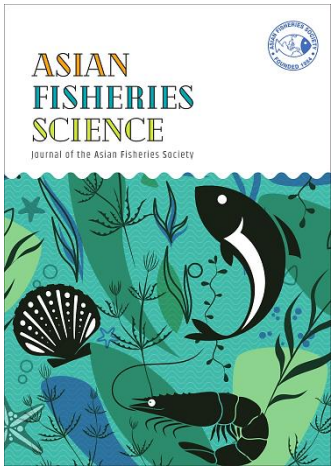

The Reduction of $CuSO_4$ Toxicity in Common Carp (*Cyprinus carpio* Linnaeus, 1758) After Pre-exposure to $CaCO_3$

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ISSN: 0116-6514
E-ISSN: 2073-3720
https://doi.org/10.33997/j.afs.2019.32.3.003

Abstract

Appropriate usage of $CuSO_4$ as a herbicide with no toxicity mainly depends on water calcium hardness. This study aims to investigate the effect of initial exposure to $CaCO_3$ for the reduction of $CuSO_4$ toxicity in *Cyprinus carpio* (Linnaeus, 1758). One group of 300 fish (6 ± 0.05 g) were placed in a tank containing 200 $mg.L^{-1}$ of $CaCO_3$ for 2 weeks while another group of 300 fish was maintained at normal conditions. All 600 fish were then exposed to $CuSO_4$ at 0.0, 1.5, and 3.0 $mg.L^{-1}$ for 60 days so that the fish weight, length and condition factor decreased significantly at increasing $CuSo_4$ with higher values seen in the fish pre-exposed to calcium ($P < 0.01$). The highest mortality was observed in 3 $mg.L^{-1}$ of copper treatment without calcium exposure. The feed conversion ratio was elevated with an increase copper concentration. Also, the red and white blood cells, haemoglobin, and haematocrit decreased significantly ($P < 0.05$) at increasing copper concentration in the group that were pre-exposed to calcium. The amount of copper in muscle was elevated at increasing copper concentration, but was less in the group pre-exposed to $CaCO_3$. Thus, pre-exposure to $CaCO_3$ could reduce copper toxicity, especially at low concentrations of $CuSO_4$.

Keywords: calcium hardness, common carp, copper accumulation

Introduction

Copper sulphate ($CuSO_4$) has been widely used as a herbicide in fish ponds to control phytoplankton and filamentous algae growth and single-celled pathogens as well as to restrict some specific diseases (Boyd 1990). Using $CuSO_4$ in preventing the growth of new phytoplankton can result in copper accumulation reaching toxic levels. When the copper concentration exceeds in the environment or continually enters the pond, it is absorbed by the fish and stored in organs such as muscle, liver and kidneys. This process can develop a chronic form of poisoning and, if continued, clinical signs of this type of poisoning appear (Abdel-Tawwab et al. 2007). Therefore, bioaccumulation of heavy metals such as copper in fish can make their consumption unsafe for humans (Marchetti 2013).

Based on previous reports, the influence of copper in aquaculture is complicated, depending on the physico-chemical characteristics of water (Takasusuki et al. 2004). For appropriate usage of $CuSO_4$ with no toxic effect will depend on water hardness and alkaline condition (Wurts and Perschbacher 1994). Moreover, calcium hardness has significant effects on copper toxicity (Perschbacher and Wurts 1999) such that high calcium levels in the water as a component of hardness may reduce the toxic effects of copper. Due to higher toxicity of $CuSO_4$ in soft water compared to hard water, precaution should be taken before use of $CuSO_4$ in soft water (Wurts and Perschbacher 1994). The reduction of copper toxicity in the aquatic environment can be achieved by adding calcium which will improve the ion exchange in the gills thus overcoming the destructive effects of copper (Abdel-Tawwab et al. 2007). Perschbacher and Wurts (1999) showed that in the channel catfish, *Ictalurus punctatus* (Rafinesque, 1818), the hardness of calcium was more efficient in decreasing copper intoxication than the hardness of magnesium. In *Prochilodus scrofa* (Steindachner, 1881), 10 °C increase of temperature caused higher toxicity of copper (Carvalho et al. 2004). In the channel catfish,

$CuSO_4$ is used more frequently in the fish ponds during spring while the toxicity of copper sulphate may increase with increase of temperature. According to Bat et al. (2000) report, the toxicity of metals was increased in freshwater invertebrates at a temperature range of 10 to 30 °C.

