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IMPACT OF CHRONIC EXPOSURE TO HEAVY METAL MIXTURES ON SELECTED BIOLOGICAL PARAMETERS OF FRESHWATER FISH SPECIES

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ABSTRACT

Heavy metal contamination usually causes depletion in feed utilization in fish, and such disturbance may result in reduced metabolic rate and hence causing reduction in their growth. The present study assessed theharmful effect of sub-lethal concentrations of four different heavy metal mixturesi.e., Mix-I (Mn+Ni), Mix-II(Mn+Ni+Pb), Mix-III (Mn+Pb+Zn+Fe), and Mix-IV (Mn+Ni+Pb+Zn+Fe) on five fish species including *Labeorohita*, *Catla catla,Cirrhinamrigala, Hypophthalmichthys molitrix*, and *Ctenopharyngodonidella.*The experiment was carried out in triplicatefor each species separately in glass aquarium for 12 weeks. The results showed a significant*($p < 0.001$)*effect on the treated group as compared to the control group. The average weight gain was as *C. catla*>*L. rohita*>*C. mrigala*>*C. idella*>*H. molitrix.* The condition factor was as *C. idella*>*C. mrigala*>*L. rohita*>*C. catla*>*H. molitrix.* Similarly, the feed intake also reduced significantly *($p>0.001$)* in the treated group, and the feed conversion efficiency showed significant *($p>0.05$)* effect of metal mixtures on fish metabolism and growth activity. The current study concluded that the studied heavy metals are toxic to different fish species, even at sub-lethal concentrations, and affect different biological parameters of fish growth.

Keywords:heavy metals; chronic exposure;freshwater fishes;growth performance; feed conversion efficiency

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INTRODUCTION

Heavy metal pollution is a serious problem for the environment because of their harmful properties like toxicity, insolubility, bioaccumulation, and biomagnification (Garai *et al.*, 2021). They are not easily biodegradables;therefore, they mayaccumulate and stay actively persistent in the environment and possibly flow to the aquatic environment, threaten fish health, and pose human health problems via food-web (aquatic consumption). Recently, heavy metal contamination of aquatic pollution becamea global problem and gained high research interest due to their direct/indirect adverse effects on the aquatic environment and their possible effect on human health. In today's advanced era, a thousand complex health hazard substances enters into the aquatic environment from human consumption which alter the natural aquatic biota (Sallam *et al.* 2018;Akinkuolie *et al.*, 2021). The routes of toxicities cause by heavy metals in aquatic food chainis well understood (dietary consumption, direct water and biotaconsumption and non-dietary consumption, indirectfish epithelial absorption), as a result, aquatic organism accumulate heavy metal concentration many time higher than the surrounding medium and play a key role in transfer of these health hazard metals from one tropic level to another (Rakib *et al.*, 2021).Toxic levels of these heavy metals significantly affect an organism's physical and chemical processes; thus, pollution levels in both water bodies and fish are clearly monitored to retain aquatic food quality(Azmat and Javed, 2011; Naz *et al.*, 2021).

Freshwater fishes are considered more vulnerable and exposed as compared to marine fishes. As freshwater fish gain more water and lose salt while marine fish gain more salt and lose water due to their surrounding water salt concentration (Nikinmaa, 2014). Fish gets heavy metals either by ingestion, gills and skin (Ion exchange) or by tissue absorption (Ahmed *et al.*, 2014). These heavy metal accumulation in fish different body parts depends on many factors, such as feeding behavior, water solubility, physiology and ecology of fish, including fish size, age, health, species, reproductive health, different habitats, and bioavailability (Perugini *et al.*, 2014; Anandkumar *et al.*, 2017)**.** Different studies have been conducted on the heavy metals contamination effect on aquatic environments and fish fauna. Like, Adewoye *et al.* (2010) reported that various substances released from modern complex human societies that enter the aquatic ecosystems alter survivability of aquatic biota.Chronic exposure of fish to water-borne Cu, Cd or Zn has been shown to cause a variety of physiological and behavioral changes, including loss of appetite, reduced growth, lower feed conversion ratio, ionic loss and increased fish mortality(Gharedaashi *et al.*, 2013). Growth is a sensitiveand reliable endpoint in chronic toxicological investigations(De Boeck *et al.*, 1997; Hafeez *et al.* 2020).

