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Toxic effects of a mixture of heavy metal pollutants on fresh water fish species *Cirrhina mrigala* L.

Muhammad Zia ur Rehman[1, 2], Inaam Ullah[3], Sajid Abdullah[1]

1. *Department of Zoology and Fisheries, University of Agriculture, Faisalabad, Pakistan*
2. *Industrial Biotechnology Division, National Institute for Biotechnology and Genetic Engineering, Faisalabad, Pakistan*
3. *Department of Agriculture, Shaheed Benazir Bhutto University, Sheringal Dir (Upper), Pakistan*

Abstract

This study was performed to determine acute toxicity of a mixture of five water-borne metals (iron, zinc, lead, nickel and manganese) to fresh water fish species *Cirrhina mrigala* L. We performed acute toxicity assays to determined LC_{50} (concentration at which 50% fish die) and LC_{95} (concentration at which 95% fish die) of the metals mixture after 96 hours of exposure to one-month old fish specimens. All the assays were performed in glass aquaria of 100 liters volume. We found significant difference between LC_{50} (26.84 mg L^{-1}) and LC_{95} (70.42 mg L^{-1}) of metals mixture for *C. mrigala*. The amount of total sodium, potassium and calcium increased with increasing concentration of the metals mixture, as did total ammonia in the test medium however, concentration of magnesium decreased significantly. We also observed increased use of oxygen by the fish under stress of heavy metals mixture, and in turn accelerated carbon dioxide release into the water that increases water pollution. We conclude that the tested metals mixture is highly toxic to *C. mrigala*. Further work is recommended to unearth molecular basis of, and synergistic effects of the metals toxicity to fish fauna.

Keywords: *Cirrhina mrigala*; Environmental Pollution; Heavy Metals; Toxicity; LC_{50}.

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***Correspondence:**
Dr. Inaam Ullah
Department of Agriculture, Shaheed Benazir Bhutto University, Sheringal Dir (Upper), Pakistan.
Email: inaamullahpbg@gmail.com

Introduction

Aquatic systems are exposed to a number of pollutants that are mainly released from effluents discharged from industries, sewage treatment plants and drainage from urban and agricultural areas (Lopez *et al*., 2002). Heavy metals are natural trace components of the aquatic environment, but their levels have increased due to domestic, industrial, mining and agricultural activities (Kalay and Canli, 2000). Heavy metals as micronutrients are important for animal life, and are integral part of many enzymes, hormones and other biological compounds. Furthermore, they are critical in optimizing many biochemical processes including metabolic regulation, growth, reproduction and erythropoiesis (Nozdriuchina, 1977). However, when in excess of biological needs, heavy metals become harmful to aquatic biota and tend to accumulate inside the organisms. The ions of heavy metals exert toxicity by generating reactive oxygen species (ROS), causing oxidative stress to aquatic life. Furthermore, they are toxic and carcinogenic in nature and pose a threat to human health and the environment (Damien *et al*., 2004; Farombi *et al*., 2007). They pose serious hazards to aquatic fauna because of their toxicity, bio-accumulation, long persistence, and bio-magnification in the food chain (Eisler, 1988).

At elevated concentrations, heavy metals can be absorbed through biological membranes of cell, system, organ or organism. Organisms can also take up these pollutants through respiratory organs, and food ingestion (Phillips and Rainbow, 1994). Furthermore, at higher levels of biological organization (tissue, organ and whole organism) heavy metals induce changes in metabolism, biochemistry, physiology, histology; inhibit synthesis of proteins and nucleic acids (Choz, 1983; Mur and Ramamurti, 1987; Diuga and Penni, 1989; Wicklund *et al*., 1992; Wilson and Taylor, 1993).

Fish are the most important aquatic animals, and are used as food, pet and also for aesthetic beauty and gaming. Fish are highly nutritious, easily digestible and have high protein content, low saturated fats and also contain omega-3 fatty acids. However, nutritional value of fish depends upon its biochemical composition which may be affected by the physico-chemical characteristics of water they are raised in (Burger *et al*., 2002; Rauf *et al*., 2009). The most successful species of polyculture in Pakistan are *Catla catla*, *Labeo rohita* and *Cirrhina mrigala*, due to optimum environmental conditions for their culture, and tasty meat (Rauf et al. 2009).

the polyculture of these major carps has assumed much popularity among the private as well as the public sector farms in the recent past.

