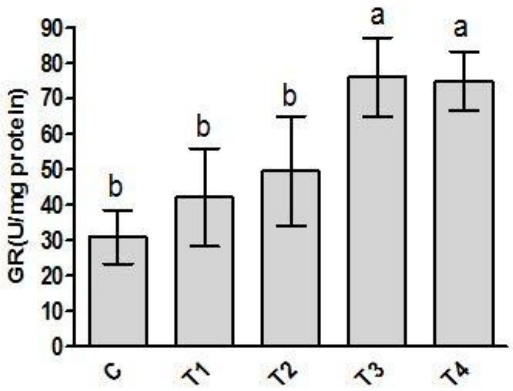

Figure 7: Liver GR value in *Oreochromis niloticus* (mean±SD, n=9) exposed to different ammonia and salinity concentrations. Non-similar letters show significant differences at $p<0.05$.

Figure 8 Shows ICDH activity was significantly increased in T1, T2 and T4 groups compared to Control group ($p<0.05$) following 96 h exposed to ammonia and salinity.

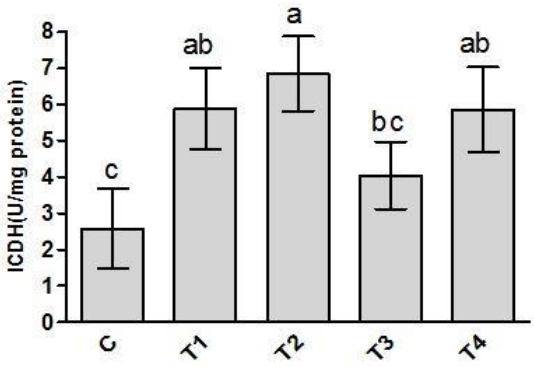

Figure 8: Liver ICDH value in *Oreochromis niloticus* (mean±SD, n=9) exposed to different ammonia and salinity concentrations. Non-similar letters show significant differences at $p<0.05$.

Figure 9 Shows ME activity was significantly increased in T1, T2 and T4 groups compared to Control group ($p<0.05$) following 96 h exposed to ammonia and salinity.

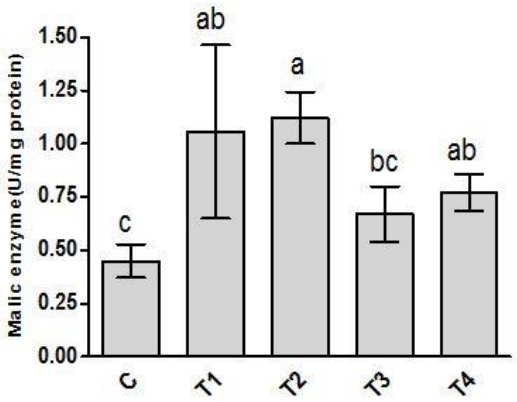

Figure 9: Liver ME value in *Oreochromis niloticus* (mean±SD, n=9) exposed to different ammonia and salinity concentrations. Non-similar letters show significant differences at $p<0.05$.

Discussion

It is widely accepted that ammonia toxicity and salinity stress can induce injuries in fish physiological functions and oxidative stress (Zeng *et al.*, 2017; Bal *et al.*, 2021). However, fish, like other organisms, has antioxidant defense mechanisms to protect against the toxic effects of ROS by enhancing the activities of enzyme and non-enzyme antioxidants. On the other hand, there are controversial reports about the modulating effect of salinity on ammonia toxicity (Sinha *et al*., 2015; Bal *et al*., 2021). To find how aquatic animals cope with environmental mixed-stresses, and to understand their mode of interaction, we assessed the short-term effects of combined exposures of ammonia and salinity on oxidative stress responses in Nile tilapia.

