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Acute toxicity of nitrite on tilapia (*Oreochromis niloticus*) at different external chloride concentrations

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Abstract

Tilapias (*Oreochromis niloticus*) juveniles (total length 4.9 ± 0.2 cm and weight 1.8 ± 0.2 g) were exposed to several nitrite concentrations (0, 10, 18, 32, 56 and 100 mg l^{-1}) for 96 h, using a semi-static renewal method at chloride levels of 35.0 and 70.0 mg l^{-1}. At the end of the 96-h period, the median lethal concentration (LC_{50}) of nitrite was 28.18 mg l^{-1} in water with low chloride content (35.0 mg l^{-1}) and 44.67 mg l^{-1} with high chloride content (70.0 mg l^{-1}, respectively). It indicated that high concentrations of chloride ions could reduce the toxicity of nitrite. During the toxicity experiments, the behaviour and clinical signs of tilapias were also observed. Furthermore, the test of toxic mechanism was designed taking five test concentrations, viz., 5, 10, 15 and 20 mg l^{-1} and a nitrite-free control. Nitrite exposure produced high levels of methaemoglobin (MHb) but did not seem to cause mortality, as surviving tilapias showed high levels (85.37 ± 2.23 and 53.82 ± 3.44 at 35.0 and 70.0 mg l^{-1} chloride, respectively). The percentage of MHb exposed to nitrite was significantly higher ($P < 0.05$) than the control (0 mg l^{-1} nitrite) and increased with the increasing nitrite concentration. However, the percentage of MHb decreased with the increasing chloride concentration.

Introduction

Aquaculture is an important business in many countries around the world (Lin and Wu 1996). The economics of most modern aquaculture operations require that aquatic animals be cultured at high densities. The rapid nitrite accumulation in the pond even up to 11.65 mg l^{-1} was one of the greatest difficulties of high-density aquacultural system (Wei et al. 2001). The increase of nitrite concentrations in water can result in many physiological disturbances (Hagopian and Riley 1998; Hargreaves 1998). In addition, nitrite can enter natural waters from ponds and have several adverse effects upon human health (Connolly and Paull 2001). For example, the *in vivo* reaction between nitrite and secondary or tertiary amines produces *N*-nitrosamines, which are potential carcinogens, mutagens and/or teratogens.

Nitrite (NO_2^-) is a naturally occurring intermediate product in two bacteria-mediated process involving transformations of nitrogen in water and soils (Mevel and Chamroux 1981). Nitrite accumulates in aquaculture systems and can be toxic to aquatic animals (Colt and Armstrong 1981; Tomasso 1994; Hargreaves 1998; Cheng and Chen 1999; Huertas et al. 2002; Svobodová et al. 2005). Acute toxicity of nitrite has been investigated in a number of fishes, crustaceans, molluscs and echinoderms (Handy and Poxton 1993; Basuyaux and Mathieu 1999; Sampaio et al. 2002; Lin and Chen 2003). However, very little is known about the acute toxicity of nitrite on small tilapia (*Oreochromis niloticus*) at different external chloride concentra-

tions, one of the most farmed species in China. Therefore, the present study was designed to investigate the effects of nitrite on tilapia (*Oreochromis niloticus*) at 35.0 and 70.0 mg l^{-1} chloride, respectively, and determine medianlethal concentrations (LC_{50}) of environmental nitrite. The toxic mechanism of nitrite on tilapia was also discussed based on the haematological changes.

