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THE EFFECT OF SUB-LETHAL LEVEL OF DIETARY COPPER AND CADMIUM ON THE GROWTH PERFORMANCE AND OXIDATIVE STRESS IN *Cirrhina mrigala*

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Heavy metals are common aquatic pollutants and their importance is increasing due to anthropogenic activities. These pollutants reduce fish metabolic rate and hence reduce their growth. Heavy metals produce reactive oxygen species which cause oxidative stress. The fish, *Cirrhina mrigala* was given the sub-lethal concentrations of copper and cadmium in the diet to check growth performance and oxidative stress. Three diets (2% of body weight) were fed to fish on daily basis to satiation. The diet # 1 and 2 were sub-lethal doses of copper and cadmium while diet # 3 was without metal. The weight gain in fish fed with diet 1 and 2 was significantly lower than that of the fish that was fed with diet # 3. The similar trend was observed for gain in fish fork and total length. The fish feeding behavior was significantly better on diet # 3 as compared to diet # 1 and 2 in the test mediums. The feed conversion ratio and condition factor of fish varied significantly among treatments. Specific growth rate in treated mediums (T_1 and T_2) decreased as compared to control medium. The enzyme peroxidase activity was maximum in liver of fish in control group and minimum in kidney of fish fed with copper containing diet. The activities of peroxidase and catalase in fish organs and treatments were significant. The highest superoxide dismutase activity in the liver of copper fed fish and least in kidney of control fish were recorded. Results indicated that heavy metals (copper and cadmium) reduced the growth and increased the oxidative stress in fish.
Keywords: Fish, heavy metal, sub-lethal exposure, oxidative stress, aquatic ecosystem

INTRODUCTION

Natural water bodies consist of various types of impurities which differ in amount and nature in different water sources. Metals get into aquatic systems via a number of processes including, dissolution of aerosol particles from the atmosphere, weathering of rocks, leaching of soils and from various human activities (Asaolu *et al*., 1997). In agricultural revolution, increase in the use of metal-based fertilizer could cause rise in the concentration of metal pollution in fresh water reservoir as a result of water run-off (Adewoye, 1998).
Heavy metals in aquatic ecosystem are either accumulated in the sediments or in the aquatic organisms (Matagi *et al*., 1998). Among pollutants, the heavy metals are thought to be most dangerous at global level because of their toxicity (Vuren *et al.,* 1999; Tawfik, 2013; Batool *et al*., 2014). Heavy metals contamination usually showed less food consumption by fish (Vincent *et al.,* 2002).
Fish which are mostly at the peak of aquatic food chain may accumulate metals in their bodies from the water (Mansour and Sidky, 2002). Growth rates are also affected by heavy metals in major carps (Hayat *et al.,* 2007). Any such resentment could result in decreased metabolic rate of fish and automatically decreased their growth (Sarnowski, 2003). Fish exhibits reduction in weight in metal polluted waters. Similar trends like weight escalations are also shown by average fork and total length (Javed, 2006).
Oxidative stress is described as a disruption of the antioxidant balance in favor of the former, leading to potential damage (Sies, 1991). It results from one of these factors: (1) an increase in ROS (reactive oxygen species) (2) male functioning of antioxidant defense system (3) inability to repair oxidative damage. Redox homeostasis is maintained in organisms by antioxidant defense system. Reactive oxygen species and oxygen free radicals can react with the main components of cell, thus causing oxidative stress, by damaging tissues which includes oxidation of DNA, protein, as well as peroxidation of unsaturated lipids in the cell membranes (Swann *et al*., 1991).
Peroxidase has been postulated to protect the erythrocytes from damage by H_2O_2. Therefore, it is hypothesized that this enzyme may protect tissues against oxidative damage due to lipid peroxidation. Environmental pollutants may enhance the peroxidase activity (Li *et al*., 2003). Catalase is a primary antioxidant defense element that protects the fish from harmful effects of oxidative stress by changing the hydrogen peroxide (H_2O_2) to water and oxygen (Bernet *et al*., 2001). Superoxide dismutase is involved in the reduction of superoxide radicals into H_2O_2 (hydrogen peroxide) which is converted by catalase into water and oxygen (Sanchez *et al*.,

2005). The activities of superoxide dismutase may be affected by a variety of environmental factors such as salinity, temperature, age, feeding habits and season (Rocha *et al*., 2003).

