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Synergistic Effects of Metals (Cobalt, Chromium and Lead) in Binary and Tertiary Mixture Forms on *Catla catla*, *Cirrhina mrigala* and *Labeo rohita*

Ummara Batool* and Muhammad Javed
Department of Zoology and Fisheries, University of Agriculture, Faisalabad.

Abstract.- Acute toxicity tests, in terms of 96 h LC_{50} and lethal concentrations of individual metals (Co, Cr and Pb) and their mixtures were conducted under controlled laboratory conditions to evaluate the sensitivity of major carps viz. *Catla catla*, *Cirrhina mrigala* and *Labeo rohita*. All tests were performed under constant water temperature (30ºC), pH (7.50) and total hardness (225mgL^{-1}). Fish fingerlings (110-day age) were first acclimatized to laboratory conditions and then shifted to glass aquaria for toxicity experiments. During the whole acute toxicity trails, fish mortality was observed at 8 h intervals, while physico-chemical variables of water *viz.* carbon dioxide, dissolved oxygen, pH, potassium, sodium, temperature, total hardness and total ammonia were checked at 12 h intervals. Fish mortality data were analyzed through probit analysis method at 95% confidence interval to compute 96 h LC_{50} and lethal concentrations of individual metals and their mixture for each fish species. Among the major carps, *Catla catla* showed significantly ($p<0.05$) higher sensitivity to all metals with the mean 96 h LC_{50} and lethal concentrations of 40.16±22.72 and 71.33±39.98mgL^{-1}, respectively, followed by that of *Labeo rohita* and *Cirrhina mrigala*. Among all treatments, Co+Cr+Pb mixture appeared significantly most toxic to the fish than that of individual metals and their binary mixtures. Results suggested that metals (Co, Cr and Pb) in a mixture form showed synergistic effects on the fish. However, there existed 2x synergistic effects for Co+Cr, Co+Pb and Co+Cr+Pb while that for Cr+Pb mixture was 3x.

Key words: Acute toxicity, cobalt, chromium, lead, metal mixtures, major carps.

INTRODUCTION

In spite of wastewater management efforts in Pakistan, heavy metals are still posing immense health hazards to aquatic organisms due to rapid industrialization and use of agricultural chemicals (Azmat *et al*., 2012). The metallic ions pollution of freshwater bodies is a serious problem for human health directly, because the aquatic organisms especially fish is an integral part of human diet (Subathra *et al*., 2007).

Some metals are essential for normal physiological functioning of fish but become toxic when they accumulate in their body tissues and are not metabolized. In addition, significantly higher concentrations of metals in fish can alter its physiological functioning that could lead to high mortality and ultimately loss of indigenous fish biota (Avenant-Oldewage and Marx, 2000). Cobalt, an essential metal, performs important biochemical functions but its higher concentration in aquatic ecosystems becomes toxic to fish as it interferes with the enzyme systems (Yaqub and Javed, 2012). It is reported to be a potential carcinogenic compound and has been included recently in group 2A carcinogens (*i.e.*, probably carcinogenic to humans) (I.A.R.C., 2003). Chromium is used extensively in industries to produce pigments and dyes. Chromium is dangerous as it can accumulate in fish body as much as 4000 times greater than that of their surroundings (Avenant-Oldewage and Marx, 2000). Lead is highly toxic metal as it is reported to be responsible for death or sub-lethal changes in reproduction, growth and behavior of the fish (Ramsdorf *et al*., 2009). Lead in the form of Pb^{2+} is most common and stable ion in aquatic environments and has strong tendency to get bioaccumulate in fish organs like gills, kidney, liver, muscles, scales and skin (Spokas *et al*., 2006; Ahmed and Bibi, 2010). Under the exposure of waterborne lead, fish exhibits a wide range of neurological and muscular abnormalities, growth inhibition, reproductive problems and mortality (Tekale, 2003).