Several studies have examined the influence of initial contact with calcium on the reduction of heavy metal in fish. Abdel-Tawwab and Mousa (2005) and also Abdel-Tawwab et al. (2007) illustrated the positive effect of calcium pre-exposure on copper toxicity in Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758). Moreover, Song et al. (2013) indicated the effect of calcium pre-exposure on cadmium toxicity in *Synechogobius hasta* (Temminck & Schlegel, 1845) while Chen et al. (2012) reported calcium effect on the copper accumulation in several organs of *Pelteobagrus fulvidraco* (Richardson, 1846). They also demonstrated that calcium pre-exposure reduced copper toxicity in that species (Chen et al. 2012). Singhadach et al. (2009) showed that calcium pre-exposure reduced histopathological alteration in Nile tilapia, *O. niloticus*. In addition, Gill and Epple (1992) indicated the reduction of cadmium toxicity in water with high calcium concentration (200 $mg.L^{-1}$ $CaCO_3$) and with calcium pre-exposure which prolonged the survival of *Fundulus heteroclitus* (Linnaeus, 1766). The present study investigates the effect of exposing constant dose of $CaCO_3$ to different copper concentrations of water on the growth and feeding parameters, blood factors and copper accumulation in common carp.

Materials and Methods

Experimental design

A total of 600 carp fish (6.00 ± 0.05 g) were obtained from the Jahad Daneshgahi's fish farm, transferred to the Aquaculture Research Center (Chapakrood, Jooybar, Iran), and maintained in aquaria for 1-week. Three hundred of them were placed in an aquarium containing 200 $mg.L^{-1}$ of $CaCO_3$ (Merck, Germany) for 2 weeks while the rest of them were kept in normal conditions for the same period. Afterwards, all fish were exposed to 0.0, 1.5, and 3.0 $mg.L^{-1}$ of $CuSO_4$ for 60 days (Table 1). As the next step, six treatments were applied; each consisted of three replicates, and each replication included 20 fish. The fish were stocked randomly in 18 aquariums (100 × 40 × 40 cm) with 140 L of water.

Table 1. Sample treatments exposure (+Ca) or non-exposure (-Ca) to $CaCO_3$ at different concentrations of $CuSO_4$ in common carp *Cyprinus carpio* for 60 days.

Treatments	T1	T2	T3	T4	T5	T6
Ca exposure	-	-	-	+	+	+
$CuSO_4$ ($mg.L^{-1}$)	0	1.5	3.0	0	1.5	3.0

The fish were fed three times a day during the experiment. The food (Behdaneh, Iran) included 32.23 % crude protein, 5.5 % total fat, 8 % ash and 3 % fibre. Dead fish were daily removed from each aquarium and recorded and finally, the growth and feeding parameters were measured (Abdel-Tawwab et al. 2007).

Water physico-chemical parameters

The physico-chemical parameters of water are shown in Table 2.

Growth and feeding parameters

Body weight gain (g) = Final weight – Initial weight

Total length gain (mm) = Final length – Initial length

Condition factor (CF, %) = $[W.L^{-3}] \times 100$
Where, W = Fish weight (g) and L = Fish total length (cm)

Mortality (%) = (Dead fry/Total fish) × 100

Feed conversion ratio (FCR) = Feed given (g)/Weight gain (g)

Blood parameters

At the end of the experiment, five fish from each treatment were anaesthetised by 1 $mg.L^{-1}$ of clove extract and then, the blood samples were taken from the caudal peduncle (Farhadi et al. 2014) and stored in sterile tubes containing EDTA for blood cells evaluations (Mckenzie and Williams 2014). The red and white blood cells (RBC and WBC) were counted by Neubauer haemocytometer (Blaxhall and Daisley 1973) while blood haematocrit levels were measured through microhaematocrit method (Rehulka 2003). Blood haemoglobin was also determined by a kit and spectrophotometer (Unico, USA) at the wave length of 540 nm (Blaxhall and Daisley 1973).