Materials and methods

Tilapia acclimation

This study concerns healthy tilapia (*Oreochromis niloticus*) obtained from the Fish Hatchery of Hangzhou, China and transferred to the Laboratory of Aquaculture Department, Zhejiang University. The tilapias were divided randomly into two groups and acclimated for 15 days in two rectangular 640-l aquaria (filled with 560 l) with continuously aerated clean tap water at different external chloride concentrations, of 35.0 and 70.0 mg l^{-1}, respectively. During acclimation, the fishes were fed commercial diet (Huangguan Co., Hangzhou, China) twice a day until 2 days before the beginning of the experiment. At the end of the acclimation period, tilapia used averaged total length of 4.9 ± 0.2 cm and weighed 1.8 ± 0.2 g with no significant difference ($P > 0.05$) between two treatments. Water temperature was maintained at 25 ± 1 °C, dissolved oxygen (DO) at 6.8 ± 0.4 mg l^{-1}, and pH ranged from 7.94 to 8.02 with an average of 7.98. Total hardness and total alkalinity were 139 mg l^{-1} as $CaCO_3$ and 76 mg l^{-1} as $CaCO_3$. The tilapias were exposed to a 14-h light and 10-h dark photoperiod using overhead fluorescent lamps and no nitrite was recorded in the aquaria.

Experimental procedure

Short-term LC_{50} (median lethal concentration) toxicity tests were carried out according to the methods described by the American Public Health Association et al. (1985) and Buikema et al. (1982). Sodium nitrite ($NaNO_2$) was used as a source of nitrite and the stock solutions were prepared. Initially, a range-finding test was carried out to determine the main experimental concentrations. The test concentrations were obtained by serial dilution of the nitrite stock solution. The tests were conducted for different chloride concentrations, 35.0 and 70.0 mg l^{-1}, respectively. Then, after being starved for 2 days in acclimatization aquaria, 10 tilapias were placed in each of the continuously aerated 25-l glass tanks (filled with 20 l) for the 96-h acute toxicity test (semi-static). At each group of different chloride concentration level, the experiments consisted of control tanks (0 mg l^{-1} of NO_2^-) and five concentrations of nitrite (10, 18, 32, 56 and 100 mg l^{-1} of NO_2^-), and three replicate trials at each level were performed. Tilapias were not fed during the experiment in order to reduce nitrogen excretion and maintain water quality, especially nitrite concentration and the dead tilapias were removed daily. The experimental medium was changed every 24 h with fresh solution to maintain the initial concentration in a semi-static system for 96 h. Death was assumed when the tilapias were immobile and showed no response when touched with a glass rod. Water temperature was 25 ± 1 °C, dissolved oxygen (DO) was 6.5 ± 0.3 mg l^{-1}, and the pH 7.98 ± 0.08.

According to the 96-h LC_{50} value of nitrite, the test of toxic mechanism was designed taking five test concentrations, viz., 5, 10, 15 and 20 mg l^{-1} and a nitrite-free control. Three replications were also maintained for each of these nitrite concentrations including the control and 10 tilapias were placed in each of the glass tanks. The chloride concentration of each glass tank was 35.0 or 70.0 mg l^{-1} and water quality was just like that of the 96-h acute toxicity test. The tilapia used in the test of toxic mechanism averaged total length of 6.2 ± 0.3 cm and weighed 4.0 ± 0.4 g with no significant difference ($P > 0.05$) between the treatments. Samples of surviving tilapias were taken and analysed after being exposed to glass tank water of different nitrite and chloride concentrations for 96 h. The toxicity of nitrite was studied through the haematological changes in the blood of the tilapias.

Sampling and analytical methods

Temperature, dissolved oxygen (DO), pH, total hardness, total alkalinity, chloride and nitrite were measured using the Hach kit model DREL 2400 (Hach Company, Colorado, USA). The LC_{50} values of nitrite-N and other related statistical

analysis for 96 h of exposures were based upon the method described by Reish and Oshida (1987) and Hamilton et al. (1978) through probit analysis and calculation using both computer software and probit paper method.

During the test of toxic mechanism, samples of surviving tilapias blood were taken from each treatment including the control at the end of 96 h and then immediately analysed. The blood was drawn from the vena of tilapia tail using a 250 μl-glass syringe and placed in plastic Eppenddorf tubes on ice. The pretreatment of the samples and methaemoglobin (MHb) percentage studies were carried out following standard methods for blood analysis (Institute of Shanghai Medicine 1979). All chemicals used in experiments were purchased from Sigma, Roche, Sangon or Merck.

Statistical analysis, using one-way ANOVA (Statistical Analysis System, SAS, version 6.03), was performed to find significant difference of the parameters between treated and control groups (Ming 2002). A significance level of $P < 0.05$ was used.