It is, therefore, essential to undertake studies to document the consequence of heavy metals exposure in freshwater fishes that adversely affect their physiology, growth, and survival(Akinkuolie *et al.*, 2021).Major and Chinese carps were included in this study due to their significance in fisheries and suitability for various culture systems. They can tolerate harsh aquatic conditions, easy to reproduce, and are resistant to several diseases(Hayat, 2009; Zubair *et al.*, 2020). The present study aimed to assess the effect of various metal mixtures (iron, nickel, zinc, lead, and manganese) on the growth performance of five different fish species, i.e., *Labeorohita, Catla catla, Cirrhinamrigala, Hypophthalmichthys molitrix, and Ctenopharyngodonidella*.

MATERIALS AND METHODS

Fish Culture: Ninety days old (post-fertilization) fingerlings of *Labeorohita*, *Catla catla,Cirrhinamrigala, Hypophthalmichthys molitrix*, and *Ctenopharyngodonidella*were obtained from fish seed hatchery inFaisalabad, Punjab, Pakistan.Priorto the start of experiment, the obtained selected fish species were measured for their weight, fork length, and total length and acclimatized at 25±2°C, and 12:12; L:D period under laboratory conditions for 15 days in a self-made glass aquarium (30″ × 12″ × 18″) having capacity of 60 liters. The aquarium was equipped with a thermostatic electric heater (26º C). The fish were reared in reverse osmosis (RO) purified water maintained at neutral pH to minimize the effect of water quality parameters during theexperimentation. Fresh air was supplied to each aquarium through an air pump (Sobo, Model: AP-200) fitted with a capillary system.

Preparation of stock and treatment solutions: Stock solutions (mgL^{-1}) of studied metals, i.e., Lead (Pb), Nickel (Ni), Zinc (Zn), Manganese (Mn), and Iron (Fe) were prepared by dissolving required quantities of their respective Chlorides,i.e., $PbCl_2$, $NiCl_2.6H_2O$, $ZnCl_2$, $MnCl_2.H_2O$, and $FeCl_2.4H_2O$ (Sigma Aldrich, 99% Purity), in double distilled, deionized water. The four metals mixtures viz, Mix. I (Mn+Ni), Mix. II (Mn+Ni+Pb), Mix. III (Mn+Pb+Zn+Fe), and Mix. IV (Mn+Ni+Pb+Zn+Fe) were prepared by mixing suitable quantities (in equal ratio from each metal) from stock solutions of each metal based on metallic ion equivalents. The chronic concentrations of prepared mixtures (Mix. I, II, III, & IV) for each fish species were calculated by the following formula:

$$HMMC_E = \frac{HMMC_{LC50}}{3}$$

Whereas, $HMMC_E$is the exposed concentration of heavy metals mixtureto each fish species, while$HMMC_{LC50}$ is the 90 hours LC_{50} concentration of heavy metal mixture for each fish species(**Table 1**). LC_{50} concentrations of studied heavy metal mixtures were based on the study ofNaz and Javed (2013).

Chronic exposure of fishes to metals mixtures: The study was conducted on five selected fish species reared in different aquariums with a stocking density of 1 fish / 6 L of water. The studied fish species were divided into five groups (one control and four treatments) for each fish species. The control group was kept untreated while the other four groups were exposed to chronic concentrations of four different metal mixtures in a glass aquarium for 12weeks (Table 1b), according toJaved and Yaqub (2010). The experiment was conducted ata controlled temperature (25 ± 2ºC) and relative humidity (65%) under 14: 10, L: D photoperiod. The fish were fed twice a day with a diet prepared in the lab **(Table 2)**. During the experiment, physiological parameters like specific growthrate, feed intake, change in wet weights, feed conversion efficiency, and condition factor were measured weekly by carefully taking the fish out of the tank and releasing them back to their respective tank after recording the required data. The trial ended after 12 weeks, and weekly data was analyzed. Each group had three replications.