Heavy metal in fish flesh

Three samples were randomly selected from each replicate and kept in the laboratory at -20 °C for 5 days. To digest frozen flesh, the fish were placed in a dryer for 48 h until they completely dried. Dried tissues were separately ground by a meat grinder (Pars Khazar, MG 1400, Iran) and powdered by electric mill (Pars Khazar, Iran). A 0.2 g of each powder was then poured into the test tubes separately. After adding nitric acid (5 mL), all samples were placed in ambient temperature for 1 h and were completely digested on a hot plate at 100 °C for 3 h. After cooling down, the volume of all samples reached 25 mL by

Table 2. Physico-chemical parameters of water of aquaria stocked by common carp *Cyprinus carpio* exposed to different copper concentrations for 60 days with and without calcium pre-exposure.

Parameters	T (°C)	pH	DO (ppm)	Total alkaline condition (mg.L^{-1} $CaCO_3$)	Total hardness (mg.L^{-1} $CaCO_3$)	Ca (mg.L^{-1})	Mg (mg.L^{-1})	Cu (mg.L^{-1})
Amount	22 ± 1.00	7.5 ± 0.30	13.11 ± 0.19	140.2 ± 0.61	159 ± 0.18	144.00	115.20	0.12

adding distilled water (Okoye 1991). In order to estimate the amount of copper, all samples were filtered by acetate cellulose filter (0.2 µ). The concentrations of heavy metals in all samples were evaluated by an atomic absorption device (Model ICP-OES) and were calculated as:

$C = (Gs \times V)/ W$
where:
C: metal concentration in solid sample (mg.kg^{-1})
Gs: metal concentration in solution from digestion (ppm)
V: dilution volume (mL)
W: dry weight of sample (g)

Statistical analysis

The data of completely randomised block experiment were analysed by one-way ANOVA and were compared by Duncan's test using SPSS software. A confidence level of 99 % was determined for the amount of copper, the growth, feeding, and mortality parameters. For blood factors, however, the confidence level was 95 %.

Results

The effect of exposure to $CaCO_3$ and copper sulphate on growth and feeding parameters

The results showed that increasing copper concentration reduced the weight and length of fish with different behaviour in each treatment ($P < 0.01$). As can be seen in Table 3, the calcium-exposed fish had higher value of both length and weight. Also, by increasing copper in both groups of fish, CF decreased with a significant fall at 1.5 and 3.0 mg.L^{-1} of copper in the first group (without pre-exposure to $CaCO_3$) ($P < 0.01$). Moreover, by increasing copper concentrations, FCR increased so that less value of FCR was observed in calcium treated fish significantly (This was also obvious from comparing T2 and T3 to T4 and T6, respectively) ($P < 0.01$). The minimum value of FCR (2.31 ± 0.028) was achieved in T4 while T3 had the highest value (5.5 ± 0.566). Except T1, T4 and T5 treatments which had no mortalities, increasing copper in other treatments increased the number of mortalities. This fact led to significant differences between T2 and T3 and also T5 and T6 treatments ($P < 0.01$, Table 3). In addition, the number of mortalities in fish exposed to $CaCO_3$ (T5, T6) was less than those without any calcium exposure (T2, T3).

The effect of exposure to $CaCO_3$ and copper sulphate on haematological parameters

Decreasing haemoglobin level at increasing copper concentration resulted in significant differences between fish with and without exposure to calcium ($P < 0.05$, Table 4). Higher haemoglobin amount was seen in the fish exposed to $CaCO_3$. Moreover, decrease in haematocrit amount due to increase copper concentration showed statistical differences between fish with (T4, T5, and T6) and without (T1, T2, and T3) exposure to calcium. Although increasing copper levels led to haematocrit fall of in both groups, it was higher in fish exposure to calcium. In addition, the number of RBCs declined by adding copper in water, especially in the groups without calcium. Meanwhile, those fish which were treated with calcium showed a higher number of RBCs (Table 4). Also, WBCs decreased by adding copper so that its maximum value was observed in the fish with calcium (Table 4).

