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Assessment of Toxic Interactions of Heavy Metals and Their Effects on Accumulation in Tissues of Freshwater Fish*

¹Lopa Ghosh, ¹S. Adhikari and ²S.Ayyappan

¹Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar, India

²KAB-II, Pusa, Indian Council of Agricultural Research, New Delhi 110012

Abstract: The present study deals with the interaction between accumulation of waterborne lead and chromium in *Labeo rohita* fingerlings in the laboratory. There was no mortality occurred in individual and binary treatment. Kidney, liver, gill, skin and muscle of *L. rohita* exposed to sublethal concentration 0.10 µg Pb L⁻¹ alone and 0.10 µg Pb L⁻¹+0.15 µg Cr L⁻¹ accumulated Pb linearly with time over 90 days and significant differences occurred with Pb alone. Lead accumulation in binary mixture Pb+Cr significantly decreases than Pb alone in all tissues of fish. So, the interactive effect of Cr on Pb accumulation was of antagonistic in nature. Cr is accumulated linearly with time in Kidney, liver and gill of *L. rohita* exposed to 0.15 µg Cr L⁻¹ alone and 0.10 µg Pb L⁻¹+0.15 µg Cr L⁻¹ for 90 days. Muscle reached a steady state Cr levels after 60 days of exposure. The interactive effect of Pb on Cr accumulation was synergistic in nature in Pb+Cr mixture. The presence of Cr in a mixture decreases the accumulation of Pb. Though kidney, liver and gill tissues of *L. rohita* accumulated Pb and Cr above permissible level but muscle accumulates below human consumption level.

Key words: Lead, chromium, toxic interactions, *Labeo rohita*

Introduction

Heavy metal contaminants in aquatic ecosystem pose a serious environmental hazard because of their persistence and toxicity. Wastewater discharging into natural waters frequently carries more than one toxic or potentially toxic substance. Majority of the studies on the biological action of pollutants have concentrated on the action of single compounds against organisms (Don Pedro, 1996). Due to the presence of multiple pollutants including heavy metals in natural environment, joint-action accumulation studies are becoming increasingly popular in measuring and predicting impacts of chemical pollutants on organisms (Don Pedro, 1996). Among myriad of heavy metal pollutants, Pb and Cr merits a special attention due to its potential health hazard to fish themselves or the aquatic organisms that consume them, including top level receptors, including human life in particular. Among different toxicity tests bioaccumulation is one of the most important to assess the impact of low doses of a toxicant. The frequent presence of Pb and Cr in industrial wastes and its high toxicity along with considerable bioaccumulation in freshwater fishes make it a toxicant that should be given due consideration in aquatic toxicology. Fish accumulate xenobiotic chemicals, especially those with poor water soluble occurs because of the very intimate contact with the medium that carries the chemicals in solution or suspension and also because fish have to extract oxygen from the medium by passing enormous volumes of water over their gills. For fish, the gills, skin and digestive tract are potential sites of absorption of water borne chemicals. The chemical once it is absorbed is transported by the blood to either a storage point, such as bone, or to the liver for transportation. If transported by the liver,

Corresponding Author: Lopa Ghosh, Centre for Energy Studies, Indian Institute of Technology Delhi, Hauz Khas, New Delhi 110016, India

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it may be stored there, excreted in the bile, or passed back into the blood for possible excretion by the kidney or gills or stored in extra hepatic tissues such as fat. Metal-metal combinations can reduce the toxic effects of metals by decreasing their accumulated amounts (Fargasova *et al.*, 1997; Kargin and Cogun, 1999; IR Ilgen, 2001). So, there is no clear insight in the interaction between Pb and Cr that determine the accumulation of the metals during combined exposure. The aim of this study was to determine the effects of metal interaction on the accumulation of Pb and Cr in the muscle, gill and liver and kidney tissues of *Labeo rohita*.

Materials and Methods

Experimental Set-up

Freshwater fish e.g., *Labeo rohita* were acclimatized in the laboratory at Central Institute of Freshwater Aquaculture, Bhubaneswar, India for one month at $27\pm 1^\circ\text{C}$, which was the temperature of experimental conditions. After acclimatization period mean length and weight of fish were measured 9 ± 0.42 cm and 7.0 ± 0.32 g, respectively. A total of three sets of glass jars 100 L capacity of each, with three replications were maintained and in each jar 10 numbers of fish were released in a dechlorinated tap water.

