Response of Acid and Alkaline Phosphatase Activities to Copper Exposure and Recovery in Freshwater Fish *Carassius auratus gibelio var*

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Abstract: Phosphatase is known to be sensitive to metal exposures and can be used to predict metal toxicity. In this study, freshwater fish *Carassius auratus gibelio var* were exposed to different concentrations (0.1, 0.2, 0.5, 1.0 and 2.0mg/L) of copper for 96 h, and the group of 2.0 mg/L exposure was then transferred to clean water for different days (1, 4, 8 and 12d) to assess recovery profile. Responses of acid phosphatase (ACP) and alkaline phosphatase (ALP) activities from kidney, liver, gill, spleen, muscle and brain to copper exposure and recovery were investigated. As shown from the results, after a 96-h copper exposure, ACP and ALP activities in different organs/tissues appeared to be different. At the highest copper concentration (2.0 mg/L), compared with the control, ACP activity decreased significantly in kidney, liver, gill and spleen, but increased significantly in muscle and brain. ALP activity decreased significantly in kidney, liver, gill, spleen and brain. However, after removing 2.0 mg/L copper exposure, ACP and ALP activities in different organs/tissues all normalized within 12 days. The observed data suggest that ACP and ALP in spleen of *Carassius auratus gibelio var* are most sensitive to copper stress and might be used as suitable biomarkers for copper contamination in aquatic environment.
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Keywords: Copper exposure;acid phosphatase;alkaline phosphatase;recovery;*Carassius auratus gibelio var*

1. Introduction

The increasing industrial, agricultural and anthropogenic activities have caused a rise of metal emissions and aquatic environments have been continuously contaminated by heavy metals. In particular, environmental concentration of copper has been increasing in aquatic environments recently because the wide application of $CuSO_4$ as a pesticide in agricultural practices and as an algicide, fungicide and bactericide in the control of algae and pathogens in aquaculture. Although copper, as a trace element, is essential to perform the functions of specific proteins and enzymes, it becomes toxic and has a potential hazard to aquatic organisms when beyond the normal level (Cerqueira and Fernandes, 2002; Li. et al., 2006). The toxicity of copper to fish has been widely studied in recent years and a wide range of effects on biotransformation, histology, haematology, osmoregulation, immunological modulations and behaviour of fish have been reported (Shariff et al., 2001; Cerqueira and Fernandes, 2002; Oliveira et al., 2004; Liu. et al., 2010). Besides, the knowledge of effects of contaminants on enzymatic activities in fish is also very important to describe the health of fish status and to understand the ecological impacts (Radhaiah et al., 1987). There are some studies that focussed on copper induced the changes of enzymatic activities have highlighted the importance of using enzymatic activities in biomonitoring programs as an Early warning system reflecting the copper pollution in aquatic environments (Antognelli et al., 2003; Carvalho and Fernandes, 2008; Atli et al., 2006). These studies involved in different enzymes and fish species, however, none of the reports focused on the effect of copper on phosphatase activities in different organs/ tissues of freshwater fish *Carassius auratus gibelio var*.

Phosphatase is a hydrolytic enzyme, leading to the release of *ortho*-phosphate from phosphorus compound and based on the optimum pH of action environment, classified into acid phosphatase (ACP, EC 3.1.3.2, optimum pH≤6.0) and alkaline phosphatase (ALP, EC 3.1.3.1, optimum pH≥8.0) (Jansson et al., 1988). Acid phosphatase is identified as a marker enzyme for the detection of lysosomes in cell fraction (Cajaraville et al., 2000) and alkaline phosphatase is a intrinsic plasma membrane enzyme found in almost all animal cells (Mazorra et al., 2002). Both enzymes are metalloenzyme, involved in various metabolic processes, such as permeability, growth and cell differentiation, protein synthesis, absorption and transport of nutrients, and gonadal maturation (Ram and Sathayanesan, 1985). Any change in acid and alkaline phosphatase activities can affect the metabolism of the fish. In fisheries sciences, changes in phosphatase activities have been regarded as indices of growth, illness and spawning of fish (Goldemberg et al., 1987; Matusiewicz and Dabrowski, 1996). And

in the assessment of ecotoxicology, these enzymes have also been used as bioindicators of heavy metals intoxication because of their sensitivity to metal pollution (Anan et al., 2002; Mora et al., 2004).

Carassius auratus gibelio var, a triploid freshwater fish with natural gynogenesis, being close relationship to the crucian carp, is only distributed in Qihe river of Henan province, China. The fish is famous for its delicious taste, rich nutrition and high commercial value. In recent years, this fish has been widely cultured throughout Henan province of China, but the copper pollution of farming water reduced its quality and affect the health of consumers through the food chain. At present, the studying of physiology, growth and reproduction of this fish has become a important task because of its economic importance. However, little research has been carried out on the relationship of this fish and heavy metal pollution for the protection of the aquaculture environment and consumer health.

In this study, the responses of acid and alkaline phosphatase activities to copper exposure in kidney, liver, gill, spleen, muscle and brain of *Carassius auratus gibelio var* were investigated. Furthermore, after removed the copper exposure, recovery process of ACP and ALP activities was evaluated in different periods. The recovery research from copper stress in fish has been investigated by some authors in recent years (Cerqueira and Fernandes, 2002; Shaw and Handy, 2006; Zahner et al., 2006), but these studies all focused on the histopathological and physiological changes in recovery process, the recovery of enzyme activity was not mentioned. Therefore, the phosphatase recovery phase of this experiment will extend our knowledge of the reversibility of enzyme activity in fish. In short, the main objective of this study was to assess the toxicity of copper to *Carassius auratus gibelio var*, investigate the reversibility of phosphatase activity after removal from copper exposure, evaluate the effectiveness of ACP and ALP as early biomarkers to monitor copper pollution in aquatic ecosystems and provide the useful database for healthy breeding of *Carassius auratus gibelio var*.