The presence of heavy metals such as iron (Fe), zinc (Zn), lead (Pb), nickel (Ni) and manganese (Mn) beyond the permissible limits in the untreated wastewater have adversely affected fresh water fish fauna (Javed & Mahmood, 2001). The potential routes for a pollutant to enter in a fish are the food, non-food particles, gills, oral consumption of water and through the skin. After uptake, metals are transported by the blood to either a storage point or to the liver for transformation and/or storage. After transformation by the liver, metals may be stored there or excreted in the bile, passed back into the blood for possible excretion by the gills and kidneys, or stored in the fat tissue (Heath, 1991).

Toxicological studies with heavy metal on fish have reported toxic effects, altering physiological activities and biochemical parameters both in tissue and in blood of fish (Larsson *et al*, 1984). The mixture of Al, Cd, Cu, Fe, Mn, Ni, Pb, and Zn reduced survival of brown trout (*Salmo trutta* L.) larvae. They have also lowered levels of body Na^+, K^+, and Ca^{++} ions, and impaired bone calcification with exposure to more than one metals. Reduced body ion level usually results from their inhibited uptake by the gills. The gills are obviously very susceptible to waterborne metals, and often show various metal induced lesions (Reader *et al.,* 1989). Rainbow trout respiration rate was one of the parameters which were most sensitive to lethal and sub-lethal intoxication with metals mixture (Petrauskiene, 1999). The respiratory functions are also very susceptible to metal intoxication and sub-lethal exposures often result in a reduced oxygen consumption rate (Vosyliene *et al.,* 2003). Besides the direct effect of trace metals, their interaction to seawater variables such as temperature, pH, dissolved oxygen, salinity, nutrients and biological factors may contribute to enhance the toxicity to fish fauna (USEPA, 2003).

Heavy discharge of metals and their compounds in to the river systems of Pakistan has adversely affected the freshwater fish fauna. The heavy metals affect the aquatic life in both single and mixture forms. While, most of the research experiments have been conducted to find the effects of single metal. Thus, only limited information is available on the effect of mixture of metals on *Cirrhina mrigala*. Therefore, our objective was to study the acute and chronic toxicity of a mixture of five water-borne metals; iron, zinc, lead, nickel and manganese on fresh water fish species *C. mrigala* L.

Materials and Methods

Fish Seeds

The fish seed was collected from Government Fish Seed Hatchery, Faisalabad. Seed was transported to Fisheries Research Farms, Department of Zoology and Fisheries, University of Agriculture, Faisalabad. Fish were acclimated to laboratory conditions for two weeks, during which time they were fed with crumbled feed (25% digestible protein and 2.94 Kcal g^{-1} digestible energy). Ten fish were kept in each aquarium for acclimatization, and were kept unfed for 24 h before the start of the experiment.

Heavy Metals

Five heavy metals; iron (Fe), zinc (Zn), lead (Pb), nickel (Ni) and manganese (Mn) were purchased in the form of pure metal chlorides; Iron chloride ($FeCl_3.6H_2O$), Zinc chloride ($ZnCl_2$), Lead chloride ($PbCl_2$), Nickel chloride ($NiCl_2.6H_2O$), and Manganese chloride ($MnCl_2.4H_2O$) were purchased in powder form from commercial supplier in Faisalabad.

Preparation of solutions

The above mentioned pure chloride compounds were dissolved in distilled water and stock solution was prepared. Molecular mass of specific metal chloride was divided by the atomic mass of its metal to obtain the amount of metal chloride required for 1 gram of metal. For reference, 1X stock solution, containing all metals in the ratio of 1:1:1:1:1 by weight (1mg of each metal per mL), can be prepared by mixing 4.83g of $FeCl_3.6H_2O$, 2.09g of $ZnCl_2$, 1.34g of $PbCl_2$, 4.08g of $NiCl_2.6H_2O$, and 3.6g of $MnCl_2.4H_2O$ in 1L of water. A 50X stock solution was prepared, here on termed as “metals mixture”. Fresh metals mixture was prepared daily.