Contamination of aquatic environments with heavy metals likewise cadmium (Cd) is a global problem due to the stability and continuing addition of these elements. Cadmium is a non-essential toxic metal usually detected in terrestrial and aquatic environments where it is discharged from both anthropogenic and natural sources, including mining, agricultural and industrial activities (Burger, 2008). Copper (Cu) is an essential trace element that is needed in little amounts by fish and shellfish, humans and other mammals for the functioning of greater than 30 enzymes and the carbohydrate metabolism. However, the concentrations of copper can be toxic when they rise above twenty micrograms per gram. The effects of copper on aquatic organisms can be directly or indirectly lethal (Bradl, 2005). The present project was planned to study the effect of sub-lethal level of dietary copper and cadmium on growth performance and oxidative stress in *Cirrhina mrigala*.

MATERIALS AND METHODS

A 90-day experiment was conducted at fisheries research farm, University of Agriculture, Faisalabad to study the effect of sub-lethal level of dietary copper and cadmium on growth performance and oxidative stress in *Cirrhina mrigala*. For acclimation prior to the experiment, the fish fingerlings were kept in the laboratory for 14 days in 500 liter cemented tanks. The growth parameters viz. wet body weight; fork and total lengths of fingerlings were measured and recorded at stocking and at the end of the experiment. After acclimation period, fish were transferred to 70-liter glass aquaria to study growth performance and oxidative stress. Each tank stocked with twenty fish. The water temperature (30ºC), pH (7.0) and total hardness (200 mg L^{-1}) maintained throughout the experimental period. However, calcium, magnesium, sodium, potassium, total ammonia, carbondioxide and electrical conductivity were measured on daily basis by following the method described in APHA (1998).

Chemically extra pure chloride compounds of copper and cadmium were used to prepare stock solutions of desired dilution. The sub-lethal (1/3 of LD_{50}) dietary copper and cadmium dose used for *Cirrhina mrigala* were 50.17 and 48.39 µg g^{-1} in T_1 and T_2, respectively (Yaqub and Javed, 2012). While, in control aquarium metal free diet was given to the fish. The fish were fed to satiation daily at 09:00 am and 17:00 p.m. hours with the experimental feeds. During sub-lethal exposure of dietary copper and cadmium the feed intake, increase or decrease in average wet weight, fork and total length, feed conversion ratio, condition factor and specific growth rate of the fish were calculated and recorded. After collecting data, fish were released back into their respective aquarium.

Table 1. Percent feed composition of fish

Sr.#	Ingredients	Percentage
1	Fish meal	50.00
2	Corn gluten(30%CP)	34.27
3	Rice polish	05.51
4	Wheat flour	03.00
5	Oil (sun flower)	03.22
6	Vitamin and mineral mixture	04.00

Digestible protein (DP) =40%

Digestible energy (DE) =3.10 K $calg^{-1}$

Water temperature and dissolved oxygen were measured and recorded by electronic meter HANNA HI-9146 while pH and electrical conductivity were measured by the digital meters WTW inolab. However, total ammonia, hardness, calcium, magnesium, CO_2 were measured by following the method of APHA (1998).

After growth trial of 90 days, fish from all three groups were dissected and organs viz. liver, gills and kidney were used to analyze antioxidant enzymes viz. peroxidase, catalase and superoxide dismutase. To remove the blood the dissected organs were rinsed with phosphate buffer having pH 6.5 (0.2 M) and the organs were homogenized in cold buffer (1:4 w/v) using a blender. After the homogenization, organ homogenates were centrifuged for 15 minutes at 10,000 rpm and at 4ºC temperature. After centrifugation process, clear supernatants of liver, gills and kidney were stored at -80 ºC for enzyme assay while residue was discarded.

***Assay of peroxidase*:** The activity of peroxidase was determined by measuring its ability to reduce the concentration of H_2O_2 at A_{470} nm (Civello *et al*., 1995).A cuvette containing the 3 ml of blank solution was placed into the spectrophotometer and set it to zero at wavelength of 470 nm. In a cuvette containing buffered substrate solution (0.2 M phosphate buffer, pH 6.5, 750 µL Guaiacol, 0.3 ml Hydrogen peroxide) 0.06 ml of enzyme extract was added and put into the spectrophotometer. The reaction time was 3 minutes and so the absorbance was noted after 3 minutes.

***Assay of catalase*:** Catalase activity was determined by its ability to decrease the H_2O_2 concentration at 240 nm (Chance and Mehaly, 1977). A cuvette containing the 2 ml of blank solution (buffer) was placed into the spectrophotometer and set it to zero at wavelength of 240 nm. In a cuvette containing buffered substrate solution (60 mM sodium phosphate buffer, pH 7.0, 10 mM hydrogen peroxide) 0.05 ml of enzyme extract was added and put into the spectrophotometer. The reaction time was 3 minutes and the absorbance was noted after interval of 1 minute.