2. Material and Methods

Fish collection and care

Experimental fish (*Carassius auratus gibelio var*) were obtained from aquatic farm of Henan Normal University (Xinxiang, China), with body lenghth 12.36±1.54 cm and body weight 100.54±1.25 g, and then were transferred to laboratory and acclimatized for 14 days before copper exposure. The fish were reared in experimental tanks sized 100×50×40 cm, each containing 10 fish in 100 L of test solution or tap water only for controls. The tap water used for the experiments had a pH value of 7.9±0.20, conductivity of 578.8±17.8 μs/cm, total hardness of 305.2±19.3 mg $CaCO_3$/L and alkalinity of 140.1±12.5 mg $CaCO_3$/L. Supplemental aeration was provided to maintain dissolved oxygen levels near saturation, the temperature was kept at 25±1 ℃ and the photoperiod controlled (12D: 12L). The fish were fed with commercial fish pellet once a day during acclimation, and without food during experimentation.

Experimental protocol

According to 96 h LC_{50} value (6.02 mg/L) of copper for *Carassius auratus gibelio var* obtained by the acute toxicity test, in this study, sublethal copper concentrations were assigned as 0, 0.1, 0.2, 0.5, 1.0 and 2.0 mg/L (corresponding to 0%, 1/60, 1/30, 1/12, 1/6 and 1/3 of 96 h LC_{50}). Copper was added to the exposure tanks through diluting the stored solution of $CuSO_4$, prepared by analytical grade $CuSO_4 \cdot 5H_2O$ (from Chemical Reagent Company of China). The tap water without adding copper ions was considered as 0 mg/L of copper concentration (the control). In pre experiment, fish exposed to 2.0 mg/L of copper concentration（1/3 96h LC_{50}）for 96h did not show acute and subacute copper intoxication symptoms. So, experimental fish (n=100) were randomly divided into six groups (the control group and 2.0 mg Cu^{2+} /L group each had 30 fish, and the other four groups each had 10 fish.) and exposed to the different copper concentrations for 96h. Then, 5 fish of each group were sampled to be used to biochemical analysis. The surplus fish exposed to 2.0 mg Cu^{2+} /L for 96h were transferred to the clean water to conduct the recovery experiment, and then 5 fish in the recovery group and the control group were taken out randomly and sampled on Day 1, 4, 8, 12 respectively. The experiments were carried out using a static-renewal regimen, 50% of the experimental solution was replaced daily to ensure the relative stabilization of copper concentrations.

Sample prepration

The experimental fish were dissected carefully in ice, and the kidney, liver, gill, spleen, muscle and brain tissues were sampled immediately. The tissues were homogenized in ice-cold physiological salt water (1:9, w/v). Homogenates were centrifuged at 10000 g for 10 min at 4 ℃ in a Universal 30RF centrifuge (Hettich, Tuttlingen, Germany). the supernatant was collected and stored at -80 ℃ until biochemical analysis. All the above operations were carried out below 4 ℃.

Determination of the enzyme activity

Acid phosphatase (ACP) was analyzed according to the methods of Pennington (1961). Alkaline phosphatase (ALP) was measured based on

the method as described by Bretaudiere et al. (1977). The protein content of enzyme crude extract was determined using Coomassie Brilliant Blue (G-250) method repoted by Bradford (1976) and bovine g-globuline (BSA, purchased from Amresco) was used as the standard. The optical density was measured at 405 nm (ACP) and 400 nm (ALP) using a UV–VIS spectrophotometer (TU-1810APC, China) respectively. Enzyme activity unit was expressed as nmol/mg protein per minute.

Statistical analysis

Experimental data are presented as Mean± Standard Deviation (Mean±SD). Statistical analysis was implemented in SPSS statistical package programs. One-way ANOVA was used to compare variables among the different groups. An unpaired two-tailed Student's *t*-test was used to analyze significant differences. Significant level was assigned at $P = 0.05$ (significant difference) and $P = 0.01$ (highly significant difference).

3. Results

Response of ACP and ALP activities to copper exposure and recovery in kidney

After a 96-h copper exposure, compared with the control, ACP activity in kidney significantly decreased at 0.5, 1.0 and 2.0 mg Cu^{2+}/L exposures ($P<0.01$), with the inhibition rate at 5%, 9% and 12%, respectively. The lowest value of ACP activity appeared at 2.0 mg Cu^{2+}/L exposure (Figure 1a). ALP activity increased at lower copper concentration, and decreased with the elevated copper concentration, and reached the peak value at 0.2 mg Cu^{2+}/L. Compared with the control, ALP activity significantly increased by 31% at 0.2 mg Cu^{2+}/L exposure ($P<0.01$), but significantly decreased by 38% and 39% at 1.0 and 2.0 mg Cu^{2+}/L, respectively ($P<0.01$) (Figure 1c).

During the recovery period, compared with the control, ACP activity in kidney was significantly lower than that in the control on Day 1 and 4 ($P<0.05$). On Day 8 and 12, ACP activities were approximate to the control value ($P>0.05$), and it was suggested that ACP activity was restored to the normal physiological level on the 8th day (Figure 1b). ALP activity in kidney was significantly lower than that in the control on Day 1, 4 and 8 ($P<0.05$ or $P<0.01$), however, compared with the 96-h exposure, ALP activity increased continuously from Day 1 to Day 12 and was significantly higher on Day 8 and 12 ($P<0.01$). On day 12, ALP activity was similar to the control ($P>0.05$), which indicated that ALP activity returned to normal level (Figure 1d).