Effect of metal mixtures on Condition Factor: The following formula was used to calculate the effect of metals mixtures on the condition factor of various fish species:

$$K = \left(\frac{W \times 105}{L^3}\right)$$

Whereas; K = *condition Factor*, W = *Wet fish weight (g)* and L = *Wet fork length (mm)*

Effect of metal mixtures on Feed Conversion Efficiency: The effect of metals mixtures on feed conversion efficiency was calculated using the following formula:

$$FCE = G_w - F_i$$

Whereas; FCE = *Feed Conversion Efficiency*, G_w = *Gain in wet weight of the fish (g)*, F_i = *Feed Intake (g)*

The effect of metals mixture on the specific growth rate of different fish species was calculated using the following formula:

$$SGR = \left(\frac{W_f - W_i}{T}\right) \times 100$$

Whereas; SGR = *Specific Growth Rate*, W_i = *Initial weight of the fish (g)*, W_f = *Final weight of the fish (g)* and T = *Time in days*

Statistical analyses: Different physiological parameters were analyzed using One-way Analysis of variance (ANOVA) in IBM SPSS Statistics (Version 25). Tuckey's post hoc test was used to compare the means of different groups for each studied parameter in IBM SPSS Statistics (Version 25). Furthermore, Regression analyses were also carried out to quantify the relationships among various studied parameters.

RESULTS

Effect on Body Weight Gain:All four metals mixture significantly affected the gain of body weight in all five fish species compared to the control treatment. The obtained results showed that among different exposure treatments, maximum weight gain in *C. catla*was observed during the exposure of Mix. I (12.07±0.03g), followed by Mix. II (11.01±0.02g) and Mix. IV (10.97±0.02g), while minimum weight gain was found in Mix. III (10.81±0.02) exposure. In *L. rohita* the Mix. I showed the maximum (11.96 ± 0.08 g) weight gain, while fish exposed to Mix. III showed a minimum (10.84 ± 0.04 g) weight gain at the end of the trial. The results showed that *H. molitrix, C. mrigala*, and *C. idella*were most affected by Mix. IV with least weight gain of 10.07 ± 0.05 g, 10.02 ± 0.01g, and 10.05 ± 0.01g, respectively. Among 4 metals mixtures, the Mix. I (Mn+Ni) had the least effect on weight gain with mean weight gain of 12.23 ± 0.02 g in *C. idella*while Mix. IV (Mn+Ni+Pb+Zn+Fe) caused significantly lower weight gain (10.02 ± 0.01 g) in *C. mrigala*. Among the five studied fish species, the mean weight gain was significantly different and was in the order of (*C. catla*>*L. rohita*>*C. idella*>*H. molitrix*>*C. mrigala*). The results showed that *C. mrigala* was most affected by the metals mixture exposure. Furthermore, the fish in the control treatment had significantly higher weight gain than all the four metals mixture exposed groups **(Table 3)**.

Effect on Condition Factor:After exposure to various metal mixtures, different treatment groups showed significantly varied conditions factor. Fishes exposed to Mix. I showed a significantly higher value of condition factor (2.20 ± 0.22), while the lowest condition factor (1.42 ± 0.30) was observed under the influence of Mix. II. Among different fish species, *C. idella*gained a significantly higher average condition factor (1.89 ± 0.36), followed by *C. mrigala*, *L. rohita, C. catla*and *H. molitrix***(Table 3)**.

Effect on Fish Feed Intake: The feed intake among different fish species was highest in Mix. IV treatment for *C. idella* (21.92 ± 0.02g) while the lowest feed intake was recorded in Mix. II for *C. catla* (14.65 ± 0.02g). Among treated fish species the mean feed intake was in order of *C. idella*(19.83 ± 2.33g) >*H. molitrix* (19.27 ± 1.90g) >*L. rohita* (17.50 ± 1.15g) >*C. mrigala* (16.83 ± 1.72g) >*C. catla* (15.83 ± 1.15g). Furthermore, the fish in the control treatment exhibited significantly higher feed intake than the treatment groups **(Table 3)**.