Set-I

Three glass jars contained $0.10 \mu\text{g L}^{-1}$ Pb

Set-II

Three glass jars contained $0.15 \mu\text{g L}^{-1}$ Cr

Set-III

Three glass jars contained $0.10 \mu\text{g L}^{-1}$ Pb+ $0.15 \mu\text{g L}^{-1}$ Cr

All chemicals used for sample preparation were in analytical grade. Stock solution of 1000 mg L^{-1} of K_2CrO_4 was prepared in deionized water. The chromate solution was acidified with H_2SO_4 (pH = 3.5) to maintain a relatively stable Cr (VI) species (Skoog *et al.*, 1997). High purity nitrate salt of Pb was made from pure metals by dissolving it, in 1:1 (v: v) HNO_3 (16 M), followed by dilution to 1000 mg L^{-1} in deionized water.

Experimental Procedure

The experiment was continued for 90 days. During exposure period chemical parameters of water such as pH, total alkalinity and dissolved oxygen were analyzed (APHA, 1992). Water was aerated and feeding was done daily with pelleted feed. After feeding feces and uneaten food were siphoned off and 50% of water was changed every 3 days to replenish Pb and Cr. The water used for the experiments such as pH, dissolved oxygen and alkalinity were in the range of 7.8 ± 0.40 , $6\pm 0.32 \text{ mg L}^{-1}$ and of $120\pm 5.4 \text{ CaCO}_3 \text{ mg L}^{-1}$, respectively. Sampling of fish for accumulation in both single and binary mixtures was done every 15 days interval. Before sampling fish were starved for 24 h period. For each exposure period, 3 numbers of fish in each set were analyzed to determine accumulated metal concentration. Fish were dissected and muscle, skin, gills, liver and kidney were removed. Background levels of lead and chromium were measured in 3 unexposed fish that were sampled on the days the experiment started (Table 1).

Table 1: Concentrations of lead and chromium in tissues of *Labeo rohita* fingerlings before experiment. Mean \pm SD for three fish. The results are expressed as $\mu\text{g g}^{-1}$ dry wt.

Tissues	Lead	Chromium
Muscle	0.04 ± 0.006	0.12 ± 0.06
Skin	0.07 ± 0.003	0.16 ± 0.03
Gill	0.52 ± 0.11	0.74 ± 0.13
Liver	0.40 ± 0.08	0.28 ± 0.10
Kidney	0.26 ± 0.07	0.24 ± 0.08

Analysis of Total Metal Contents in Fish Tissues

Dried fish tissues were transferred to porcelain basin and put to a Heraeus Thermicon P muffle furnace at a temperature of about 550°C for 4 to 5 h. Samples were digested with tri- acid mixture (HNO₃:HClO₄: H₂SO₄ = 10:4:1) at a rate of 5 mL per 0.5 g of sample and was placed on hot plate at 100°C temperature. Digestion was continued until the liquor was clear (AOAC, 1990). All the digested liquors were filtered through Whatmann 42 filter paper and diluted to 25 mL with distilled water.

Measurement of Heavy Metals by Atomic Absorption Spectrophotometer

All heavy metals were measured with a Perkin-Elmer Atomic Absorption Spectrophotometer (Model No. 3110) by specific cathode lamp using wavelength and potential detection limit for respective heavy metals as follows:

Heavy metals	Wavelength (nm)	Detection limit (µg g ⁻¹)
Pb	283.3	1.0
Cr	357.9	1.5

Heavy metal concentration was calculated as follows:

$$\mu\text{g g}^{-1} \text{ of metal in tissue material} = \frac{\mu\text{g g}^{-1} \text{ of metal in solution} \times \text{volume of acid}}{\text{g of sample}}$$

Statistical Modeling

The interactive metal effect was assessed by comparing the Metal Accumulation (MA) in single components (x,y) at the ith test level and at the concentration (x+y) i in binary mixtures where x and y are the concentration of the first and second metal ions, respectively. Statistical testing involved the following:

- Estimating the metal accumulation (MA) diff,

$$\text{Defined as MA diff} = \text{MA}_{(x+y)_i} - \text{MA}_{(x, y)_i} \tag{1}$$

- Estimating the standard error (SE)_{diff} of MA_{diff} and estimated t-value for each test level defined as

$$\text{SE}_{\text{diff}} = \{[\text{SE}_{(x+y)_i}]^2 + [\text{SE}_{(x, y)_i}]^2\}^{1/2} \tag{2}$$

$$t_{\text{estimated}} = \text{MA}_{\text{diff}} / \text{SE}_{\text{diff}} \tag{3}$$

- Comparing the t_{estimated} in Eq. 3 with the tabulated t value (Student's t) to determine if the MA_{diff} is statistically significant at 95% confidence level.
- Assessing the type of binary interaction at each test level.

If the difference was positive and statistically significant, the interaction was called synergistic implying that the metal accumulation in the binary mixture was higher than the additive interaction. If the difference was negative and statistically significant, the interaction was called 'antagonistic i.e., metal accumulation in the binary mixture was lower than the additive interaction. If the difference was statistically insignificant, the interaction was additive.

Data are presented as means±SE. The metal accumulation was fitted by applying regression analysis (Chapra and Canale, 1989). Statistical significance is indicated as follows: *P<0.05 and **p<0.01. All tissue concentrations are given as µg Pb or Cr g⁻¹ dry wt.

Results

Water quality parameters did not varied significantly ($p>0.05$) in all the treatments. There was no mortality observed and metal concentrations in water remained constant during 90 days exposure periods. The metal accumulation was determined according to regression equation, $Y = mX+C$, where Y is metal concentration ($\mu\text{g g}^{-1}$) in fish tissue and X is the exposure periods (days) and calculated according to least squares.

Lead Accumulation

Lead accumulated in muscle of *L. rohita* linearly with time to Pb alone, $Pb_{\text{muscle}} = 0.13d+0.08$, $R^2 = 0.94$ and to Pb+Cr mixture, $Pb_{\text{muscle}} = 0.04d+0.02$, $R^2 = 0.98$ ($p<0.001$). In mixer and Pb alone treatment, Pb concentration deviated from the initial value throughout exposure period. Lead concentration was lower in the mixture relative to Pb alone. Consistent difference in accumulation occurred in Pb+Cr mixture after 30 days onwards (Fig. 1a). Lead concentration in muscle maintained in the average of 0.67 and 0.19 $\mu\text{g g}^{-1}$ dry wt in Pb alone and Pb+Cr mixer, respectively during exposure period. Lead accumulated linearly with time over 90 days in the skin of *Labeo rohita* exposed to lead alone, $Pb_{\text{skin}} = 0.17 d+0.05$, $R^2 = 0.94$ and to Pb+Cr mixer, $Pb_{\text{skin}} = 0.07 d+0.04$, $R^2 = 0.98$. Significant differences in accumulation occurred in Pb+Cr mixture after 30 days onwards (Fig. 1c). Lead accumulation in gill could be described by the linear function with time, Pb_{gill}