Environmental contaminants are frequently

* Corresponding author : ummara.batool@yahoo.com
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encountered as mixtures (Naz *et al*., 2012) but unfortunately water quality criteria are established by considering the acute and chronic bioassays of individual chemicals (Otitoloju, 2003; Shaw *et al*., 2006). Therefore, present as a mixture in ambient waters, trace metals may enter the organisms via common routes and interact with each other affecting uptake, bioaccumulation and toxicity. The type of interaction depends on the metals involved, their external concentration, availability and exposure scenario, length of exposure, studied species and examined organs (Norwood *et al*., 2003). The co-solutes may induce either synergistic (increased) or antagonistic (decreased) effects as compared to additive (independent) behavior (Altenburger *et al*., 2003). It seems that interactions among various metals are related to their competitive uptake from the environment and different patterns of distribution in fish tissues. Thus, it is necessary to consider possible multi-metal interactions, which are currently ignored by water quality guidelines, when setting site specific water quality criteria (Norwood *et al*., 2003). Acute toxicity tests (determination of LC_{50} and lethal concentration values) are the most reliable tests for assessing the potential adverse effects of toxic chemicals on aquatic life (Subathra *et al*., 2007). Assessment of individual chemicals toxicity could not predict interactions and their effects in mixture form. Therefore, the purpose of present research work is to evaluate the acute toxicity of waterborne metals and their mixtures in indigenous fish fauna of Punjab, Pakistan.

MATERIALS AND METHODS

Acute toxicity tests, in terms of 96-h LC_{50} and lethal concentrations of water-borne metals (Co, Cr and Pb) and their mixtures were conducted to evaluate the sensitivity of three fish species (*Catla catla*, *Cirrhina mrigala* and *Labeo rohita*). Fingerlings of each fish species (110-day old) were acclimatized to laboratory conditions in holding tanks, supplied with flow through aerated water, for one week. The mean average weights of fish ranged from 8.95±1.02, 9.23±0.84 and 9.05±0.67g for *Catla catla*, *Cirrhina mrigala* and *Labeo rohita*, respectively. Fish were fed with feed having 30% digestible energy, however, feeding were stopped before toxicity experiments to avoid change in metals toxicity due to fish excretory products. Experiments were conducted in the glass aquaria of 60 liter water capacity, supplied with capillary system to provide constant flow of air. All tests were performed under constant water temperature (30ºC), pH (7.50) and total hardness (225mgL^{-1}). Chemically pure chloride compounds of cobalt ($CoCl_2$. $6H_2O$), chromium ($CrCl_3$. $6H_2O$) and lead ($PbCl_2$) were dissolved, separately, in deionized water and stock solutions were prepared for required metals and their mixture concentrations (1:1 and 1:1:1 ratio) on metallic ion equivalence basis.

The following treatment combinations were used during acute toxicity determinations: 1, Cobalt (Co); 2, Chromium (Cr); 3, Lead (Pb); 4, Co + Cr; 5, Co + Pb; 6, Cr + Pb; 7, Co + Cr + Pb).

A group of ten fingerlings of each species were exposed to individual metals and their mixtures, separately, for 96 h. In order to avoid immediate stress to the fish the concentrations of individual metals and their mixtures in each aquarium were increased gradually and total test concentration maintained within seven hours. The test concentrations for each metal and their mixtures were started from zero with an increment of 5.00 to 10.00mgL^{-1}, for low and high concentrations, respectively. Each test dose was tested in triplicate. The test media were checked for their respective metallic ions concentrations, through atomic absorption spectrophotometer, on daily basis to maintain the desired treatment concentrations in each aquarium. During the acute toxicity trails, fish mortality and physico-chemical variables of water *viz*., carbon-dioxide, dissolved oxygen, pH, potassium, sodium, temperature, total hardness and total ammonia were checked at 12 h intervals by following the methods described by A.P.H.A. (1998). Fish mortality data were analyzed through Probit Analysis method (Hamilton *et al*., 1977) at 95% confidence interval to estimate the 96 h LC_{50} and lethal concentrations of metals and their mixture for each species of fish. Goodness-of-fit was estimated by using Pearson Chi-square test. Analyses of variance using factorial experiment (RCBD) and Tukey's/Student Newman-Keuls test were performed to analyze statistical differences among different treatments and fish species at $p<0.05$. Metal interactions were evaluated by calculating toxic units (T.U) following Sobrino-Figueroa (2007).

$$\text{T.U.} = \frac{[A]_{metal}}{A_{LC50}} + \frac{[B]_{metal}}{B_{LC50}} + \ldots$$

where $[A]_{metal}$, concentration of metal A in the mixture (mgL^{-1}); A_{LC50}, A metal 96 h LC_{50} value (mgL^{-1}); $[B]_{metal}$, concentration of metal B in the mixture (mgL^{-1}); B_{LC50}, B metal 96 h LC_{50} value (mgL^{-1}).

T.U. = 1, metals in a mixture show additive effect; T.U. < 1, synergistic effect; T.U. > 1, antagonistic effect.