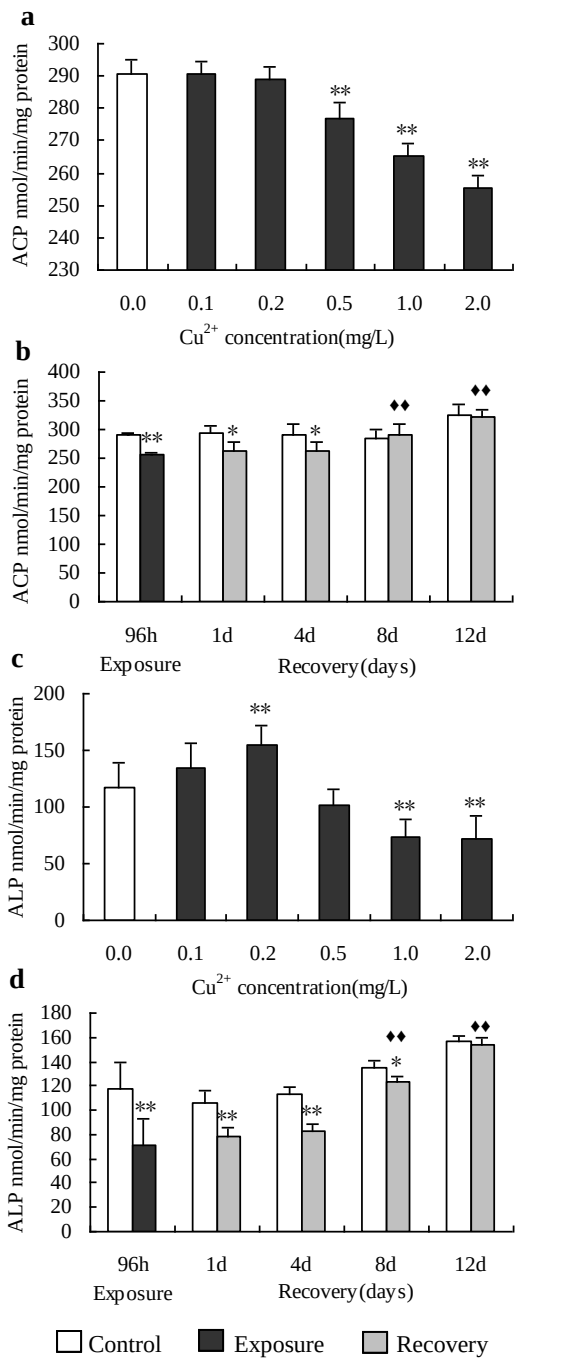

Figure 1. Changes of ACP and ALP activities in kidney of *Carassius auratus gibelio var* after a 96-h copper exposure and recovery of enzyme activity after removed 2.0 mg/L exposure. The values are expressed as mean±SD (n=5). Compared with the control, "*" respresents the significant difference (p <0.05), "**" respresents the highly significant difference (p <0.01); Compared with the 96-h copper exposure, "♦" respresents the significant difference (p <0.05), "♦♦" respresents the highly significant difference (p <0.01).

Response of ACP and ALP activities to copper exposure and recovery in liver

Figure 2a and Figure 2c illustrates the changes in ACP and ALP activities in liver after a 96-h copper exposure. With the increase of copper concentration, both ACP and ALP activities increased firstly and reached the peak values at 0.2 mg Cu^{2+}/L exposure and then decreased. Compared with the control, ACP activity significantly increased by 5% and 4% at 0.2 and 0.5 mg Cu^{2+}/L exposures, respectively (P<0.01), but significantly decreased by 3% at 2.0 mg Cu^{2+}/L exposure (P<0.05). ALP activity significantly increased by 54% and 138% at 0.1 and 0.2 mg Cu^{2+}/L exposures, respectively (P<0.01), but significantly decreased by 53% at 2.0 mg Cu^{2+}/L exposure (P<0.05).

During the recovery, after removed 2.0 mg/L copper exposure, ACP activity in liver was significantly lower than that in the control on Day 1, 4 and 8 (P<0.05 or P<0.01). Compared with the 96-h exposure, ACP activity decreased continuously and remarkably from Day 1 to Day 4 (P<0.05 or P<0.01). On Day 12, ACP activity showed no significant difference compared with the control (P>0.05), which indicated that ACP activity recovered to the normal level (Figure 2b). ALP activity in liver was significantly lower than that in the control on Day 1 and 4 (P<0.01). On Day 8 and 12, ALP activity was very close to the control (P>0.05), it was demonstrated that ALP activity in liver recovered to normal level on the 8th day. Compared with the 96-h exposure, ALP activity increased significantly on Day 4, 8 and 12 (P<0.01) (Figure 2d).

Responses of ACP and ALP activities to copper exposure and recovery in gill

After a 96-h copper exposure, ACP activity in gill decreased gradually with increased copper concentration and reached the least value at 2.0 mg Cu^{2+}/L exposure. 0.5, 1.0 and 2.0 mg Cu^{2+}/L exposures caused significant decreases in ACP activity in gill compared with the control (P<0.01), with the inhibition rate at 7%, 17% and 19%, respectively (Figure 3a). With the increased copper concentration, ALP activity gradually increased firstly and reached the peak at 0.5 mg Cu^{2+}/L. ALP activity significantly increased by 50% and 92% at 0.2 and 0.5 mg Cu^{2+}/L exposures, respectively (P<0.01), but significantly decreased by 34% at 2.0 mg Cu^{2+}/L exposure (P<0.01) (Figure 3c).