The significant increase in SOD activity in groups T1 and T2 compared to the control group is could be due to the induction of this enzyme by acute ammonia toxicity and superoxide anion

production (Murthy *et al.*, 2001; Kim *et al.*, 2017). The main source of peroxides in cells is superoxide anion, which is produced by hypoxia and converted into H_2O_2 by SOD (Birnie-Gauvin *et al.*, 2017). Evidences show that SOD activity is a function of ammonia concentration in fish (Kim *et al*., 2020). Following exposure of fish to an abnormal level of ammonia, alterations in SOD activity would be depended on the ammonia tolerance each of species. For example, *Takifugu obscurus* shows an increase in SOD activity in case of exposure to Ammonia stress (Wang *et al*., 2015), while *Litopenaeus vannamei* Shows a decrease (Lin and Chen, 2001) and *Dicentrarchus labrax* does not show any changes (Sinha *et al*., 2015).

Our findings on reduced catalase activity coupled with reduction in GSH and GSH/GSSG ratio, which was accompanied by an increase of MDA in T1 and T2 groups, could be a line of evidence for excessive production of H_2O_2 in these groups. It was reported that excess H_2O_2 inhibits catalase and the glutathione redox enzymes activity, which leads to peroxidation of cell membrane lipids and release of MDA in blood stream (Barata *et al*., 2005).

Kim *et al.* (2017) showed a significant decrease in SOD and CAT activities in *Anoplopoma fimbria* exposed to low salinity after four months. Moreover, Kim *et al.* (2019) showed a significant increase in SOD and CAT activities in *Paralichthys olivaceus* exposed to ammonia after 96 h. They reported that SOD and CAT activities seem to be affected by ammonia concentration, exposure time, and the interaction between these two factors. GSH as a non-enzymatic antioxidant directly scavenges singlet oxygen and hydroxyl radicals modifies the redox state of protein thiols and also as a cofactor of GPX, detoxifies H_2O_2 and hydroperoxides (Xia and Wu, 2018). Therefore, at the present study, lower GSH levels in fish exposed to ammonia and salinity might be due to its depletion in the second stage of biotransformation.

We have shown that the glutathione redox system is stimulated against ammonia toxicity. This is supported by a slight increase in the activity of GPX and GR in groups T1 and T2, along with a significant increase in the activity of cytosolic isocitrate dehydrogenase and malate dehydrogenase. On the other hand, high MDA and low GSH indicate that induced oxidative stress by ammonia dominates the glutathione redox system. This is in a good agreement with Yu *et al.* (2020) findings on increased MDA levels in the gill, brain, and spleen of *Rhynchocypris lagowski* following 96h exposure to ammonia. In this respect, Zeng *et al.* (2017) also showed an increase in MDA levels and a decrease in SOD and GPx values following 48h exposure to hyper-salinity group in *Pseudosciaena crocea fish*.

In the present study, despite the increase in the activity of the malic enzyme and isocitrate dehydrogenase, the ratio of GSH/GSSG decreased in groups T1 and T2. This phenomenon

may be due to insufficient NADPH produced for GR activity, or its use for biosynthetic pathways (Hegazi *et al*., 2010). GR is an NADPH-dependent oxidoreductase that catalyzes the conversion of GSSG to GSH (Sirikanth *et al*., 2013). High level of GSH/GSSG ratio could detoxify ROS (Sies, 1999), which demands availability of NADPH-producing enzymes such as G6PD, cytosolic malic enzyme, and cytosolic isocitrate dehydrogenase (Tomanek, 2014; Moreno-Sánchez *et al*., 2018).

Increase in catalase and SOD activity and decrease in MDA in T3 and T4 groups shows that the antioxidant defense system of the liver has been able to overcome ammonia-induced oxidative stress in Tilapia. These findings show that the glutathione redox system play an important role in protection of liver against ammonia-induced oxidative stress. Importantly, increase of salinity was accomapied by an increase in hepatic SOD, catalase, ME and IDH values; that support the notion on improvement in antioxidant defense system following exposure to a balanced level of ammonia and saline.

We conclude that in acute poisoning caused by high doses of ammonia, the antioxidant defense system is suppressed. However, in sub-acute ammonia poisoning at 4 ppt salinity, glutathione-dependent antioxidant system inhibits oxidative stress. It could be assumed that either the salinity reduced ammonia toxicity, or that ammonia toxicity was dose-dependent. To determine which of the two factors have a more significant role, it is suggested to investigate a wide range of salinities in the presence of different amounts of ammonia in future studies.

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