Experimental Arena

All the assays were performed in glass aquaria of 100-liter water capacity. The aquaria and glassware used in this experiment were washed thoroughly with hydrochloric acid, and rinsed with deionized water prior to use.

Environmental conditions

Environmental conditions were regularly observed throughout the experiment; pH, total hardness and water temperature by using bioassay systems. While, total hardness of water was maintained by using the salts of calcium and magnesium sulphate, and ethylene diamine tetra acetic acid (EDTA) and its sodium salts. However, pH was maintained by NaOH (to increase pH) and HCl (to decrease pH). The water heaters were used to maintain temperature in aquaria. Continuous air supply was maintained to all the test mediums with an air pump through capillary system. Physico-chemical variables viz. temperature, pH, total hardness, dissolved oxygen, total ammonia, electrical conductivity, sodium, potassium and carbon dioxide were monitored every 12h during the course of the experiment.

Exposure to heavy metals

30 days old fish were transferred to the experimental arena. Fish were tested at 12 different concentrations of the metals mixture; 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 and 55 mgL^{-1}. Dead fish were counted after every 12h interval. Experiment was terminated after 96h from the start. At least 30 fish were tested on each concentration.

Determination of physico-chemical parameters

Water temperature and dissolved oxygen of the test medium were measured and recorded by Dissolved Oxygen Meter, modelHI 9146 (HANNA, Australia) while pH and electrical conductivity by the Portable Oxi-Meter MultiLine® Multi 3410 IDS (WTW, Germany). However, total ammonia, hardness, calcium, magnesium, carbondioxide, were measured by following the methods of American Public Health Association (APHA. 1998).

Total Hardness: A 50 mL sub-sample of water taken in an Erlenmyer's flask and its pH was raised up to 12, by adding appropriate volume of the buffer. The reaction mixture was stirred, and 0.1 mL of Erichrome Black-t (EBT) indicator was added to it. The solution was, then, titrated against EDTA (1.0 N) to reach the end point of blue colour. Total hardness was estimated by using following formula:

$$\text{Total Hardness} = \frac{\text{Volume of EDTA used for titration x A x1000}}{\text{Volume of sample (mL)}}$$

Where: A= mg of $CaCO_3$ equivalent to 1.0 mL EDTA titrant at Ca^{++} indicator end point.

Total Ammonia: In 10 mL of water sample, 1-2 drops of sodium-potassium tartrate solution were added and mixed well, 0.5 mL of Nessler's reagent was added for the development of colour and it was allowed to settle for 15 minutes. Concentrations were measured with Spectrophotometer. Standards and samples were run at 420 nm for 1 cm light path. Calibration curve was prepared at the same temperature and reaction time used for samples. Concentration readings were measured against a reagent blank and parallel checks were run frequently against standards in the nitrogen range of samples.

Carbon dioxide: Carbon dioxide concentration was determined by titrating the water sample with sodium carbonate by using phenolphthalein as indicator, using established protocol of Method No. 4500-CO_2 of APHA (1998). Carbon dioxide concentration was calculated using following formula:

$$\text{Carbon dioxide (mgL}^{-1}) = \frac{\text{Volume of } Na_2CO_3 \text{ used x 1000}}{\text{Volume of the samples (mL)}}$$

Calcium: Calcium was estimated according to standard methods of APHA (1998). The following formula was used to calculate the calcium contents of the sample:

$$\text{Calcium (mgL}^{-1}) = \frac{\text{Volume of EDTA used for titration x 400.8}}{\text{Volume of the sample (ml)}}$$

Magnesium: Magnesium was measured after analysing the calcium and total hardness by the following formula:

$$A - B = C$$
$$C/4 = Mg\ (mg\ L^{-1})$$

Where, A = Total hardness, B = Calcium x 2.5

Sodium and Potassium: Sodium, and potassium were determined with the help of Flame Photometer (PFPI) by using methods – 10a and -11a of "Hand Book- 60", respectively (Richards, 1954).