***Assay of superoxide dismutase*:** The activity of superoxide dismutase was determined by measuring its ability to inhibit the photo reduction of Nitroblue tetrazole (NBT) following

the method of Giannopolitis and Ries (1977). 1 ml buffer was taken in cuvette as blank and placed into spectrophotometer to note the readings of blank, after taking reading spectrophotometer was adjusted to zero at A_{560} nm. Then 5-6 cuvettes were taken and set in a light box with an internally mounted light bulb of 30 Watt.

One ml of buffer (0.067 mM, pH 7.8), 0.05 ml enzyme extract and 0.016 ml of riboflavin (0.12 mM) was added in each cuvette. All the cuvettes were incubated in light box for 12 minutes. In cuvettes containing illuminated reaction mixture 0.067 ml of EDTA/NaCN solution (0.1 M/0.3 mM) and 0.033 ml of NBT (1.5 mM) were added. The absorbance was noted after 20 s of reaction. Activity of superoxide dismutase was determined by measuring the % age inhibition of NBT.

***Data analyses*:** Three replicates were used for this experiment and data on the different parameters of the fish growth, antioxidant enzymes and Physico-chemical characteristics were subjected to statistical analysis by following Steel *et al*. (1996). Statistical differences among different treatments were measured by Analysis of Variance. Comparisons of means were used to measure the differences within the treatments. Correlation analysis was also performed to find-out relationship among various parameters under experimentation.

RESULTS AND DISCUSSION

The wet weight of the fish *Cirrhina mrigala* showed statistical variations between control and two treatment groups (cadmium and copper). Overall control fish had significantly higher weight of 1.15±0.10 g followed by cadmium and copper fed diet i.e. 0.76±0.05 and 0.59±0.03 g, respectively. Fish showed less increase in fork and total length gain in both treated mediums as compared to the control medium throughout the experimental period. The control fish had significantly higher increase in fork length of 1.75±0.11 mm followed by 1.32±0.11 and 1.07±0.09 mm in cadmium and copper fed mediums, respectively. The increase in total length of control fish was 2.06±0.10 mm followed by that of cadmium and copper fed fish having total length increments of 1.19±0.07 and 0.93±0.07 mm, respectively (Table 1).

These results were similar with the findings of Hollis *et al*. (2000) which investigated the effect of long term sub-lethal cadmium exposure on rainbow trout (*Oncorhynchus mykiss*) which proved poor growth rate, swimming performance and oxygen utilization. Hayat *et al*. (2007) also recorded similar results in major carps. They reported that the fingerlings of three major carps viz. *Catla catla, Labeo rohita* and *Cirrhina mrigala,* exposed to sub-lethal concentrations of manganese for 30 days showed negative growth. They concluded that stressed major carps showed significantly lower values of weight, fork and total length gain than fish kept in control group.

The overall feed intake by the fish in control medium (1.38±0.09 g) was significantly better as compare to treated mediums. The feed conversion ratio and condition factor also showed significant difference between control and treated groups. The control fish had significantly better specific growth rate (SGR) of 16.39±1.42 as compare to rates of growth performance in treated groups (Table 1).

Vincent *et al.* (2002) investigated that the heavy metals contamination usually showed depletion in food utilization parameters. Murai *et al.* (2003) investigated effects of dietary copper on channel catfish. During study reduced growth and feed conversion ratio were noted in fish species. Shaw and Handy (2006) reported that there were no statistically significant differences in condition factor although their trend was higher in the copper fed fish by the end of the experiment.

Analysis of variance on wet weight, fork length, total length, feed conversion ratio and specific growth rate showed statistically significant differences among weeks and treatments. The statistical interaction i.e. weeks × treatments also showed highly significant differences. Metal fed diet exerted a significant ($p<0.01$) negative impact on fish growth in terms of average weight, fork length and total length increase. Feed intake and condition factor showed significant differences among weeks and treatments while the interaction i.e. weeks ×treatments was statistically non-significant (Table 2).

***Correlation studies*:** Data regarding the correlation coefficient among the water quality parameters and increase in wet weight of *Cirrhina mrigala* cultured in sub-lethal dietary cadmium and copper fed mediums are presented in Tables 3 and 4. Among the Physico-chemical parameters of the sub-lethal dose of cadmium treatment, the negatively

Table 2. Growth performance of treated and control fish.