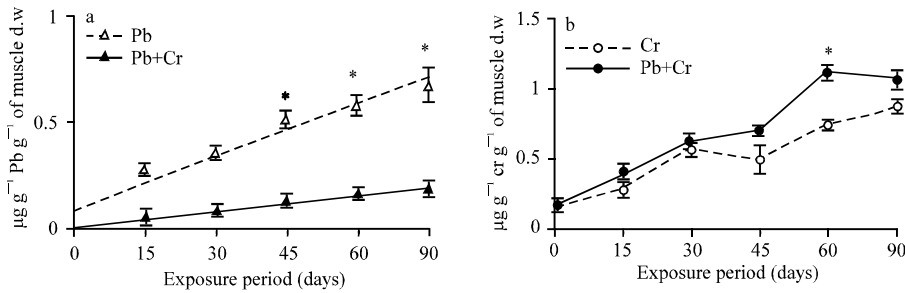


Fig. 1a,b: (a) Lead accumulation in muscle of *Labeo rohita* exposed to $0.10 \mu\text{g Pb L}^{-1}$ and $0.10 \mu\text{g Pb L}^{-1}+0.15 \mu\text{g Cr L}^{-1}$ (b) chromium accumulation in muscle of *Labeo rohita* exposed to $0.15 \mu\text{g Cr L}^{-1}$ and $0.10 \mu\text{g Pb L}^{-1}+0.15 \mu\text{g Cr L}^{-1}$ for 90 days. Each point indicates mean \pm SE for 3 numbers of fish. * indicate the difference between both the groups is statistically significant at 0.05 level

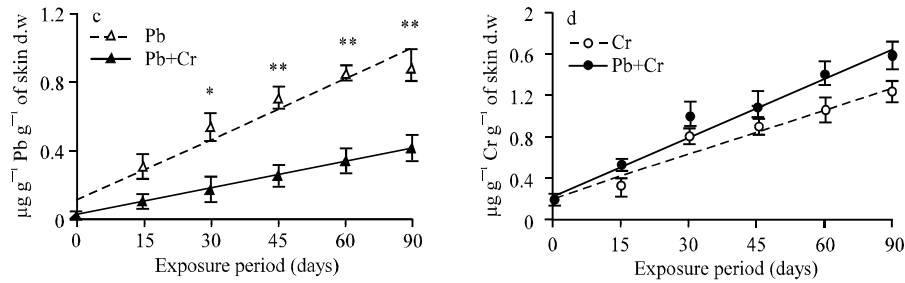


Fig. 1c,d: (c) Lead accumulation in skin of *Labeo rohita* exposed to $0.10 \mu\text{g Pb L}^{-1}$ and $0.10 \mu\text{g Pb L}^{-1}+0.15 \mu\text{g Cr L}^{-1}$ (d) chromium accumulation in skin of *Labeo rohita* exposed to $0.15 \mu\text{g Cr L}^{-1}$ and $0.10 \mu\text{g Pb L}^{-1}+0.15 \mu\text{g Cr L}^{-1}$ for 90 days. Each point indicates mean \pm SE for 3 numbers of fish. * and ** indicate the difference between both the groups is statistically significant at 0.05 and 0.01 levels

=1.09 d+0.84, $R^2 = 0.98$ and to Pb+Cr, $Pb_{gill} = 0.58 d + 0.38$, $R^2 = 0.90$. Lead concentration in mixture was lower than that of Pb alone. The mixture showed an increasing trend during the entire period but accumulation was significant only after 60 and 75 days compared to Pb alone (Fig. 1e). Lead accumulated linearly with time over 90 days in the liver of *Labeo rohita* exposed to lead alone, $Pb_{liver} = 0.48 d + 0.63$, $R^2 = 0.88$ and to Pb+Cr mixer, $Pb_{liver} = 0.22 d + 0.34$, $R^2 = 0.74$. Lead concentration in liver tissue in Pb alone treatment maintained higher values throughout the exposure than the mixture (Fig. 1g). Only significant difference observed in 75 days in Pb+Cr mixture. Lead accumulated linearly with time over 75 days in the kidney of *Labeo rohita* exposed to lead alone, $Pb_{kidney} = 0.18 d + 0.46$, $R^2 = 0.88$, to Pb+Cr mixer, $Pb_{kidney} = 0.08 d + 0.21$, $R^2 = 0.37$. In mixer and Pb alone treatment, Pb concentration deviated from the initial value throughout exposure period. Lead concentration was consistently lower in mixer than that of Pb alone treatment. The difference in Pb accumulation in Pb alone was significant only at 60 days ($p < 0.05$) while in Pb+Cr a consistent significant difference observed at 30, 45 and 60 days (Fig. 1i). Maximum lead concentration in kidney maintained in the average of 1.18 and $0.61 \mu g g^{-1}$ dry wt in Pb alone and Pb+Cr mixer, respectively during exposure period.