Magnification factor (M.F.) was determined according to Marking (1977): M.F. = 1÷ (T.U.-1)

RESULTS

Mortality data of each treatment and each fish species were analyzed through Probit analysis method. The mean 96 h LC_{50} and lethal concentration values with 95% confidence intervals obtained by probit analyses and their calculated chi-square are presented in Table I. The calculated chi-square (χ^2) with higher p-values (ranged from 0.921-1.000) indicated higher precision of all these regression models. Analysis of variance showed highly significant differences among treatments and all the three fish species.

Catla catla

Catla catla showed differential sensitivity towards metals and their mixtures. Significantly higher sensitivity of this fish was observed for Co+Cr+Pb mixture with mean 96 h LC_{50} and lethal concentration values of 21.57±0.34 and 42.07±0.22 mgL^{-1}, respectively. Co appeared least toxic metal with lowest mean LC_{50} and lethal concentration values of 86.32±0.37 and 157.86±0.36mgL^{-1}, respectively. The toxicity of metals and their mixtures for this fish species increases in following order: Co < Cr < Co+Cr < Pb < Cr+Pb < Co+Pb < Co+Cr+Pb (Table I).

Cirrhina mrigala

The mean 96 h LC_{50} and lethal concentration values for *C. mrigala* showed that Co+Cr+Pb mixture was more toxic for this fish than that of individual metals and their binary mixtures. The highest and lowest mean 96 h LC_{50} values for this fish were observed as 117.39±0.36 and 33.30±0.40mgL^{-1} for Co and Co+Cr+Pb, respectively. However, mean highest and lowest lethal concentrations of 192.17±0.29 and 50.86±0.32mgL^{-1} were observed for Co and Co + Cr + Pb, respectively. For all the treatments, sensitivity of *C. mrigala* in terms of LC_{50} and lethal concentration values increased in following order: Co < Cr < Co+Cr < Pb < Co+Pb < Cr+Pb < Co+Cr+Pb (Table I).

Labeo rohita

The sensitivity of *L. rohita* varied significantly under the exposure of metals and their mixtures (Table I). Co. appeared least toxic metal among all the treatments with higher mean 96 h LC_{50} and lethal concentrations of 106.12±0.38 and 183.57±0.41mgL^{-1}, respectively. However, lowest mean LC_{50} and lethal concentrations of 26.57±0.27 and 47.07±0.15mgL^{-1}, respectively, were observed for Co+Cr+Pb. The toxicity of metals and their mixture followed the order: Co < Cr <Co+Cr < Pb < Cr+Pb < Co+Pb < Co+Cr+Pb in terms of LC_{50} values while Co < Cr <Co+Cr < Pb < Co+Pb < Cr+Pb < Co+Cr+Pb in terms of lethal concentrations.

Regarding overall responses of major carps, three fish species showed significantly variable sensitivity towards metals and their mixtures. *C. catla* showed significantly higher sensitivity for all the treatments (in terms of both LC_{50} and lethal concentrations), followed by that of *L. rohita* and *C. mrigala* (Table II). Among treatments, Co+Cr+Pb mixture showed significantly higher toxicity than that of individual metals and their binary mixtures. However, cobalt appeared least toxic metal amongst all treatments.

Comparison of mixtures toxicity

Comparison of individual metals and their mixture is presented in Figure 1. It was observed that Co, Cr and Pb in all combinations revealed

Table I.- **Responses of three fish species for their 96-h LC_{50} and lethal concentrations (mgL^{-1}) under the exposure of individual metals and their mixtures.**