Effect on Feed Conversion Efficiency: Regarding feed conversion efficiency, a significant effect of metals mixture exposure was observed in all five fish species against all four treatments andthe control treatment. Overall, Mix. I was observed to have the least effect on feed conversion efficiency (73.23±2.87%) while Mix. IV (Mn+Ni+Pb+Zn+Fe) exhibited the most pronounced effect of metals mixture exposure on feed conversion efficiency (59.20±8.18%). *Catla catla*attained significantly higher feed conversion efficiency (71.11±5.61%) among the treated fish, followed by *C. mrigala* (67.30±8.18%) *L. rohita* (66.04±5.83%), *H. molitrix*(59.80±9.25%), and *C. idella*(58.02 ± 10.8 %). The results showed that the highest feed conversion efficiency was observed in *C. mrigala* (76.33±0.05%) for Mix. I, while the least feed conversion efficiency was found in *H. molitrix*(48.09±0.02%) for the Mix IV. Furthermore, the feed conversion efficiency of the control group was significantly higher than all exposed groups **(Table 3)**.

Effect on Specific Growth Rate: Results showed a significantly lower specific growth rate in treatment groups than in control. Among exposed fishes, *C. catla* exhibited the highest mean specific growth rate (17.28±5.80), while *C. idella*, *L. rohita,C. mrigala* and *H. molitrix* showed a non-significant difference among mean values of their respective specific growth rate. Mix. III showed a significantly lower effect (18.14±0.02) on the specific growth rate of *C. catla* while the effect of Mix. II was more pronounced, causing a significantly lower specific growth rate (7.29±0.01) in *H. molitrix* **(Table 3)**.

Effect of Feed Conversion Efficiency and Feed Intake on Fish Growth: Effect of feed conversion efficiency and feed intake on the growth rate of five fish species exposed to four different metals mixtures was studied **(Table 4)**. The results showed that *C. catla* exhibited 30.01% dependency on feed conversion efficiency, while the combined effect of feed conversion efficiency along with feed intake was 81.19% on the growth performance of this fish species. In a two-step regression model, the first step of the regression equation for *C. mrigala*produced 18.40% change in fish growth due to feed conversion efficiency with a non-significant positive regression coefficient. While step-two of the regression equation showed a significantly positive impact of both feed conversion efficiency and feed intake on the growth rate of this fish species. The effect of metals mixtures (Mix I, II, III, and IV) on growth rate of *C. idella*exposed to these metal mixtures was found to be 34.90% caused by feed conversion efficiency. Furthermore, the combined contribution of feed conversion efficiency and feed intake was 75.86% for the growth performance of this fish species. However, the defined variables for *L. rohita* and *H. molitrix* did not meet the criteria of step-wise regression analyses, so the output could not be constructed for these fish species.

Table 1a: Average weight (g), fork length (mm), and total length (mm) of five fish species exposed to sub-lethal concentrations of metals mixtures (Mix I, II, III, IV).

Mixture	Metals in mixture	Fish Species	Average weight (g ± S.D)	Average fork length (mm ± S.D)	Average total length (mm± S.D)
		Catla catla	3.37±1.86	57.13±11.86	65.95±12.06
		Labeorohita	5.65±2.09	75.17±11.83	84.24±11.30
Mix. I	Ni+Mn	*Cirrhinamrigala*	4.45±2.01	72.87±11.82	81.51±11.31
		Ctenopharyngodonidella	4.06±1.65	70.30±12.28	80.06±12.02
		Hypophthalmychthys molitrix	3.76±11.60	65.01±11.52	74.87±11.72
		Catla catla	3.35±1.86	57.14±11.85	65.92±12.08
		Labeorohita	5.67±2.07	75.15±11.87	84.19±11.25
Mix. II	Pb+Ni+Mn	*Cirrhinamrigala*	4.50±2.03	72.92±11.82	81.64±11.37
		Ctenopharyngodonidella	4.15±1.67	70.42±12.24	80.15±12.04
		Hypophthalmychthys molitrix	3.95±11.84	65.17±11.57	74.99±11.76
		Catla catla	3.35±1.85	57.09±11.86	65.90±12.01
		Labeorohita	5.67±2.08	75.14±11.83	84.21±11.26
Mix. III	Fe+Zn+Pb+Mn	*Cirrhinamrigala*	4.56±2.01	72.98±11.89	81.62±11.37
		Ctenopharyngodonidella	4.17±1.67	70.45±12.28	80.19±12.02
		Hypophthalmychthys molitrix	3.80±11.80	65.04±11.57	74.88±11.74
		Catla catla	3.26±1.89	57.02±11.88	65.84±12.01
		Labeorohita	5.56±2.07	75.03±11.84	84.11±11.28
Mix. IV	Fe+Zn+Pb+Ni+Mn	*Cirrhinamrigala*	4.43±2.03	72.84±11.81	81.50±11.33
		Ctenopharyngodonidella	4.12±1.67	70.32±12.27	80.12±12.02
		Hypophthalmychthys molitrix	3.83±11.87	65.05±11.58	74.90±11.74