The effect of exposure to $CaCO_3$ and copper sulphate on copper concentration in muscle tissue

The amount of copper in the fish muscle was zero in T1 and T4 treatments and it increased at an increasing copper concentration in water from 0.00 mg.kg^{-1} in the control group to 0.12 ± 0.002 mg.kg^{-1} and 0.11 ± 0.00 mg.kg^{-1} in T3 and T6 treatments, respectively. Furthermore, significant differences were observed between T2 and T5 in 1.50 mg.L^{-1} and T3 and T6 in 3 mg.L^{-1} concentration of copper sulphate ($P < 0.01$) (Fig. 1).

Discussion

According to the results of the present study, greater weight, length and condition factor were achieved when the fish received calcium treatment prior to high copper concentration exposure. Moreover, comparing the growth of fish in T4, T5, and T6 to T1, T2, and T3 demonstrated that because of the reduction of copper toxicity, increasing calcium

Table 3. The growth and feeding parameters of common carp *Cyprinus carpio* fry exposed to different copper concentrations after 60 days.

	Without Ca^{+2}			With Ca^{+2}		
$CuSo_4$ (mg.L^{-1})	0	1.5	3	0	1.5	3
Treatment	T1	T2	T3	T4	T5	T6
Weight gain (g)	9.44 ± 0.326^{e}	5.67 ± 0.297^{c}	1.83 ± 0.287^{a}	11.55 ± 0.261^{f}	8.48 ± 0.248^{d}	2.74 ± 0.229^{b}
Length gain (mm)	50.84 ± 2.492^{e}	34.87 ± 2.066^{c}	18.74 ± 3.032^{a}	53.14 ± 1.882^{f}	44.31 ± 1.660^{d}	21.39 ± 1.731^{b}
Condition factor (%)	1.45 ± 0.026^{c}	1.29 ± 0.097^{ab}	1.20 ± 0.054^{a}	1.44 ± 0.044^{c}	1.43 ± 0.037^{c}	1.39 ± 0.031^{bc}
Mortality (%)	0.00 ± 0.00^{a}	4.45 ± 1.851^{b}	22.22 ± 1.851^{d}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	11.11 ± 1.845^{c}
Feed conversion ratio	2.61 ± 0.038^{a}	3.52 ± 0.052^{b}	5.5 ± 0.566^{d}	2.31 ± 0.028^{a}	2.75 ± 0.049^{a}	4.86 ± 0.232^{c}

* Values in the rows having similar superscript letters are not statistically different ($P > 0.01$)

Table 4. Haematological parameters of common carp *Cyprinus carpio* fry exposed to different copper concentrations after 60 days.

	Without Ca^{+2}			With Ca^{+2}		
$CuSo_4$ (mg.L^{-1})	0	1.5	3	0	1.5	3
Treatment	T1	T2	T3	T4	T5	T6
Hb (g.dL^{-1})	5.85 ± 0.153^{b}	5.83 ± 0.114^{b}	5.15 ± 0.094^{a}	9.55 ± 0.661^{d}	8.88 ± 0.078^{c}	8.4 ± 0.285^{c}
HCT (%)	41 ± 5.043^{c}	33.53 ± 1.885^{a}	32.27 ± 2.404^{a}	63 ± 6.199^{d}	40.8 ± 5.894^{c}	36.87 ± 1.685^{b}
RBC (10^{6}.mm^{-3})	92915.732 ± 1.47^{d}	25166.115 ± 1.43^{b}	20000 ± 1.40^{a}	140414.52 ± 3.56^{f}	105039.675 ± 2.35^{e}	30550.505 ± 1.68^{c}
WBC (n.mm^{-3})	6420 ± 1400^{bc}	6020 ± 600^{bc}	3820 ± 400^{a}	7220 ± 200^{c}	6020 ± 1000^{bc}	4820 ± 1000^{ab}

* Values in the rows having similar superscript letters are not statistically different ($P > 0.05$).