Chromium Accumulation

Chromium accumulation in muscle tissue of *Labeo rohita* showed an increasing trend in both the mixture and Cr alone treatment during the entire experimental period. In Pb+Cr mixture, the

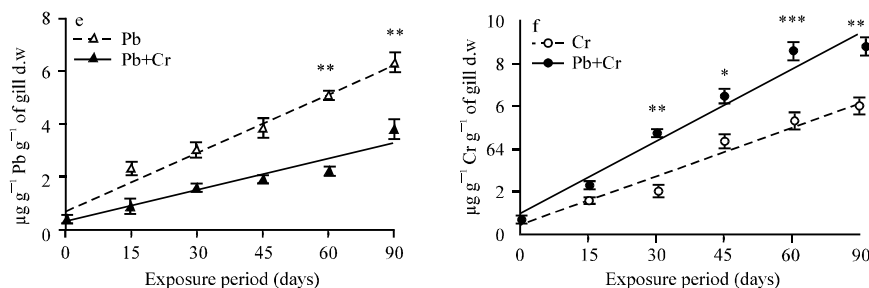


Fig.1e,f: (e) Lead accumulation in gill of *Labeo rohita* exposed to $0.10 \mu g Pb L^{-1}$ and $0.10 \mu g Pb L^{-1} + 0.15 \mu g Cr L^{-1}$ (f) chromium accumulation in gill of *Labeo rohita* exposed to $0.15 \mu g Cr L^{-1}$ and $0.10 \mu g Pb L^{-1} + 0.15 \mu g Cr L^{-1}$ for 90 days. Each point indicates mean \pm SE for 3 numbers of fish. and ** indicate the difference between both the groups is statistically significant at 0.05 and 0.01 level

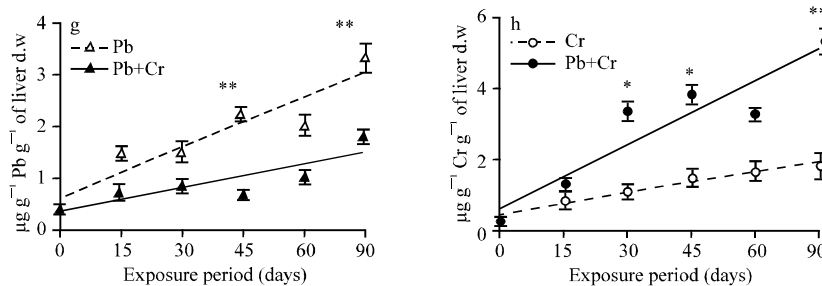


Fig.1g,h: (g) Lead accumulation in liver of *Labeo rohita* exposed to $0.10 \mu g Pb L^{-1}$ and $0.10 \mu g Pb L^{-1} + 0.15 \mu g Cr L^{-1}$ (h) chromium accumulation in liver of *Labeo rohita* exposed to $0.15 \mu g Cr L^{-1}$ and $0.10 \mu g Pb L^{-1} + 0.15 \mu g Cr L^{-1}$ for 90 days. Each point indicates mean \pm SE for 3 numbers of fish. * and ** indicate the difference between both the groups is statistically significant at 0.05 and 0.01 levels

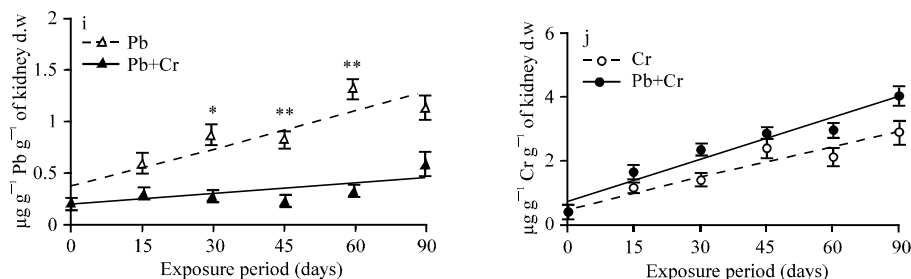


Fig. 1i,j: (I) Lead accumulation in kidney of *Labeo rohita* exposed to $0.10 \mu\text{g Pb L}^{-1}$ and $0.10 \mu\text{g Pb L}^{-1} + 0.15 \mu\text{g Cr L}^{-1}$ (j) chromium accumulation in kidney of *Labeo rohita* exposed to $0.15 \mu\text{g Cr L}^{-1}$ and $0.10 \mu\text{g Pb L}^{-1} + 0.15 \mu\text{g Cr L}^{-1}$ for 90 days. Each point indicates mean \pm SE for 3 numbers of fish. * indicate the difference between both the groups is statistically significant at 0.05 level