Data Analysis

The LC_{50} and LC_{95} were estimated by Probit Analysis (Hamilton *et al.*, 1977). Correlation analysis was performed to find relationships among various parameters. Statistical analyses were performed using computer software SPSS version 16 (IBM, USA).

Results

Acute toxicity tests

Probit Analysis on mean values of percent mortality data showed LC_{50} concentration of 26.84±2.46 mgL^{-1} (confidence interval = 21.72-31.92 mg L^{-1}, at 95% confidence) while, the LC_{95} was 70.42±7.53 mg L^{-1} (confidence interval values of 59.04-91.80 mg L^{-1}, at 95% confidence) (Table 1). Fish mortality increased with increasing concentration of the metal mixture (Figure 1).

Physico-chemical variables studied during acute toxicity tests

Physical Properties: Physical properties such as temperature, pH, total hardness and electrical conductivity, were analysed periodically during the metals mixture toxicity tests. During these toxicity trials, mean water temperature, pH and total hardness were maintained at 30°C, 7 and 224 mg L^{-1}, respectively at all concentrations. The electrical conductivity of the solution, however, increased significantly with increase in concentration (correlation coefficient 0.95) from 2.65 micro-Siemens per centimetre or μScm^{-1} at 5 mg L^{-1} to 3.24 μScm^{-1} at 55 mg L^{-1} (Table 2).

Total Ammonia: Total ammonia contents of the test mediums increased along with increase in concentration of metals mixture during acute toxicity tests. Total ammonia contents of test mediums ranged from 1.70to 3.05 mg L^{-1} at 5 and 55 mg L^{-1}, respectively (Table 2).

Dissolved Oxygen: Maximum and minimum dissolved oxygen concentrations in the mediums used for *Cirrhina mrigala* were recorded as 5.17 and 4.58 mg $L^{-1}$for metals mixture concentrations of 5 and 55 mg L^{-1}, respectively. The dissolved oxygen contents of the test mediums declined as the concentration of metals mixture increased (Table 2).

Carbon Dioxide: Carbon dioxide contents of the test medium increased with the increase of metals mixture concentration. However, mean maximum carbon dioxide concentration for *C. mrigala* test mediums was recorded as 2.60 mg L^{-1} at 55 mg L^{-1} metals mixture concentration while the same was as lowest as 1.63 mg L^{-1} at 5 mg L^{-1} (Table 2).

Table 1: Lethal concentrations (LC_{50} and LC_{95}) of metals mixture (iron, zinc, lead, nickel and manganese) for *Cirrhina mrigala* L. calculated using probit analysis. 30 days old fish were tested at 12 different concentrations of the metals mixture; 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 and 55 mgL^{-1}. At least 30 fish were tested at each concentration

Cirrhina mrigala	LC_{50} (mgL^{-1})	CI (95%)	LC_{95}(mgL^{-1})	CI (95%)	X^2
Mean±SD	26.84±2.46	21.72-31.92	70.42±7.53	59.04-91.80	0.989

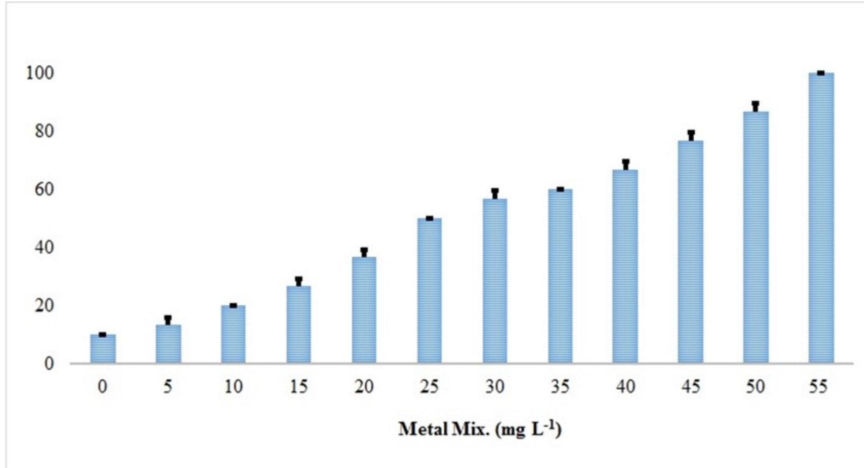

Figure 1: Percent mortality of *Cirrhina mrigala* at different concentrations of the metal mixture