Table 1b: Used Concentrations ($mgL^{-1} \pm$ S.E) and 96-hours LC_{50} of heavy metals mixtures used in this study for five fresh water fish species.

Fish Species	Fish Species							
	Mix. I		Mix. II		Mix. III		Mix. IV	
	$HMMC_E$ ($mgL^{-1} \pm$ S.E)	$HMMC_{LC50}$ ($mgL^{-1} \pm$ S.E)	$HMMC_E$ ($mgL^{-1} \pm$ S.E)	$HMMC_{LC50}$ ($mgL^{-1} \pm$ S.E)	$HMMC_E$ ($mgL^{-1} \pm$ S.E)	$HMMC_{LC50}$ ($mgL^{-1} \pm$ S.E)	$HMMC_E$ ($mgL^{-1} \pm$ S.E)	$HMMC_{LC50}$ ($mgL^{-1} \pm$ S.E)
C. catla	25.32±1.97	75.95±0.45	22.87±1.05	68.62±0.62	15.53±0.91	46.60±0.59	14.09±0.55	42.26±0.58
L. rohita	29.15±1.93	87.45±0.63	24.07±1.07	72.20±0.75	22.90±0.50	68.70±0.43	20.65±0.61	61.94±0.42
C. mrigala	22.66±2.01	67.99±0.46	22.05±2.01	66.15±0.39	14.44±1.32	43.32±0.57	14.45±1.09	43.35±0.78
C. idella	30.64±1.48	91.92±1.10	21.48±1.34	64.45±0.94	17.20±1.26	51.60±0.87	17.45±1.39	52.36±1.17
H. molitrix	25.43±1.98	76.28±1.23	19.55±1.23	58.66±0.58	13.82±1.12	41.45±1.03	10.42±1.66	31.25±1.24

† $HMMC_E$ = Exposed concentration of heavy metals mixtures to the fish species; $HMMC_{LC50}$ = 96-hours LC_{50} of heavy metals mixtures use

Table 2: Fish feed prepared in the lab.

Ingredients of diet	Percentage (%)	
Corn Gluten (30% CP)	39.27	
Wheat flour	5.00	
Fish meal	40.00	
Rice polish	07.51	
Vitamin and Mineral Mixture	05.00	
Oil (Sun flower)	03.22	
Digestible Protein (DP) = 35%	**Major Profile (%)**	
Digestible Energy (DE) = 2.90 $Kcalg^{-1}$		
	Methionie	0.8205
	Lysine	1.9365
	Ca^{++}	2.2659
	Na^{+}	0.3244
	PO_4^{--}	1.2023

Table 3: Effect of chronic exposure of different metals mixtures on various physiological Parameters of fish Growth.