Treatment	Inc. in Weight (g)	Inc. in F.L. (mm)	Inc. in T.L. (mm)	Feed Intake (g)	FCR	K	SGR
Copper	0.59±0.03c	1.07±0.09c	0.93±0.07c	0.86±0.07c	1.57±0.13a	1.87±0.01b	8.41±0.43c
Cadmium	0.76±0.05b	1.32±0.11b	1.19±0.07b	0.98±0.07b	1.35±0.11b	1.95±0.01a	10.89±0.82b
Control	1.15±0.10a	1.75±0.11a	2.06±0.10a	1.38±0.09a	1.51±0.14a	1.79±0.02c	16.39±1.42a

Means sharing similar letters in a row are statistically non-significant at $p>0.05$. Feed Conversion Ratio =Feed intake ÷ Increase in weight; $K= W\times10^5 \div (L)^3$ Where W= Average weight and L= Average fork length; SGR=Weight increase×100/Duration

Table 4. Correlation coefficients among Physico-chemical parameters and increase in weight (g) in cadmium fed fish.

Parameters	Inc. Wt	NH_3	DO	CO_2	EC	Na	K	Ca
NH_3	0.372							
DO	0.251	0.041						
CO_2	0.310	0.225	0.249					
EC	-0.352	0.124	0.186	-0.221				
Na	0.279	0.094	0.228	0.098	-0.351			
K	0.054	0.150	-0.119	0.246	-0.134	-0.536		
Ca	-0.136	-0.281	0.098	0.306	0.372	-0.240	-0.194	
Mg	0.113	0.279	-0.111	-0.305	-0.354	0.231	0.186	-0.999**

* = Significant (P<0.05); ** = Highly significant (P<0.01). Temp = Temperature (ºC); T.H = Total hardness (mg L^{-1}); NH_3 = Total ammonia (mg L^{-1}); D.O = Dissolved oxygen (mg L^{-1}); CO_2 = (mg L^{-1}); E.C = Electrical conductivity (mS cm^{-1}); Na = Sodium (mg L^{-1}); K = Potassium (mg L^{-1}); Ca = Calcium (mg L^{-1}); Mg = Magnesium (mg L^{-1}); Inc. wt. = Increase in weight (g)

Table 5. Correlation coefficients among Physico-chemical parameters and increase in weight (g) in copper fed fish.

Parameters	Inc.Wt	NH_3	DO	CO_2	EC	Na	K	Ca
NH_3	0.110							
DO	-0.079	0.221						
CO_2	-0.054	0.088	0.642*					
EC	0.192	0.026	-0.105	-0.014				
Na	0.049	0.021	0.513	0.086	-0.484			
K	0.197	-0.047	0.038	0.140	0.140	-0.329		
Ca	-0.031	0.229	0.001	-0.115	-0.481	0.026	0.159	
Mg	0.053	-0.213	-0.010	0.111	0.483	-0.056	-0.160	-0.997**

* = Significant (P<0.05); ** = Highly significant (P<0.01). Temp = Temperature (ºC); T.H = Total hardness (mg L^{-1}); NH_3 = Total ammonia (mg L^{-1}); D.O = Dissolved oxygen (mg L^{-1}); CO_2 = (mg L^{-1}); E.C = Electrical conductivity (mS cm^{-1}); Na = Sodium (mg L^{-1}); K = Potassium (mg L^{-1}); Ca = Calcium (mg L^{-1}); Mg = Magnesium (mg L^{-1}); Inc. wt. = Increase in weight (g)

significant relationship was observed between magnesium and calcium while all other parameters showed statistically non-significant relationship with each other (Table 3). During the sub-lethal dose of copper fed medium carbondioxide and dissolved oxygen showed significantly positive relationship while the relationship between calcium and magnesium was significantly negative (Table 4).

***Enzyme activity analysis*:** The activity of peroxidase during control, cadmium and copper fed mediums to *Cirrhina mrigala* is shown in Figure 1. Highest peroxidase activity was observed in the liver of control medium (0.28±0.03 U/mL) and lowest peroxidase activity was observed in kidney of copper fed fish (0.12±0.02 U/mL). The activity of peroxidase in the fish reared in control, cadmium and copper fed mediums were observed in the following order i.e. control>cadmium>copper. The peroxidase activity in the organs of fish showed the following order liver>gills>kidney in all treatments.

The catalase activity in different organs of *Cirrhina mrigala* during control, cadmium and copper fed mediums is shown in Figure 2. Highest catalase activity was observed in the liver of fish reared in control medium (131.87±0.62 U/mL) and lowest catalase activity was observed in kidney of cadmium fed fish (86.07±0.31 U/mL). The activity of catalase in the fish exposed to control, cadmium and copper mediums were observed in the following order control>cadmium>copper. The catalase activity in the organs of fish showed the following order liver>gills>kidney in all treatments.