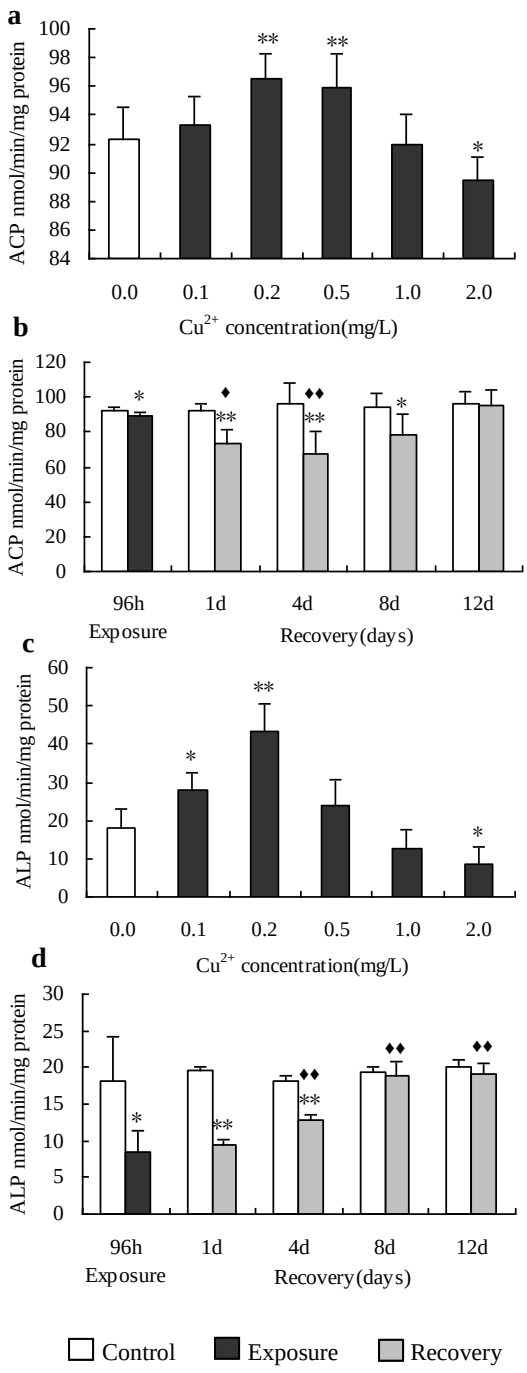

Figure 2. Changes of ACP and ALP activities in liver of *Carassius auratus gibelio var* after a 96-h copper exposure and recovery of enzyme activity after removed 2.0 mg/L exposure. The values are expressed as mean ±SD (n=5). Compared with the control, "*" respresents the significant difference (p <0.05), "**" respresents the highly significant difference (p <0.01); Compared with the 96-h copper exposure, "♦" respresents the significant difference (p <0.05), "♦♦" respresents the highly significant difference (p <0.01).

As shown from Figure 3b and Figure 3d, after 2.0 mg/L copper exposure was removed, ACP activity in gill showed no significant changes on Day 1, 4, 8 and 12, in contrast with the control (P>0.05). It was suggested that ACP activity in gill returned to normal level on the first day. ALP activity was lower significantly than that in the control on Day 1 and 4 (P<0.05 or P<0.01). Compared with the 96-h exposure, ALP activity slightly decreased on Day 1 (P>0.05), but significantly increased on Day 4 (P<0.05). On Day 8 and 12, ALP activity reached the control level (P>0.05), which showed that ALP activity in gill recovered to the normal level on the 8th day.

Response of ACP and ALP activities to copper exposure and recovery in spleen

As seen in Figure 4a and Figure 4c, after a 96-h copper exposure, ACP and ALP activities in spleen decreased gradually with the increasing copper concentration and reached the minimum value at 2.0 mg Cu^{2+}/L exposure. Compared with the control, ACP activity significantly decreased by 2%, 5%, 6%, 7% and 11% at 0.1, 0.2, 0.5, 1.0 and 2.0 mg Cu^{2+}/L exposures, respectively (P<0.01). It was demonstrated to have a significant negative correlation between the ACP activities (A) and the exposure concentrations (X). The regress equation is $A = -6.1513X + 293.26$ (R^2=0.9748). Compared with the control, ALP activity significantly decreased by 18%, 35%, 50%, 60% and 62% at 0.1, 0.2, 0.5, 1.0 and 2.0 mg Cu^{2+}/L exposures, respectively (P<0.05 or P<0.01). A significant negative correlation was also found between ALP activities (Y) and the exposure concentrations (X), and the regress equation is $Y = -8.5151X + 70.113$ (R^2=0.9447).

After 2.0 mg/L exposure was free, ACP activity in spleen was still significantly lower than that in the control on Day 1 and 4 (P<0.01). Compared with the 96-h exposure, ACP activity decreased continuously from Day 1 to Day 4, and appeared to be significantly lower on Day 4 (P<0.05). But on Day 8 and 12, ACP activity indicated no significant difference compared with the control (P>0.05), and it was shown that ACP activity in spleen recovered to normal level on the 8th day (Figure 4b). ALP activity was significantly lower than that in the control on Day 1 (P<0.01), but significantly higher than that in 96-h exposure group (P<0.01). On Day 4, 8 and 12, ALP activity was close to the control (P>0.05), and it was suggested that the normal level of ALP activity in spleen was restored on the 4th day (Figure 4d).

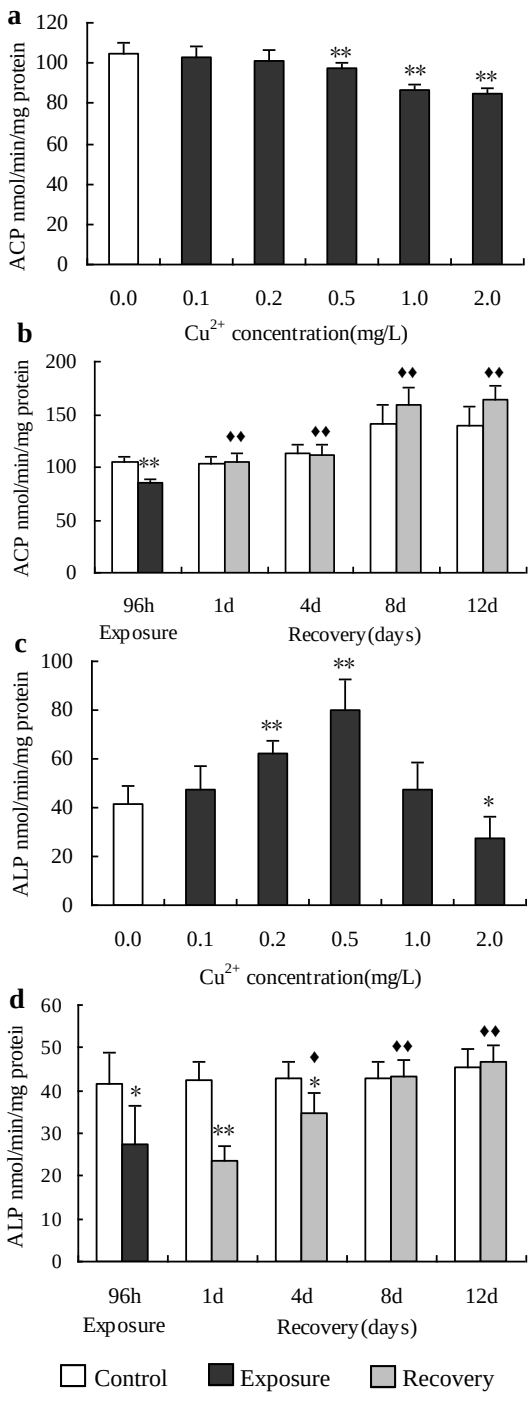

Figure 3. Changes of ACP and ALP activities in gill of *Carassius auratus gibelio var* after a 96-h copper exposure and recovery of enzyme activity after removed 2.0 mg/L exposure. The values are expressed as mean ±SD (n=5). Compared with the control, "*" respresents the significant difference (p <0.05), "**" respresents the highly significant difference (p <0.01); Compared with the 96-h copper exposure, "♦" respresents the significant difference (p <0.05), "♦♦" respresents the highly significant difference (p <0.01).