Parameters studied	Metals Mixture (Mix.)	Fish Species					Treatment Means
		C. catla	*L. rohita*	*C. mrigala*	*C. idella*	*H. molitrix*	
Increase in Weight (g)							
	I	12.07±0.03^{b†}	11.96±0.08^{b}	11.93±0.04^{b}	12.23±0.02^{b}	12.01±0.03^{b}	**12.04±0.12^{b}**
	II	11.01±0.02^{c}	11.05±0.01^{c}	11.10±0.01^{c}	11.12±0.02^{c}	11.17±0.07^{c}	**11.09±0.06^{c}**
	III	10.81±0.02^{e}	10.84±0.04^{e}	10.87±0.07^{d}	10.92±0.04^{d}	10.93±0.03^{d}	**10.87±0.05^{d}**
	IV	10.97±0.02^{d}	10.99±0.08^{d}	10.02±0.01^{e}	10.05±0.01^{e}	10.07±0.05^{e}	**10.82±1.28^{e}**
	Control	**23.77±0.02^{a}**	**28.03±0.02^{a}**	**27.66±0.05^{a}**	**23.65±0.02^{a}**	**23.87±0.04^{a}**	**25.40±2.24^{a}**
	Treated Fish Means		**11.21±0.57^{A‡}**	**11.21±0.50^{A}**	**10.98±0.78^{D}**	**11.08±0.89^{B}**	**11.04±0.79^{C}**
Condition Factor							
	I	2.16±0.04^{b}	2.05±0.05^{b}	2.55±0.02^{a}	2.28±0.02^{a}	1.98±0.03^{a}	**2.20±0.22^{a}**
	II	1.32±0.02^{e}	1.25±0.04^{e}	1.36±0.03^{d}	1.95±0.01^{c}	1.20±0.02^{e}	**1.42±0.30^{e}**
	III	1.73±0.02^{c}	1.83±0.02^{d}	1.88±0.03^{c}	1.63±0.02^{e}	1.85±0.05^{b}	**1.78±0.10^{c}**
	IV	1.47±0.01^{d}	2.01±0.03^{c}	1.34±0.04^{e}	1.71±0.02^{d}	1.46±0.03^{d}	**1.60±0.27^{d}**
	Control	**2.55±0.04^{a}**	**2.09±0.04^{a}**	**2.21±0.05^{b}**	**2.22±0.04^{b}**	**1.73±0.02 ^{c}**	**2.16±0.27^{b}**
	Treated Fish Means	**1.67±0.42^{C}**	**1.78±0.41^{B}**	**1.78±0.59^{B}**	**1.89±0.36^{A}**	**1.62±0.41^{D}**	
Feed Intake (g)							
	I	16.04±0.02^{c}	17.18±0.08^{d}	15.63±0.06^{d}	16.50±0.04^{e}	16.98±0.03^{e}	**16.47±0.64^{e}**
	II	14.65±0.02^{e}	19.12±0.02^{b}	17.95±0.04^{c}	20.65±0.04^{c}	18.44±0.02^{d}	**18.16±2.21^{c}**
	III	15.29±0.03^{d}	16.40±0.04^{e}	15.10±0.02^{e}	20.28±0.02^{d}	20.68±0.02^{c}	**17.55±2.72^{d}**
	IV	17.33±0.03^{b}	17.33±0.02^{c}	18.62±0.02^{b}	21.92±0.02^{b}	20.98±0.01^{b}	**18.88±2.60^{b}**
	Control	**22.21±0.03^{a}**	**22.11±0.02^{a}**	**19.48±0.03^{a}**	**23.49±0.02^{a}**	**22.97±0.02^{a}**	**22.05±1.51^{a}**
	Treated Fish Means	**15.83±1.15^{E}**	**17.50±1.15^{C}**	**16.83±1.72^{D}**	**19.83±2.33^{A}**	**19.27±1.90^{B}**	
Feed Conversion Efficiency (%)							
	I	75.25±0.25^{b}	69.73±0.05^{c}	76.33±0.05^{b}	74.12±0.04^{b}	70.73±0.04^{b}	**73.23±2.87^{b}**
	II	75.15±0.05^{c}	57.79±0.04^{e}	61.78±0.03^{d}	53.89±0.02^{c}	60.57±0.02^{c}	**61.84±8.04^{d}**
	III	70.75±0.05^{d}	66.09±0.01^{d}	71.99±0.02^{c}	53.75±0.01^{d}	59.83±0.02^{d}	**63.08±9.21^{c}**
	IV	63.30±0.05^{e}	70.58±0.02^{b}	59.12±0.03^{e}	50.32±0.02^{e}	48.09±0.02^{e}	**59.20±8.18^{e}**
	Control	**107.02±0.02^{a}**	**126.77±0.01^{a}**	**141.99±0.0^{a}**	**100.68±0.0^{a}**	**103.91±0.01^{a}**	**114.25±16.06^{a}**
	Treated Fish Means	**71.11±5.61^{A}**	**66.04±5.83^{C}**	**67.30±8.18^{B}**	**58.02±10.8^{E}**	**59.80±9.25^{D}**	
Specific Growth Rate							
	I	17.14±0.04^{d}	15.14±0.04^{c}	12.00±2.00^{c}	17.14±0.04^{c}	15.00±0.05^{b}	**17.28±5.80^{b}**
	II	17.57±0.02^{c}	14.29±0.02^{d}	10.71±0.02^{e}	18.00±0.01^{b}	7.29±0.01^{e}	**13.57±4.58^{c}**
	III	18.14±0.02^{b}	15.29±0.01^{b}	15.29±0.02^{b}	15.43±0.03^{d}	11.86±0.06^{c}	**13.20±4.95^{d}**
	IV	15.14±0.02^{e}	11.14±0.03^{e}	11.57±0.03^{d}	12.71±0.03^{e}	11.71±0.02^{d}	**12.45±1.61^{e}**
	Control	**46.87±0.03^{a}**	**29.44±0.03^{a}**	**28.44±0.03^{a}**	**26.58±0.03^{a}**	**44.72±0.03^{a}**	**35.21±9.74^{a}**
Treated Fish Means		**16.99±0.50^{A}**	**13.97±5.54^{C}**	**12.39±2.36^{D}**	**15.82±1.30^{B}**	**11.46±3.88^{E}**	