concentration of Cr was greater than Cr alone treatment but the differences was not significant (Fig. 1b). Chromium concentration in muscle tissue in Cr alone and Pb+Cr mixture increased 0.12 to 0.85 and $1.36 \mu\text{g g}^{-1}$ dry wt, respectively. Chromium accumulated in skin of *Labeo rohita* linearly with time to Cr alone, $\text{Cr}_{\text{skin}} = 0.27\text{d} + 0.02$, $R^2 = 0.97$ and to Pb+Cr mixture, $\text{Cr}_{\text{skin}} = 0.22\text{d} + 0.02$, $R^2 = 0.92$ ($p < 0.001$). In the mixer Cr accumulation was more than Cr singly treatment but it was not significant. Higher Cr accumulation occurred in $1.56 \mu\text{g g}^{-1}$ dry wt. in Pb+Cr compared to $1.18 \mu\text{g g}^{-1}$ dry wt in Cr alone (Fig. 1d). Exposure to Cr alone resulted in a linear increase with time in the gill, Cr concentration in gill tissue i.e., $\text{Cr}_{\text{gill}} = 1.12 \text{d} + 0.82$, $R^2 = 0.95$. Chromium accumulated linearly with time in gill exposed to Pb+Cr mixture, $\text{Cr}_{\text{gill}} = 1.67 \text{d} + 1.12$, $R^2 = 0.95$. The slope of regression line was significantly higher for group exposed to Pb+Cr than for the group exposed to Cr alone. In the mixture, Cr was highly accumulated and significant difference resulted after 30 days and maintained during the rest of the exposure (Fig. 1f). Higher Cr accumulation occurred in $8.35 \mu\text{g g}^{-1}$ dry wt in Pb+Cr compared to $6.0 \mu\text{g g}^{-1}$ dry wt in Cr alone. Chromium concentration in the liver resulted linearly with time exposed to Cr alone and Pb+Cr, $\text{Cr}_{\text{liver}} = 0.31 \text{d} + 0.48$, $R^2 = 0.94$ and $\text{Cr}_{\text{liver}} = 1.23 \text{d} + 0.89$, $R^2 = 0.93$ ($p < 0.001$), respectively. Chromium accumulation was higher in mixture than that of Cr alone. Higher significant slope of regression line of the mixture resulted than for the group exposed to Cr alone. The mixture showed significant difference after 30 days and continued during the exposure period (Fig. 1h). Chromium concentration in the liver increased from 0.25 to 1.84 and $5.28 \mu\text{g g}^{-1}$ dry wt in Cr alone and Pb+Cr, respectively. The accumulation of Cr in kidney linearly elevates with time exposed to Cr alone, $\text{Cr}_{\text{kidney}} = 0.48 \text{d} + 0.49$, $R^2 = 0.90$ and to Pb+Cr mixture, $\text{Cr}_{\text{kidney}} = 0.66 \text{d} + 0.65$, $R^2 = 0.93$. Chromium concentration in Pb+Cr mixture showed an increasing trend but not statistically significant (Fig. 1j). There were no consistent differences between groups exposed to Cr and Pb+Cr. Maximum chromium concentration in kidney maintained in the average of 2.88 and $4.71 \mu\text{g g}^{-1}$ dry wt. in Cr alone and Pb+Cr mixer, respectively during exposure period.