Results

Median-lethal concentration (LC_{50})

All tilapias survived in the control (nitrite-free) after 96-h exposure at 35.0 and 70.0 mg chloride per litre. The mortality percentage during the 96-h nitrite toxicity tests at 35.0 and 70.0 mg l^{-1} chloride is shown in Figure 1 and the LC_{50} values of nitrite and their 95% confidence limits at different external chloride concentration are presented in Table 1. Mortalities were caused by increased nitrite concentrations for the tilapias and the fishes were susceptible to nitrite at low external chloride concentration.

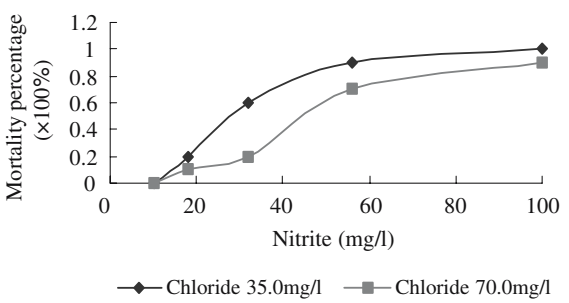

Figure 1. The mortality percentage of tilapia during the 96 h of nitrite toxicity tests at 35.0 and 70.0 mg l^{-1}.

During the toxicity experiments, the tilapias treated with nitrite at different external chloride concentration levels showed similar reactions, nevertheless the behaviours at 35.0 mg l^{-1} chloride were observed more clearly than those at 70.0 mg l^{-1} chloride. An increase in movements, convulsions, sideways and spiral swimming, disbalance and death of tilapias fingerlings were observed compared to the control group. The external observations of tilapias allowed us to define the following clinical signs compared to the control groups: brown gills, haemorrhage in the muscles and darkening of the skin.

Haematological changes

The percentage of MHb increased significantly ($P < 0.05$) in all tilapias exposed to different nitrite concentrations compared to the controls, and reached concentrations of MHb between 2.16 and 89.56% of the total haemoglobin (Hb) (Figure 2). The percentage of MHb also increased with the concentration of nitrite in glass tank water. After 96-h exposure, the percentage of MHb in surviving fish (85.37 ± 2.23) exposed to 20 mg l^{-1} nitrite was significantly higher ($P < 0.05$) than in others including the control (Figure 2).

Discussion

In the present study, increasing external chloride from 35.0 to 70.0 mg l^{-1} raised the 96-h LC_{50} from 28.18 to 44.67 mg nitrite per litre (Table 1). Russo and Thurston (1977) found that there was a negative linear relationship between nitrite toxicity and chloride concentration. The observed LC_{50} and behaviour of tilapia, *Oreochromis nilotica*, in

Table 1. Acute toxicity of nitrite (mg l^{-1}) to tilapias exposed over 96 h at different external chloride concentration under semi-static condition ($n = 3$)

Chloride (mg l^{-1})	Exposure time (h)	LC_{50} (mg l^{-1})	95% Fiducial limit (mg l^{-1})	
			Upper limit	Lower limit
35.0	96	28.18	35.58	20.79
70.0	96	44.67	57.09	32.25

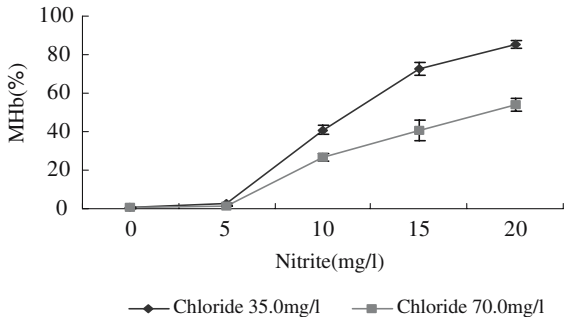

Figure 2. MHb percentage of tilapias exposed to several nitrite concentrations at 35.0 and 70.0 mg l^{-1} chloride of each glass tank.