† The means with different letters (a – e) in a single column are statistically different at $p< 0.05$ according to Tuckey's Student Newman-Keul tests.
‡ The means with different letters (A – E) in a single row are statistically different at $p< 0.05$ according to Tuckey's Student Newman-Keul tests.

Table 4: Step-wise (Two-Steps) Regression (R^2) Analysis to Quantify the Effects of Feed Conversion Efficiency (Alone and in Combination with Feed Intake) on the Growth Rate of Different Fish Species.

Fish species	Step-wise regression	r/Mr	R^2
C. catlaC. catla	Increase in weight (g) = 9.6691-0.0248 (FCE)………..Step 1 S.E. = 0.092 *	0.5478	0.3001
	Increase in weight (g) = -5.7131+0.1127 (FCE) +0.5642 (Feed intake) ………..Step 2 S.E. = 0.0142 ** 0.0855**	0.9011	0.8119
Labeorohita	**No variables meet the criteria**		
Cirrhinamrigala	Increase in weight (g) = 9.1125+0.043 (FCE) ………..Step 1 S.E . = 0.0206 ^{NS}	0.4290	0.1840
	Increase in weight (g) = -4.9641+0.1252 (FCE) +0.4683 (Feed intake) ………..Step 2 S.E. = 0.0203** 0.0875 **	0.8411	0.7074
Ctenopharyngodon Idella	Increase in weight (g) = 8.9595+0.431 (FCE) ………..Step 1 S.E. = 0.0143*	0.5908	0.3490
	Increase in weight (g) = -8.0648+0.1639 (FCE) +0.4928 (Feed intake) ………..Step 2 S.E. = 0.0246 ** 0.094**	0.8709	0.7585
Hypophthalmicthys molitrix	**No variables meet the criteria**		

Note: FCE = Feed conversion efficiency; * = Significant at $p<0.05$; ** = Significant at $p<0.01$; ^{NS} = Non-significant; R^2 = Coefficient of determination