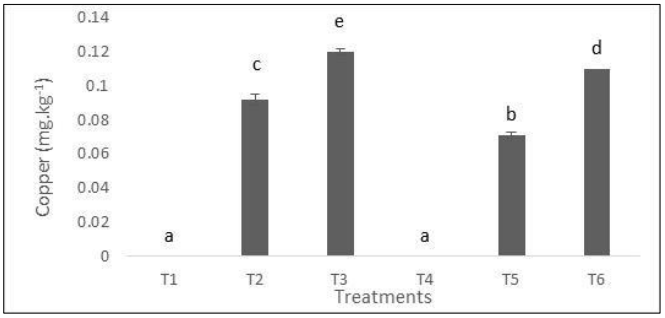

Fig. 1. The amount of copper in the muscle tissue of common carp *Cyprinus carpio* fry exposed to different copper concentrations after 60 days.

improved the growth of fish. According to Matsuo et al. (2004), binding gill surface with calcium and its permeability along with its competition with binding gill surface with copper occurred simultaneously. It should be noted that obtained results in common carp are in agreement with those of Farhadi et al. (2014) who reported the reduction of carp's weight after increasing copper level in Ca (OH) $_2$ treatment. Spry and Wiener (1991) found a reverse relationship between copper toxicity and calcium concentration in water. Moreover, Dutta and Kaviraj (1996) reported that by exposing *C. carpio* to 100 mg.L^{-1} of quicklime, the LC50 of cadmium increased from 165 to 235 mg.L^{-1}. Furthermore, Abdel-Tawwab and Mousa (2005) illustrated that adding calcium (50-100 mg.L^{-1}) could affect the mitigation of acute copper poisoning in Nile tilapia, *O. niloticus* larvae. These consequences might arise from the fact that environmental pollutants resulted in a decrease in fish appetite and growth, which caused the fish body to store less nutrients leading to growth retardation.

The percentage of fish mortality increased with increase copper concentration. As can be seen, the percentage of mortality in T6 was half of T3 which confirmed the effect of calcium on copper poisoning, especially at low concentrations of copper sulphate (1.5 mg.L^{-1}). The results indicated the protective role of calcium carbonate against copper toxicity which was more significant in low copper concentration because although the above protocol improved their growth in 3 mg.L^{-1} of copper sulphate concentration, it could not improve their survival rate. In addition, mortality might be due to a significant decrease in the number of RBCs, mainly in T3 and T6 treatments which caused respiratory restrictions and anaemia, especially in weak fish (Thomas and Egee 1998). Perschbacher and Wurts (1999) reported that in *I. punctatus* fry, fish mortality decreased from 90 % in 10 mg.L^{-1} of $CaCO_3$ to 5 % in 400 mg.L^{-1} of $CaCO_3$. The reason is that when they experienced 1.25 mg.L^{-1} of $CuSO_4$ for 48 h, the hardness of water increased. They also estimated lower fish mortality by increasing

water hardness caused by calcium than magnesium. Furthermore, the results of the present study were similar to Song et al. (2013) whereby in the same calcium concentration, the survival rate of *S. hasta* decreased at increasing cadmium concentration of water. However, by increasing calcium concentration of water, the survival rate also increased. In addition, the toxicity of cadmium in *F. heteroclitus* was lowered in water with high calcium concentration (200 $mg.L^{-1}$ $CaCO_3$) while pre-exposure to calcium prolonged the survival rate in cadmium- waterborne (Gill and Epple 1992). Also, in common carp, no mortalities were observed due to the effect of $Ca(OH)_2$ on copper toxicity (Farhadi et al. 2014). Carvalho et al. (2004) reported that by increasing $CuSO_4$, the death rate of *Prochilodus scrofa* (Steindachner, 1881) increased. The worse fact was that by increasing temperature from 20 to 30 °C at pH of 4.5, the death rate increased because of a thermal change. However, at pH of 8.0, the death rates at 20 and 30 °C were not different significantly. Also, Nile tilapia without calcium treatment suffered greater mortality (Abdel-Tawwab et al. 2006).

In this study, increase of copper levels resulted in higher values of FCR with less effect in the fish exposed to $CaCO_3$, suggesting calcium role in intoxication. Accordingly, the effect of calcium in low dosage (1.5 $mg.L^{-1}$) of copper was more sensitive than its high dosage (3 $mg.L^{-1}$). It could be also demonstrated that the initial stocking duration (14 days) of fish in the calcic water was not sufficient and hence, more pre-exposure times were required. Also, the FCR values in Nile tilapia were boosted with an increase of copper levels which was more considerable in fish lacking calcium exposure (Abdel-Tawwab et al. 2007).