Response of ACP and ALP activities to copper exposure and recovery in muscle

After a 96-h copper exposure, compared with the control, ACP activity in muscle showed no significant changes at 0.1, 0.2, 0.5 and 1.0 mg Cu^{2+}/L exposures (P>0.05), but significantly increased by 14% at 2.0 mg Cu^{2+}/L exposure (P<0.05) (Figure 5a). ALP activity indicated no significant difference between the exposure and the control (P>0.05) (Figure 5c).

During the recovery span, in 2.0 mg/L exposure group, ACP activity in muscle was significantly higher than that in the control on Day 1, 4 and 8 (P<0.01). Compared with the 96-h exposure, ACP activity increased continuously from Day 1 to Day 8, while on Day 12, ACP activity got to the control level (P>0.05). It was suggested that ACP activity in muscle normalized on the 12th day (Figure 5b). Compared with the control, there were no significant change in ALP activity during the recovery period (Figure 5d).

Response of ACP and ALP activities to copper exposure and recovery in brain

Changes in ACP and ALP activities in brain after a 96-h copper exposure are depicted in Figure 6a and Figure 6c. ACP activity in brain elevated gradually with the increase of copper concentration and reached the maximum value at 2.0 mg Cu^{2+}/L exposure. 1.0 and 2.0 mg Cu^{2+}/L exposures caused significant increases in ACP activity in brain compared with the control (P<0.01), with activation rate at 8% and 11%, respectively. Compared with the control, ALP activity slightly decreased at 0.1, 0.2 and 0.5 mg Cu^{2+}/L exposures (P>0.05), and obviously decreased by 18% and 32% at 1.0 and 2.0 mg Cu^{2+}/L exposures, respectively (P<0.01), and reached the lowest value at 2.0 mg Cu^{2+}/L exposure.

After 2.0 mg/L copper exposure was removed, compared with the control, ACP activity in brain significantly increased on Day 1, 4 and 8 (P<0.01). Compared with the 96-h exposure, ACP activity increased continuously from Day 1 to Day 8 and was significantly higher than that in 96-h exposure group (P<0.05 or P<0.01). However, on the day 12, ACP activity was restored to the control level (P>0.05) (Figure 6b). Compared with the control, there was no significant change in ALP activity in brain on Day 1, 4, 8 and 12 (P>0.05), and it was indicated that ALP activity in brain normalized on the first day (Figure 6d).

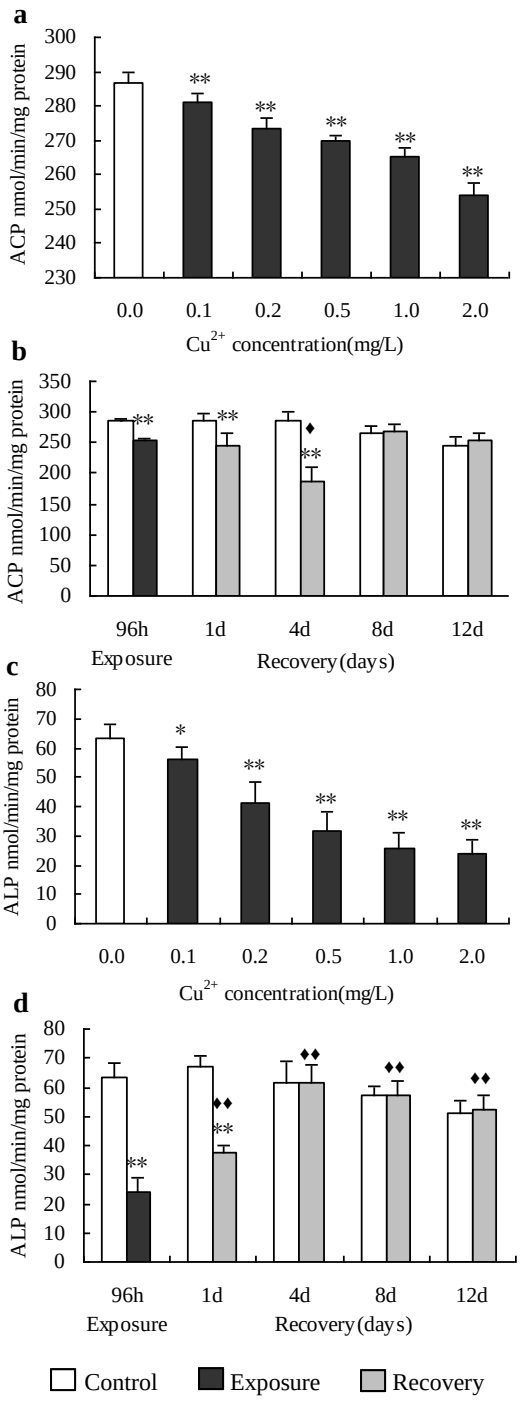

Figure 4. Changes of ACP and ALP activities in spleen of *Carassius auratus gibelio var* after a 96-h copper exposure and recovery of enzyme activity after removed 2.0 mg/L exposure. The values are expressed as mean ±SD (n=5). Compared with the control, "*" respresents the significant difference (p <0.05), "**" respresents the highly significant difference (p <0.01); Compared with the 96-h copper exposure, "♦" respresents the significant difference (p <0.05), "♦♦" respresents the highly significant difference (p <0.01)