DISCUSSION

Heavy metals and their mixtures seriously threaten aquatic organisms like fish. Modern society has recently faced a significant issue in the form of environmental contamination. The toxicity of heavy metals for living things and marine life makes them a more well-known and serious problem among environmental contaminants. Heavy metals are a special category of naturally occurring elements that last for a very long period in the environment and are not biodegradable (Chatha *et al.*, 2023). Our investigation on the effect of heavy metal mixtures (Mix I, II, III, and IV) on five fish species, demonstrated adverse effects of these mixtures at sub-lethal concentrations to different fish species. The obtained results showed that the Mix. III (Mn+Pb+Zn+Fe) and Mix. IV (Mn+Ni+Pb+Zn+Fe) significantly affected the growth of all five fish species. Naz and Javed (2012) also indicated that Mix. IV (Mn+Ni+Pb+Zn+Fe), followed by Mix. III (Mn+Pb+Zn+Fe) significantly affected the growth of *C. catla, L. rohita* and *C. mrigala* between the control and stress groups during acute toxicity trials. For both LC_{50} and lethal concentrations, *L. rohita* showed less sensitivity for Mix. IV (Mn+Ni+Pb+Zn+Fe) and a significant decrease in weight gain was observed for metal mixtures stressed fishes as compared to the control in the current work. An experiment with thirty days exposure to metal mixture(Fe, Zn, Pb, Ni and Mn) under chronic concentration (33.34 mgL^{-1}) showed reduced weight gain in *C. catla,* as compared to the control (Hussain *et al.*, 2011). Long-term exposure to heavy metal mixtures, at low concentrations greatly affected fishes in natural waters (Kazlauskiene *et al.*, 1999). In another study, sub-lethal effects of individual heavy metals and their mixtures were observed on rainbow trout. The study concluded that fish developmental stages, metal origin, and metal mixtures composition are responsible for fish behavioral responses. Toxicity effect of single and binary metal combination (Cd and Cu) on common carp developmental stages indicated that cadmium and copper mixture had significant effect compared to single metal, hence showed additive effects in metal mixtures (Ługowska, 2007).

In the current study, metal mixtures exposure did not significantly decrease fish feed intake, but lesser weight gain significantly impacted fish feed conversion efficiency. Fish weight gain, fork length, and total length give information about fish performance against the stressful environment (Javed, 2012) as fish growth decreases in contaminated water (Rowe, 2003; Hayat, 2009). The toxicity effect of copper on growth, gonad development, and reproductive performance of ornamental fish *Xiphophorus helleri* was examined in a previous study. Fish exposure to sub-lethal copper concentration reduces feed intake and feed conversion efficiency, whereas reverse trend was noticed for metabolic factors, i.e., oxygen consumption and opercular beat (James *et al.*, 2003). The physical and chemical properties of water significantly affected fish growth, feed intake, feed conversion efficiency, and the condition factor of fish (Naz *et al.*, 2013; Naz *et al.* 2022).

An increase in different metal mixtures concentration decreases fish growth performance, thus affecting fish feed conversion efficiency. Feed intake or feed conversion efficiency alone did not cause significant change in fish growth performance, but the involvement of both feed intake and feed conversion efficiency indicated a significant positive effect on fish growth in current work. A significant change in fish feeding efficiency is associated with decreased fish metabolism and activity when exposed to metal mixtures. As fish growth involves both physiological and biochemical processes, metals must bring biochemical variations before causing negative effect on fish growth (Stasiūnaitė, 1999; Jiang *et al.,* 2023). It was noticed that high concentration of heavy metals in yellow perch adversely affect feed intake, growth rate, and feed conversion ratio (Sherwood *et al.*, 2000). A significant decrease in feeding behavior was observed in larvae of common carp when exposed to copper and cadmium mixture (Jezierska *et al.*, 2005). Similarly, a reduction in the feeding activity of aquatic life was also noticed when exposed to different heavy metals either separately or in mixture (McGeer *et al.*, 2000; Jezierska *et al.*, 2006).

Conclusions: Heavy metals are among the major pollutants of the environment and the toxic effects of heavy metals are more severe for the aquatic ecosystem. Heavy metals become even more toxic when present in combination with other heavy metals. The current study demonstrated different heavy metal mixtures' adverse effects on five fish species. All the studied mixtures had significant adverse effects on fish growth and feed conversion efficiency while these mixtures did not affect the feed intake of the fish. Furthermore, the mixture with the most metals (Mix IV) had the most adverse effect on the fish growth. We concluded that heavy metals are serious pollutants of the aquatic ecosystem and have synergetic adverse effects when present in the form of mixtures. Further studies are required to find methods to control the pollution caused by heavy metal mixtures in aquatic ecosystems.

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