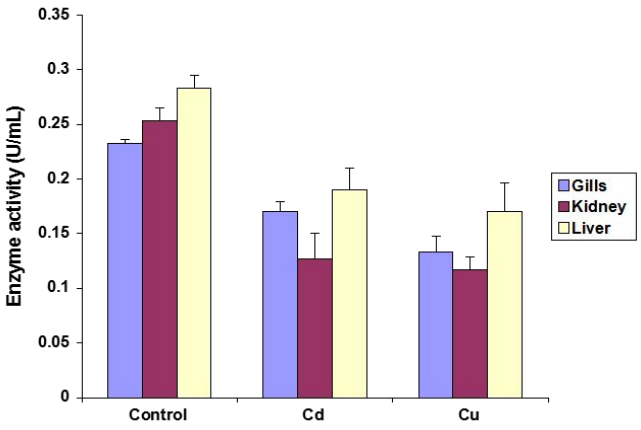

Figure 1. Peroxidase activity in different organs of *Cirrhina mrigala.*

The activity of superoxide dismutase (SOD) in different organs of *Cirrhina mrigala* during control, cadmium and

copper fed mediums is shown in Figure 3. Liver showed highest SOD activity of copper fed fish (29.55±0.26 U/mL) and lowest SOD activity was observed in kidney of control fish (11.48±0.28 U/mL). The activity of SOD in the fish exposed to control, cadmium and copper mediums were observed in the following order copper>cadmium>control. The SOD activity in the organs of fish showed the following order liver>gills>kidney. This study suggests that antioxidant enzymes have gained an importance in preventing the hazardous effects of metals, as they could be warning signals for severe damage.

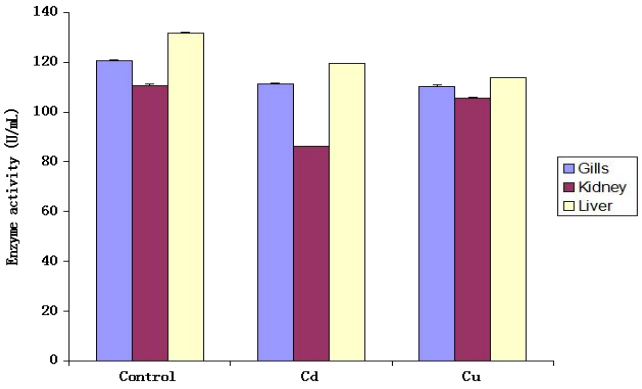

Figure 2. Catalase activity in different organs of *Cirrhina mrigala*.

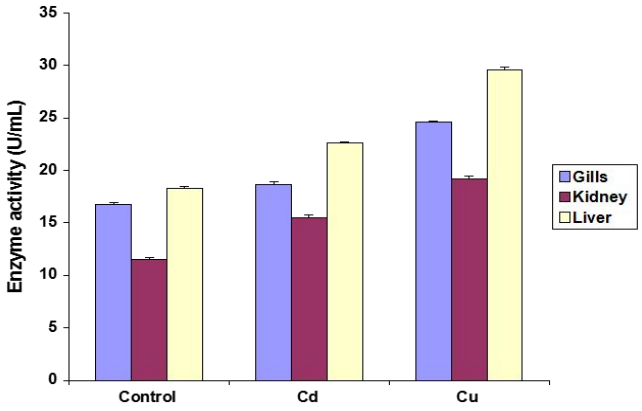

Figure 3. Speroxide dismutase activity in different organs of *Cirrhina mrigala*.

Redox active metals such as copper undergo redox cycling, whereas redox-inactive metals, such as cadmium deplete major antioxidants in the organs especially thiol containing antioxidants and enzymes. Both of these metals can cause significant increases in ROS production, followed by a situation known as "oxidative stress" leading various dysfunctions in lipids (Pinto *et al*., 2003).

***Conclusion*:** As a corollary, the current study demonstrates that the exposure to sub-lethal concentrations of copper and cadmium resulted in reduced growth rate of *C. mrigala.* The maximum peroxidase and catalase activity was in the liver of fish exposed to control medium and least peroxidase and catalase activity was observed in kidney of fish exposed to copper and cadmium, respectively. The maximum superoxide dismutase activity was observed in the liver of copper treated fish and least SOD activity was observed in kidney of control fish. Taken together, our data clearly suggest the importance of selecting the sensitive biomarkers in appropriate tissues for biomonitoring metal toxicity in an aquatic environment. Nevertheless, it is still required to examine further the antioxidant system in different aquatic animal models to understand better.

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