4. Discussions

In toxicological studies, ACP and ALP are important biochemical enzymes to be used to detect the alteration of physiological metabolism of animal induced by metal exposure (Reddy and Bhagyalakshmi, 1994; Oruc and Uner, 1999). ACP is a lysosomal enzyme, many environmental contaminates including heavy metals could be sequestered in lysosomes of eukaryotic cells, and some metals could alter the structure, permeability and integrity of lysosomal membranes and result in enzyme diffusion into cytosol (Hedayati et al., 2010). In this study, ACP activity increased significantly at 0.2 and 0.5 mg Cu^{2+}/L exposures in liver, at 2.0 mg Cu^{2+}/L exposure in muscle and at 1.0 and 2.0 mg Cu^{2+}/L exposures in brain. This elevation might be due to the activation of the enzyme which was kept in a latent state inside the membrane of lysosomes (De Duve et al., 1955) as a result of the disruption of the membrane by copper, or due to the proliferation of lysosomes in attempt to sequester copper. On the contrary, ACP activity decreased significantly at 0.5, 1.0 and 2.0 mg Cu^{2+}/L exposures in kidney and gill, at 2.0 mg Cu^{2+}/L exposure in liver and at all copper exposures in spleen. This reduction might be attributed to the escape of the enzymes from the lysosomes out of the cell due to the damage of lysosomal membrane (Malbica and Hart, 1971) by copper. The decreased lysosomal membrane stability was observed by Regoli et al. (1998) on exposure to copper.

ALP, a ubiquitous plasma membrane-bound enzyme, is often employed to assess the integrity of the plasma membrane (Akanji et al., 1993), and any perturbation in the membrane property caused by interaction with xenobiotics could lead to alteration in ALP activity (Molina et al., 2005). In the present study, ALP activity increased significantly at 0.2 mg Cu^{2+}/L exposure in kidney, at 0.1 and 0.2 mg Cu^{2+}/L exposures in liver, and at 0.2 and 0.5 mg Cu^{2+}/L exposures in gill. Such increase might be attributed to the increase in functional activity of these organs/tissues leading to the synthesis of the enzyme molecule (Umezawa and Hooper, 1982; Yakubu et al., 2001), or regard it as an adaptive response in mitigating copper toxicity at low concentration. On the contrary, ALP activity decreased significantly at 1.0 and 2.0 mg Cu^{2+}/L exposures in kidney and brain, at 2.0 mg Cu^{2+}/L exposure in liver and gill, at all copper exposures in spleen. Such reduction might be attributed to the loss of ALP from plasma membrane into the extracellular fluid (Malbica and Hart, 1971) and the reduction in concentration or total absence of specific phospholipids required by this membrane-bound enzyme to express its full activity (Yakubu et al., 2002) under the interaction of copper with plasma membrane, or due to inhibition of the enzyme activity at the cellular/molecular level (Akanji et al., 1993) and inactivation of the enzyme molecule *in situ* (Umezawa and Hooper, 1982) by the binding of copper to ALP directly. In addition, the little effect of copper on phosphatase activity in muscle revealed that the membranes of lysosomes and plasma in muscle have been little disrupted by copper.

In this study, ACP activity in liver and ALP activity in kidney, liver and gill increased at lower copper concentration and then went down with the increased copper concentration. The relation between copper concentration and enzymatic activity showed the inverted U-shaped curve, which is usually called as hormetic effect (Rodricks, 2003). Hormetic effect, a phenomenon characterized by low-dose stimulation and high-dose inhibition, has been frequently observed in organisms exposed to heavy metals (Oller and Bates, 2004) and considered to be a general rule in the study of environment toxicology, but the mechanisms underlying it induced by environmental agents still remain an enigma. Nevertheless, the mechanism of hormetic effect in this study might be interpreted as the result of instantaneous balance between the synthesis and degradation of enzyme protein under the interaction of copper with membranes of lysosomes and plasma.

In the present study, copper-induced alterations in phosphatase activities in different organs/tissues were variable, this may be relate to the different physiological functions of organs/tissues. Moreover, Atli et al. (2006) reported that the responses of enzyme activities in different organs/tissues to heavy metals also depend on metal bioaccumulation ability of these organs/tissues. Kidney is the excretory and immune organ of fish, in which the higher metal bioaccumulations were observed (Dautremepuits et al., 2004; Palaniappan and Karthikeyan, 2009). In the present study, compared with the control, ACP and ALP activity in kidney both appeared the significant change at three different copper concentrations, this suggests the sensitivity of the both enzymes to copper stress in the kidney. This sensitivity may be related to the higher copper bioaccumulation in kidney. In previous studies, the kidney was found to be a organ with the highest ALP activity by Cvancara et al. (1978), but in this study, compared the control values of the both enzymes in kidney with that in the other organs/tissues, we found the kidney of *Carassius auratus gibelio var* has not only the highest ALP activity but also the highest ACP activity.