Discussions

Lead Accumulation

Lead is accumulated significantly in gills, liver, kidney, skin and muscle tissues of *Labeo rohita* for 90 days exposure in all Pb treatments. After 90 days of exposure to Pb alone and in binary mixture to Pb+Cr, the distribution of Pb was in the order of gills > liver > kidney > skin > muscle. Gills concentrated Pb in higher amounts and muscle accumulated only small amounts in the binary mixture and Pb alone because in freshwater fishes gills might be expected to be the primary route for the uptake of waterborne pollutants (Allen *et al.*, 1988). WHO (1989) reported that all of the lead which causes

toxic effects in fish is taken up directly from the water via the gills, the present study showed a similar accumulation of Pb in the gill. Oladimaji and Offem (1989) noticed in *O. niloticus*, the gill consistently accumulated higher amounts of lead as lead nitrate. Sorensen (1991) and Allen (1995) reported very little amount of Pb is accumulated in edible muscle tissue which corroborates with the present study. Lead levels in skeletal muscle tissue than those of other tissues due to low binding rate of Pb to sulphhydryl groups in muscle (Sorensen, 1991). *Labeo rohita* exposed to binary mixture of Pb+Cr resulted in lesser content of Pb as compared to fish exposed to Pb alone in all tissues for 90 days. This may occur due to larger ionic radii of Pb (1.20 Å) can not compete with Cr (0.64 Å) from same uptake site, resulting decrease in Pb uptake. The greater the ionic radius lesser will be the chance of polarization to the protein. Lead accumulation in binary mixture Pb+Cr significantly decreases than Pb alone in all tissues of fish. The observed results of this mixture of Pb+Cr showed that interactive effects of Cr on Pb accumulation were antagonistic in nature in all tissues of fish. This implies that when two metal ions were applied together, Pb interfered with the action of the other. The presence of Cr reduces the uptake as well as accumulation of Pb in Pb+Cr mixture.

Chromium Accumulation

Chromium is accumulated significantly in gills, liver and kidney tissues of *Labeo rohita* for 90 days exposure in Cr treatment. A consistent significant difference was maintained in gills, liver and kidney tissue in binary mixture of Pb+Cr with single applied Cr, whereas in muscle and skin tissues there was no difference in accumulation. After 90 days of exposure to Cr alone and in binary mixture to Pb+Cr, the distribution of Cr was in the order of gills > kidney > liver > skin > muscle. Similar findings were reported by Buhler *et al.* (1977) that highest concentrations were in kidney, spleen, gut and bone in rainbow trout. Later on, Vander Putte *et al.* (1981) observed that maximum accumulation occurred in gill, kidney and liver tissues of rainbow trout. On the otherhand, Vincent and Ambrose (1994) investigated uptake of Cr in *Catla catla*. was in the order of kidney > intestine > gill > liver > brain tissues. Chromium accumulation in binary mixture Pb+Cr significantly increases than Cr alone in all tissues of fish. The observed results of this mixture of Pb+Cr showed that interactive effects of Pb on Cr accumulation were synergistic in nature in all tissues of fish. This implies that when two metal ions were applied together, Cr interfered with the action of the other. Chromium uptake was much higher in binary mixture than when it was applied alone. So, Pb has synergistic effect on Cr accumulation in all tissues of *L. rohita* fingerlings. This may be due to smaller ionic radii of Cr (0.64 Å) as compared to Pb (1.20 Å). The more highly charged Cr^{+6} is the more effective it is polarizing the thionene protein. Polarising power of a cation is dependent on its charge/radius ratio and is greatest for small highly charged cations. Polarising power of Cr^{+6} is greater than that of Pb^{2+} . The uptake of chromium will be more, resulting increase of accumulation. Because of very limited literature on the toxicity of aquatic organism of metal-metal interaction of Pb and Cr and there is no published studies on toxicities of Pb-Cr are available for comparison with the present result. From this study it is obvious that increasing exposure time does not result in increased chromium content in all organs because muscle reaches a steady-state after 60 days.

Conclusions

From this study it can be concluded that bioaccumulation of Pb and Cr depends on solution conditions, nature of metal ion (involving metal ions radius), exposure concentration as well as fish species and its physiological conditions. However, it is also clear from this study that lead accumulation decreases in presence of chromium which is an essential trace element at lower concentration but lead are toxic at lower concentration also in *Labeo rohita*. Levels of contaminants in fish are of particular interest because of the potential risk to humans who consume them. This study indicates though gill, liver and kidney accumulated Pb and Cr above permissible level but muscle and skin accumulated below human consumption level.

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