Electrical conductivity: Electrical conductivity is the measure of the ability of water to convey electrical current. It is directly proportional to the concentration of ions in water. The electrical conductivity of test mediums ranged between 2.65 and 3.24 μScm^{-1} at 5 and 55 mg L^{-1}metals mixture concentrations, respectively (Table 2).

Sodium: The total sodium contents of the test medium used for *C. mrigala* was observed to be 295.5 mg L^{-1} at 5 mg L^{-1} metals mixture concentration while at 55 mg $L^{-1}$was recorded as 308.58 mg L^{-1} (Table 2).

Potassium: The minimum and maximum potassium contents of 8.53 and 9.47 mg L^{-1} at 5 and 55 mg L^{-1} metals mixture concentrations, respectively were observed in the test mediums used for *C. mrigala* (Table 2).

Calcium: The highest value of calcium in the test mediums used for *C. mrigala* was recorded as 15.52 mg L^{-1} at 45 mg L^{-1} metals mixture concentration while the same was lowest as 13.69 mg L^{-1} at 5 mg L^{-1} (Table 2).

Magnesium: Magnesium is present in water as carbonates, bicarbonates, sulphates and chlorides. The highest value of magnesium contents in the test mediums used for *C. mrigala* was recorded as 47.42mg L^{-1} at 5and the lowest value as 45.56 mg L^{-1} at 20 mgL^{-1} metals mixture concentrations (Table 2).

Correlation Studies

Data regarding correlation coefficients among water quality variables and the concentrations used of metals mixture used for *C. mrigala* acute toxicity tests are presented in Table 2. There existed positive correlation of metals mixture concentration with total ammonia, carbon dioxide, electrical conductivity, calcium and sodium. However, the correlation coefficient of metals mixture with dissolved oxygen was significantly negative.

Discussion

Fish are an excellent bio-indicators of environmental pollution, and have extensively being used for the assessment of the quality of aquatic environment (Farombi et al., 2004, Abdullah et al., 2006). Heavy metals, along with toxic substances dissolved in water, often increase the sensitivity of aquatic organisms to temperature variations and changes in dissolved oxygen (Bagdonas and Vosyliene, 2006).

In present investigation, we found LC50 and LC95 of metals mixture for C. mrigala at 26.84±2.46 and 70.42±7.53 mgL-1, respectively after 96hr of exposure, with 95% confidence. Our values of lethality are significantly lower than that of Naz and Javed (2013a), who reported LC50 and Lethal concentration of a metal mixture (Fe+Ni) for 90 days old C. mrigala at 64.44 mgL-1 and 100.35 mgL-1, respectively. The lower lethal concentration in our study could be due to the younger age of the fish used in our experiment. The other factor could be the higher number of metals used in our mixture, that would have had a combined toxicity more than the Fe+Ni mixture. Witeska and jezierska, (2003) found that physic-chemical properties, such as oxygen concentration, temperature, hardness, salinity and presence of other metals may modify metal's toxicity to fish. Hypoxic conditions, temperature and increased acidification usually render the fish more susceptible to intoxication while increase in mineral contents (hardness and salinity) may reduce metal toxicity to fish.

During this study, we found positive correlation of metals mixture concentration with total ammonia, carbon dioxide, electrical conductivity, sodium and calcium while for the dissolved oxygen was negative. Similar findings have been reported by Naz and Javed (2013b), who reported higher values for total hardness and electrical conductivity, and lower values for dissolved oxygen in the metal mixture (Zn+Pb+Ni+Mn+Fe) treated media as compared to the non-treated control media.

Our results also confer to that of the Javed and Hayat (2004), who reported that the heavy metal concentration in water was inversely related to pH and the concentration of mobile magnesium, iron, and cobalt in the water. In our case pH remained constant almost throughout the experiment, and varied only slightly.