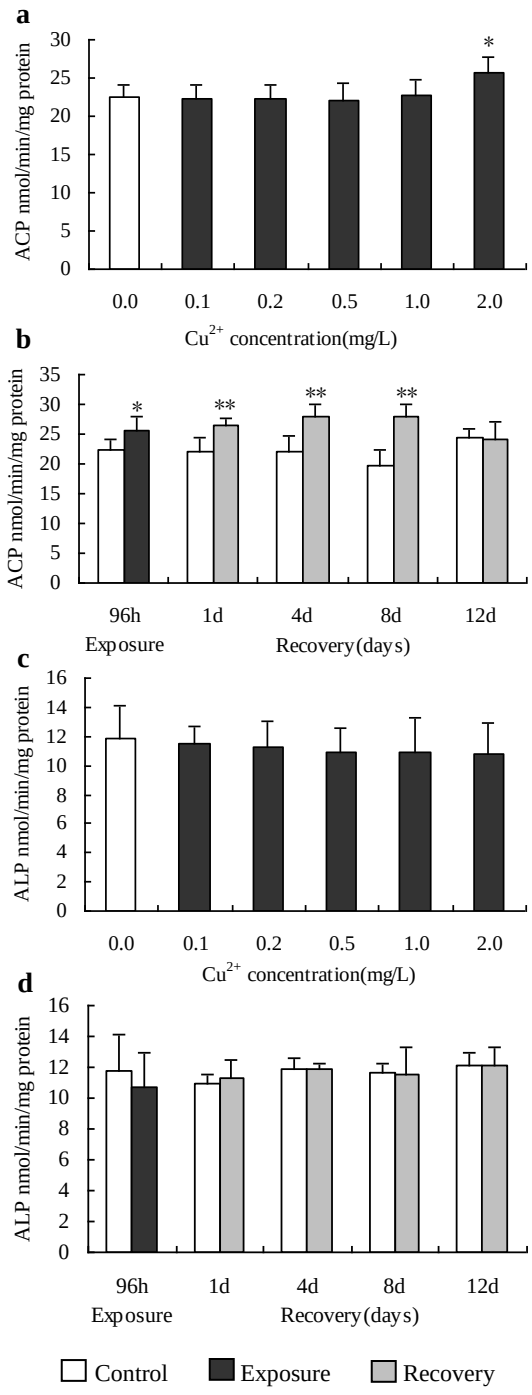

Figure 5. Changes of ACP and ALP activities in muscle of *Carassius auratus gibelio var* after a 96-h copper exposure and recovery of enzyme activity after removed 2.0 mg/L exposure. The values are expressed as mean ±SD (n=5). Compared with the control, "*" respresents the significant difference (p <0.05), "**" respresents the highly significant difference (p <0.01); Compared with the 96-h copper exposure, "♦" respresents the significant difference (p <0.05), "♦♦" respresents the highly significant difference (p <0.01).

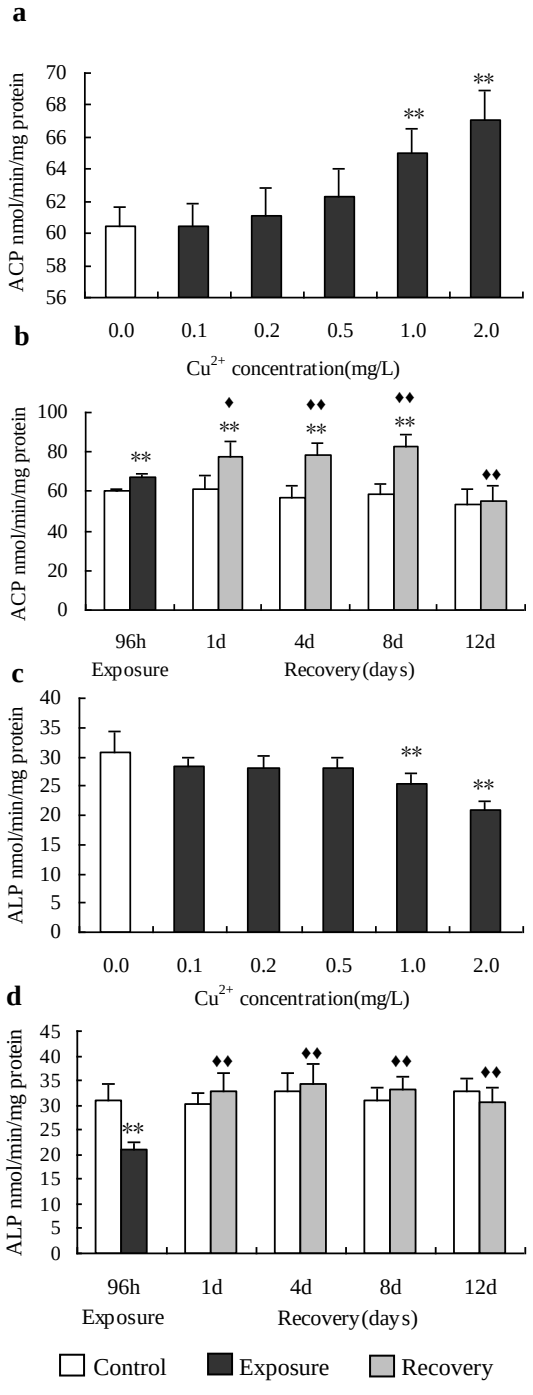

Figure 6. Changes of ACP and ALP activities in brain of *Carassius auratus gibelio var* after a 96-h copper exposure and recovery of enzyme activity after removed 2.0 mg/L exposure. The values are expressed as mean ±SD (n=5). Compared with the control, "*" respresents the significant difference (p <0.05), "**" respresents the highly significant difference (p <0.01); Compared with the 96-h copper exposure, "♦" respresents the significant difference (p <0.05), "♦♦" respresents the highly significant difference (p <0.01).

Fish liver is not only one of the vital detoxifying organs, but also the important compartment of heavy metal accumulation (Jarić et al., 2011; Fallah et al., 2011). In the present study, the effect of copper on ACP and ALP activities in liver all showed the typical hormetic dose-response, which may be associated with the detoxification function of fish liver. Marr et al. (1995) pointed out that a metal-binding protein, the metallothionein (MT), could be induced by heavy metals in liver, and there existed a positive correlation between MT and heavy metals. MT in liver can attenuate cytotoxicity induced by heavy metals by sequestering these metals and reducing their intracellular concentration. In the present study, increased ACP and ALP activities at low copper concentrations suggested the hydrolysis of phosphate esters to release energy in view of the synthesis of MT and the enhancement of the detoxification function of liver. With the increasing copper concentration, more copper ion entered into liver cells and was sequested by MT firstly, but when the higher copper concentrations in liver was beyond the regulation capacity of MT by Cu-binding, the surplus copper could direct bind to –SH groups on enzyme molecule and cause the decrease of ACP and ALP activities. At the same time, the detoxification capacity of liver decreased and this would lead to the liver damage eventually. Sharkoori et al. (1992) have suggested the decrease (or) inhibition of ACP and ALP activities might be taken as indexs of necrosis in hepatocytes.

Gill is the vital respiration organ, which was targeted by lots of xenobiotics due to their extensive surface area directly contacted with water environment and the reduced distance between the internal and external medium. In this study, in gill, ACP activity and ALP activity both changed significantly at three copper concentrations compared with the control. It was suggested that ACP and ALP in gill, like the kidney, were more sensitive to copper. In addition, ACP activity was inhibited by all copper exposures, but ALP activity was induced at low copper exposures and inhibited at high copper exposures. This result was at odds with the previous literatures which showed that ACP and ALP activities in gill were all inhibited after exposure to some contaminants (Karuppasamy, 2000; Bhavan and Geraldine, 2004). This discrepancy could be due to the difference of contaminant type and fish species.