Fish Species	Treatments	LC_{50}	95% CI	Lethal Concentrations	95% CI	Pearson Goodness of fit Test		
						χ^2	DF	p-value
Catla catla	Co	86.32±0.37 a	73.76-96.28	157.86±0.36 a	141.81-185.34	1.318	12	1.000
	Cr	52.41±0.20 b	46.74-56.65	78.36±0.32 b	71.63-91.43	1.003	8	0.998
	Pb	31.25±0.22 d	25.21-35.62	58.34±0.17 d	51.44-71.86	0.779	8	0.999
	Co+Cr	36.25±0.28 c	30.21-40.62	63.34±0.31 c	56.44-76.86	0.779	8	0.999
	Co+Pb	24.21±0.24 f	18.14-28.13	45.93±0.32 f	40.16-58.15	1.192	6	0.977
	Cr+Pb	29.08±0.12 e	23.88-33.16	53.41±0.23 e	46.95-65.84	0.786	8	0.999
	Co+Cr+Pb	21.57±0.34 g	16.21-25.31	42.07±0.22 g	36.38-54.13	1.181	6	0.978
Cirrhina mrigala	Co	117.39±0.36 a	104.43-127.69	192.17±0.29 a	175.10-221.90	1.684	12	1.000
	Cr	68.22±0.14 b	62.31-73.08	101.55±0.25 b	93.41-116.18	1.180	11	1.000
	Pb	40.54±0.32 d	34.26-45.31	71.77±0.30 d	63.68-87.69	1.676	9	0.996
	Co+Cr	42.59±0.34 c	34.34-47.77	76.07±0.17 c	67.51-94.63	1.121	8	0.997
	Co+Pb	36.25±0.23 e	30.21-40.62	63.34±0.38 e	56.44-76.86	0.779	8	0.999
	Cr+Pb	35.31±0.31 f	29.88-39.33	58.64±0.30 f	52.25-71.71	1.018	7	0.995
	Co+Cr+Pb	33.30±0.40 g	28.82-36.69	50.86±0.32 g	45.90-60.84	0.806	6	0.992
Labeo rohita	Co	106.12±0.38 a	92.67-116.69	183.57±0.41 a	165.83-214.86	1.707	12	1.000
	Cr	64.22±0.21 b	58.32-69.20	98.17±0.26 b	89.55-114.03	1.900	11	0.999
	Pb	36.72±0.37 d	30.49-41.43	68.42±0.43 d	61.00-81.72	2.313	10	0.993
	Co+Cr	39.92±0.29 c	33.17-44.57	69.31±0.53 c	61.84-84.47	1.873	8	0.985
	Co+Pb	30.12±0.31 f	20.73-35.45	63.62±0.31 e	54.27-87.19	2.584	7	0.921
	Cr+Pb	33.11±0.14 e	27.35-37.14	56.60±0.34 f	50.52-68.71	1.017	7	0.995
	Co+Cr+Pb	26.57±0.27 g	21.21-30.31	47.07±0.15 g	41.38-59.13	1.181	6	0.978

Means with similar letters in a single column for each species are statistically non-significant at $p<0.05$.n=3; 95% CI, 95% Confidence Interval Limits; χ^2, Chi-Square; DF, Degree of Freedom; p-value, Probability Values.

more toxic effects compared to toxicity observed during their individual exposures (in terms of both 96 h LC_{50} and lethal concentration values). The T.U. values of all metals mixtures ranged from 0.48470-0.71298 which showed synergistic effects of Co, Cr and Pb in all combinations. Among all the metal mixtures, Cr+Pb appeared most deleterious combination showing 3x synergistic effect of the constituent metals. However, other combinations *viz.*, Co+Cr, Co+Pb and Co+Cr+Pb showed 2x synergism based upon their T.U. values (Table III).

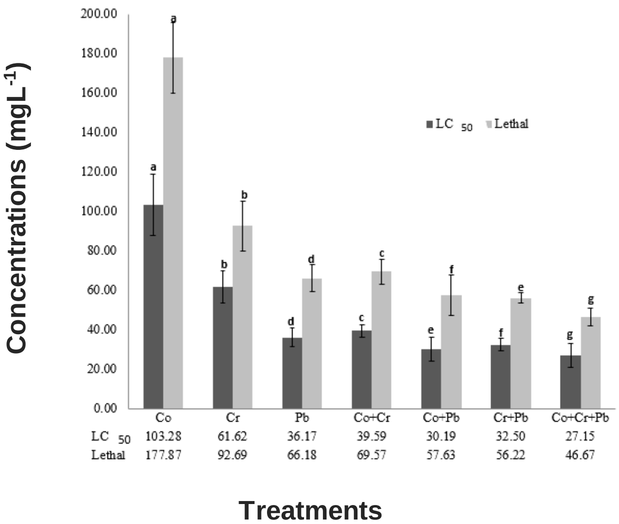

Fig. 1. Comparison of 96 h LC_{50} and lethal concentrations (mgL^{-1}±SD) of metals and their mixtures. Where LC_{50}=50% of the lethal concentration (mgL^{-1}); Lethal = Lethal concentration (mgL^{-1}).
N=10 Fish
p-value= 0.05
Error Bars= Standard Deviation
Bars with similar letters are statistically non-significant at $p<0.05$, for LC_{50} and lethal concentrations separately

Table II.- Comparison of three fish species for their 96 h LC_{50} and lethal concentrations (mgL^{-1}).