this experiment was also consistent with it. High concentrations of chloride ions reduced the toxicity of nitrite and the probable explanation was that chloride along with other anions in water provided protective action against nitrite in active branchial uptake (Williams and Eddy 1986; Stormer et al. 1996; Jensen 2003). Another hypothesis assumed that the inter-specific differences in nitrite resistance laid in chloride influx rates (Williams and Eddy 1986). Species with higher influx rates had concomitantly higher nitrite uptake rates. These species should be more susceptible to nitrite toxicosis in lower nitrite environmental concentrations. The protective effect of chloride against nitrite toxicity has been also reported in channel catfish (Tomasso et al. 1979), salmon (Crawford and Allen 1977; Perrone and Meade 1977), giant freshwater prawn (Chen and Lee 1997) and crayfish (Harris and Coley 1991). It followed from the above cases and literary data that chloride concentrations in water need to be raised if fish in aquaculture facilities were under the threat of damage by nitrites. This preventive measure was used by aquarists in aquariums where levels of nitrogenous metabolites rose as a result of insufficient water renewal (Wei et al. 2001; Svobodová et al. 2005).

The range of sensitivity to environmental nitrite was very wide among fishes depending on the test conditions and organismal traits (Lewis and Morris 1986). Thus, the comparison of toxicity values of different studies was difficult. The 96-h LC_{50} of nitrite of Tambaqui, *Colossoma macropomum*, was 0.54 mg l^{-1} and it was very sensitive to nitrite (Costa et al. 2004). The rainbow trout, *Onchorinchus mykiss*, tolerated 0.24 mg l^{-1} at 10 °C whereas *Onchorinchus tschawytscha* survived at 4.7 mg l^{-1}, and the tolerance of *Micropterus salmoides* reached 140 mg l^{-1} (Russo and Thurston 1977). The 96-h LC_{50} of nitrite in tilapia, *Oreochromis niloticus*, was 28.18 and 44.67 mg l^{-1} in a semi-static environment with chloride at 35.0 and 70.0 mg l^{-1}, respectively (Table 1). This observed LC_{50} in the present experiment was different with those reported in other fishes (Palachek and Tomasso 1984; Tomasso 1986; Atwood et al. 2001). The 96-h median lethal concentration of nitrite to small tilapia (4.4 ± 1.5 g) was up to 81 mg l^{-1} as reported by Atwood et al. (2001). Probably, the explanation for this result might be due to fish weight and concentration of chloride in experimental water and the species specific response of *Oreochromis niloticus* to nitrite.

The mode of action of nitrite in fishes was that it diffuses into red blood cells, where it oxidizes haemoglobin to methaemoglobin with a subsequent reduction of oxygen-carrying capacity and oxygen affinity (Cameron 1971; Jensen et al. 1987; Williams et al. 1993). In the present study, the conversion of haemoglobin to methaemiglobin might have created situation of oxygen shortage in the fish (Knudsen and Jensen 1997; Vedel et al. 1998). This, in turn, resulted in functional anaemia and tissue hypoxia. As expected, the exposure of tilapias to nitrite in the range of 5–20 mg l^{-1} caused an increase in MHb levels (Figure 2). In our study, the percentage of MHb increased with increasing nitrite concentration. However, several studies (Smith and Williams 1974; Brown and Mc Leay 1975; Tucker and Schwerder 1983; Huertas et al. 2002) had revealed that nitrite-induced MHb and the subsequent functional anaemia was not the primary cause of death. This was also true for tilapias, *Oreochromis niloticus*, as surviving specimens showed higher levels of MHb ($85.37 \pm 2.23\%$) in our research although there existed behaviour and clinical signs of tilapias. Chloride added to the glass tanks could decrease the toxicity of nitrite to tilapias (Figure 2) and this result was consistent with the acute toxic experiment. The concentration of chloride is usually low in freshwater culture in China (Wei et al. 2001). The experiment showed that the farmers could add chloride such as calcium chloride and sodium chloride to decrease the toxicity of nitrite to tilapias in practical culture according to aquaculture procedures in China.

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