Spleen plays an important role in immune protection in fish. In this study, ACP and ALP activities in spleen were significantly inhibited at all copper concentrations, and the significant negative correlation was found between copper exposure concentration and enzymatic activity. Nevertheless, there are few literatures to our knowledge about the ACP and ALP activities in fish spleen. The data in this study suggested that ACP and ALP in spleen were the most sensitive to copper exposure among the organs/tissues. This sensitivity may be related to the immune function of spleen, because the immune system is vital for the fish to prevent from infectious agents and immune impairment by environmental pollutants (Spromberg and Meador, 2005).

Fish muscle is an important tissue to conduct movement. Contractile proteins are rich in muscle and have a high affinity for calcium and a low affinity for heavy metals (Palaniappan and Karthikeyan, 2009). Thus, fish muscle usually has the lower accumulation ability to heavy metals. In addition, fish muscle is consumed by the general public as food. Owing to consumers healthy demand, metal bioaccumulation of fish muscle has been paid more attentions and studied extensively by many investigators (Storelli et al., 2006; Ploetz et al., 2007; Agah et al., 2009; Palaniappan and Karthikeyan, 2009). The results from these studies further confirmed that muscle had the lowest accumulation level for the most of the heavy metals among the organs/tissues in fish. In the present study, copper has little effect on ACP activity and no effect on ALP activity in muscle of *Carassius auratus gibelio var*, it was suggested to be associated with the lower copper bioaccumulation of this fish muscle.

Fish brain is the major component of the central nervous system and the main target of the pollutants (Mieiro et al., 2011). Water contaminants can effect the activities of various enzymes in brain (Bagnyukova et al., 2005; Modesto and Martinez, 2010) and even cause the neurodegenerative damage (Berntssen et al., 2003) by passing through the fish blood-brain barrier into the brain tissue. In this study, the increased ACP activity and the decreased ALP activity after an 96-h copper exposure were observed in the brain of *Carassius auratus gibelio var*. This result is in agreement with the elevation of ACP activity and the inhibition of ALP activity in brain of *Channa punctatus* after 96-h $HgCl_2$ exposure as reported earlier by Sastry and Sharma (1980). The functional significance of ACP and ALP in brain is involved in various secretory and transport processes, and the ALP also involved in blood-brain barrier mechanisms (Shaffi, 1979). The alterations in the both phosphatases activities in the present study indicate the copper-induced disturbances in the normal functioning of the brain of *Carassius auratus gibelio var*.

Studies of contaminant-induced alterations in phosphatase activities reported that the changes in ACP and ALP activities could damage the cells and organs/tissues and adversely affect their physiological

functions (Butterworth and Moss, 1966; Ramalingam and Vimaladevi, 2002; Akanji et al., 2008). In the present study, in the group of 2.0 mg/L exposure, after 96-h copper exposure, ACP and ALP activities in six organs/tissues all significantly changed except ALP activity in muscle. This showed the membranes of lysosomes and plasma have been heavy disrupted by copper at this high copper concentration. ACP activity significantly decreased in kidney, liver, gill and spleen, this decrease reflected the damage of lysosomes by copper, and the injured lysosomes would release hydrolytic enzymes into cytoplasm leading to auto degradation of cellular proteins and subsequent cell necrosis (Kågedal et al., 2001). ACP activity significantly increased in muscle and brain, this could result in indiscriminate hydrolysis of phosphate esters (Butterworth and Moss, 1966) and consequently autolysis and cell death which constitute a possible threat to the well being of the organs (Akanji et al., 2008). ALP activity significantly decreased in kidney, liver, gill, spleen and brain, such reduction would hinder adequate transportation of required ions or molecules across their cell membrane (Akanji et al., 1993) and also adversely affect other metabolic processes where the enzyme is involved such as the synthesis of nuclear proteins, nucleic acids, phospholipids and cleavage of phosphate esters (Ramalingam and Vimaladevi, 2002). However, the both enzymatic activities in the six organs/tissues all could normalize within 12 days after copper was removed, it was demonstrated that the copper toxicity on the lysosomal and the plasma membrane was transient and the enzymatic activities were reversible.

In recovery process, the recovery speed of ACP and ALP activities in different organs/tissues was different. ACP activity in gill and ALP activity in brain normalized on the first day in the fastest speed. ACP activities in liver, muscle and brain, and ALP activity in kidney normalized on the twelfth day in the slowest speed. Such discrepancy is probably related to the different regulation mechanisms of the organs/tissues. Moreover, the enzymes in differernt organs/tissues exhibited different recovery pattern. After 96-h copper exposure, ACP activities in kidney and gill, and ALP activities in kidney, liver, spleen and brain significantly decreased compared with the control, but after copper exposure was removed, enzyme activities increased continuously compared with the 96-h exposure group, which showed the consistency between the change of enzymes activities and the elimination of copper. ACP activities in muscle and brain increased significantly compared with the control after 96-h copper exposure. After copper exposure was free, they did not decrease immediately compared with the 96-h exposure group, but increased continuously from Day 1 to Day 8. The Reason might be that the higher level of acid phosphatase mRNA made the enzyme synthesis continue, although environmental factor to induce ACP activity had been eliminated. After 96-h copper exposure, ACP activity in liver and spleen, and ALP activity in gill significantly decreased compared with the control, but after copper exposure was removed, enzyme activities decreased firstly and then increased compared with 96-h copper exposure. It is difficult to explain these changes, so the further studies still need to be carried out to provide the more evidences.

In summary, our study unambiguously demonstrates for the first time that ACP and ALP in spleen of *Carassius auratus gibelio var* are very sensitive to copper stress and can be accepted as sensitive biomarkers to assess copper contamination in aquatic ecology. This study also suggests the changes of ACP and ALP activities in different organs/tissues of *Carassius auratus gibelio var* exposed to copper are different, but the changes of the both enzyme activities in the highest copper concentration group (2.0mg/L) are recoverable, and the different recovery pattern in differernt organs /tissues may be due to the physiological regulation of these organs/tissues and the enzyme complement mechanism at the cellular level. However, the more experiments are still required to carried out to provide the more evidences to better understand the response mechanism of ACP and ALP activities in fish to copper exposures and recovery process.

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