Fish species	LC_{50}	Lethal concentrations
Catla catla	40.16±22.72 **c**	71.33±39.98 **c**
Cirrhina mrigala	53.37±30.61 **a**	87.77±48.80 **a**
Labeo rohita	48.11±28.38 **b**	83.82±46.73 **b**

Means with similar letters in a single column are statistically non-significant at $p<0.05$.

Table III.- Toxicity unit, type of interaction and magnification factor in major carps exposed to mixtures of metals.

Metals mixtures	96 h LC_{50} (mgL^{-1})	Toxicity unit	Type of interaction	Magnification factor
Co+Cr	39.59±3.18	0.51278	Synergism	2x
Co+Pb	30.19±6.02	0.56368	Synergism	2x
Cr+Pb	32.50±3.16	0.71298	Synergism	3x
Co+Cr+Pb	27.15±5.89	0.48470	Synergism	2x

DISCUSSION

The present investigation reveals significantly variable tolerance limits of three fish species under the exposure of water-borne metals (Co, Cr and Pb) and their mixtures. Among the three fish species, *C. catla* showed significantly higher ($p<0.05$) sensitivity for all the treatments in terms of 96 h LC_{50} and lethal concentrations than that of *L. rohita* and *C. mrigala*. *C. catla* was reported to be the most sensitive species among major carps by Azmat *et al*. (2012) and also by Javid *et al*. (2007). *C. mrigala* appeared least sensitive species during the present investigation, however Abdullah *et al*. (2007) reported that *L. rohita* is least sensitive among major carps under the exposure of waterborne metals. Amongst all treatments, Co appeared least toxic metal for all the three fish species in terms of LC_{50} and lethal concentrations, followed by Cr and Pb. It was also noted that all combinations of metals were significantly more toxic than individual exposure of constituent metals. Tri-metal mixture (Co+Cr+Pb) appeared more toxic than bi-metal mixtures (Co+Cr, Co+Pb and Cr+Pb). Similar findings were also observed by Naz and Javed (2012). They observed that five metals mixture (Fe+Zn+Pb+Ni+Mn) caused significantly higher ($p<0.05$) toxicity to major carps as compared to four, three and two metal mixtures. The present results are also in conformity to the findings of Bagdonas and Vosyliene (2006) who reported that the toxicity caused by Cu+Zn mixture is greater than the toxicity of Cu and Zn when exposed singly.

In natural aquatic environments, trace metals are often present in the form of mixture forms. Uptake of metals in mixtures may demonstrate a series of interactive effects and modify the toxicity of individual elements. In contaminated environments, several metals are often present together at elevated concentrations (Borgmann *et al*., 2008). During the present study, it was observed that Co, Cr and Pb in all combinations revealed synergistic effects based on their toxicity units (T.U.) values. However, Cr+Pb appeared most deleterious combination showing 3x synergistic effect of these metals while other combinations (Co+Cr, Co+Pb and Co+Cr+Pb) showed 2x synergism. Similarly, Sobrino-Figueroa *et al*. (2007) reported 2x synergistic toxic effect between Cr and Pb for *Argopecten ventricosus* juveniles. They also reported 2x synergistic interaction of Cd+Cr+Pb metal mixture, however interaction between Cd+Cr and Cd+Pb was antagonistic based on their toxicity units. A similar synergistic effect of lead on copper uptake by fish (*Paracheirodon innesi*) was observed when in mixture by Tao *et al*. (1999). Less than additive toxicity of Pb+Zn and Cd+Pb mixtures to the stream resident fish were observed by Mebane *et al*. (2012).

CONCLUSIONS

The present study shows that species, *Catla catla* exhibited significantly higher ($p<0.05$) sensitivity for all the treatments, in terms of both 96 h LC_{50} and lethal concentrations, followed by that of *Labeo rohita* and *Cirrhina mrigala*. For all the treatments, the sensitivity of fish species followed the order: Co < Cr < Co + Cr < Pb < Cr + Pb < Co+Pb < Co + Cr + Pb. Among mixtures, Cr + Pb appeared most deleterious combination showing 3x synergistic effect of constituent metals. However, other combinations (Co +Cr, Co + Pb and Co + Cr + Pb) showed 2x synergism based upon their T.U. values.

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