Fish populations can be affected by aquatic pollutants, not only directly but their active retreat from polluted areas can result in disturbances in their migration and distribution. Resulting in reduction of their normal area of habitat, as well as their resources.

Table 2: Mean of physico-chemical properties of the test medium at different concentrations of metals mixture (iron, lead, zinc, nickel and manganese) used for acute toxicity assays with *Cirrhina mrigala* L. Temperature, pH and total hardness of the solution were kept constant

Metals Mixture Concentration ($mg\ L^{-1}$)	Total Ammonia ($mg\ L^{-1}$)	Dissolve Oxygen ($mg\ L^{-1}$)	Carbon Dioxide (mgL^{-1})	Electrical Conductivity (μScm^{-1})	Sodium ($mg\ L^{-1}$)	Potassium ($mg\ L^{-1}$)	Calcium ($mg\ L^{-1}$)	Magnesium ($mg\ L^{-1}$)
5	1.70± 0.59	5.17± 0.42	1.63±0.49	2.65± 0.34	295.5±10.22	8.53± 0.65	13.69±1.43	47.42± 1.04
10	1.74±0.49	5.12± 0.58	1.63± 0.59	2.79±0.51	295.66±12.48	9.16±1.16	14.19±1.34	47.06±1.14
15	1.74± 0.55	5.07± 0.40	1.85± 0.64	2.92± 0.28	296.33±12.10	8.49± 0.88	15.25±1.09	46.44±1.17
20	1.75± 0.67	5.03±0.47	1.92±0.59	2.98±0.40	296.66±29.52	8.36±1.01	15.1±0.99	45.56±1.04
25	1.91±0.47	4.97±0.30	2.00±0.73	3.00± 0.29	298±14.23	9.36±1.03	15.10±1.16	46.62±1.37
30	1.94± 0.68	4.96± 0.26	2.00± 0.72	3.02± 0.49	298±11.93	9.00± 0.90	15.60± 0.82	45.58±1.00
35	2.25± 0.61	4.87± 0.34	2.05± 0.67	3.04± 0.19	301.83±13.36	8.91± 0.93	15.01±0.24	46.72±1.20
40	2.34± 0.58	4.87± 0.55	2.06± 0.83	3.07± 0.61	301.91±12.51	8.84±1.28	15.27±1.14	46.55±1.25
45	2.48± 0.45	4.63± 0.23	2.25± 0.55	3.08±0.56	302.08±11.61	9.80±1.26	15.52±1.24	46.30± 0.84
50	2.53± 0.29	4.62± 0.40	2.25± 0.75	3.24± 0.32	307.83± 27.64	8.55± 0.63	15.19±1.26	46.52± 0.83
55	3.05± 0.59	4.58± 0.55	2.60± 0.73	3.24± 0.65	308.58±10.59	9.47± 0.90	15.13± 0.99	46.56±0.99
Cor. Coef*.	0.94	-0.97	0.95	0.95	0.94	0.44	0.65	-0.26

*Correlation between concentration of the metals mixture and physico-chemical parameters during the acute toxicity assays.

From another point of view, the avoidance response by fish is one form of phenotypic adaptation allowing fishes to survive in altered environment (Flerov, 1989). We urge researchers to focus on studying combined effects of metal pollutants on fish fauna, and to find molecular mechanism behind elevated toxicity of metal mixtures.

Conclusions

We conclude that the metals mixture is extremely toxic to fresh water fish species *C. mrigala*. The LC_{50} and LC_{95} for *C. mrigala* after 96hr of exposure to the metals mixture came to be 26.84 mgL $^{-1}$ and 70.42 mg L^{-1}, respectively. Concentration of the metals mixture showed positive correlation with total ammonia, carbon dioxide, electrical conductivity, sodium, and potassium content of the test medium while, inverse relationship was found with the dissolved oxygen. More work is required to understand the molecular mechanism behind metal toxicity to fish fauna.

Compliance with ethical standards

Conflict of Interest

The authors declare that they have no conflict of interests.

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