The study of haematological parameters is a suitable method for assessing the health status of fish, especially in stressful conditions such as heavy metals exposure. Different dosages of copper were examined in this study and confirmed a reductive effect of copper on RBCs, leading to anaemia and decrease of haematocrit and haemoglobin. A similar pattern was noted in the catfish *Heteropeneustes fossilis* (Bloch, 1794) which was affected by $CuSO_4$ and resulted in anaemia by increasing RBCs destruction and preventing RBCs production (James and Sampath 1995).The reduction of RBCs number and haemoglobin and haematocrit amounts in concentrations of more than 0.15 $mg.L^{-1}$ of copper in *Channa punctatus* (Bloch, 1793) were reported previously (Singh et al. 2008). The decrease of the number of RBCs might be due to either their accumulation in the fish gills or their death because of exposure to pollutants which reduced haemoglobin and haematocrit levels (Kudirat-Adeyemo 2007). Some studies, however, reported reverse consequences. In *P. scrofa*, for instance, copper exposure caused the increase of RBCs, haematocrit and haemoglobin (Carvalho and Fernandes 2006) because of the discharge of RBCs from the reserve storages such as the spleen at the constant volume of the cells.

Increasing copper concentration reduced haemoglobin and haematocrit in the fish blood. Compared to another group of fish, the fish with calcium treatments had higher haemoglobin levels confirming the role of $CaCO_3$ in copper intoxication. In addition, decreasing the number of WBCs by increasing copper, particularly in the group of samples without calcium treatment, could be a sign of excessive stress in the fish. It was illustrated that in non-treated fish with the addition of 0.5 $mg.L^{-1}$ $CuSo_4$ and the fish subjected to calcium, the values of haematocrit, RBCs, and haemoglobin increased (Abdel-Tawwab et al. 2006). Yadegar et al. (2011) observed decreasing trends of RBCs, WBCs, haematocrit, and haemoglobin in *Barbus sharpeyi* (Günther, 1874) treated with 0.5, 1, 2, 5, and 10 ppm of $CuSO_4$ for 1-week. The values of these blood parameters in the control group were more than others. Duplication of WBCs was a normal response of fish against the invasion of external substances such as copper which could disturb their normal physiological functions. The roles of WBCs in regulating immune activities of organisms have also been reported by Iwama and Nakanishi (1997). Further researches indicated that despite the existence of $CuSO_4$, intensive stress in alkaline pH resulted in decreasing WBCs (Wurts and Perchbacher 1994).

Due to the considerable role of the fish muscle in human nutrition and the need for ensuring its health, the fish muscle was the primary concern of this study. By increasing copper sulphate concentration, the amount of copper increased in muscle significantly such that copper amount in T6 was less than T3. Calcium kept binding sites highly saturated and prevented copper from attaching and transferring from gills to blood. Dutta and Kaviraj (1996) reported that when fish is exposed to calcium, the uptake of cadmium from water and its transfer from gill to blood were reduced. Moreover, Song et al. (2013) reported that in *S. hasta,* calcium pre-exposure tends to decrease the amount of cadmium in intestine and gill but not in muscle and liver which was not the same as the result of the present study. Also, Farhadi et al. (2014) reported that pre-exposure to CaO had no significant effect on the amount of copper in the muscles of common carp which was not in agreement with the results of the present study. The reason for this difference might be the difference in the time of calcium pre-exposure.

Conclusion

The present study showed that when common carp was pre-exposed to calcium carbonate, it could improve the growth, feeding, and haematological parameters in the fish. In addition, calcium reduced the amount of copper in the muscle of the fish.

According to the percentage of mortality obtained in this study, it is more appropriate to protect fish in lower concentrations of copper sulphate (up to 1.5 $mg.L^{-1}$). In addition, if there is a need to treat fish with an increased concentration of copper sulphate, it is suggested that the pre-exposure amount of calcium carbonate or pre-exposure time to calcium carbonate should be increased.

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