Determination of Median Lethal Concentration (LC_{50}) and Bioaccumulation of Copper in *Labeo rohita* and *Cirrhinus mrigala* Based on Acute Toxicity Test

Azam Jan Afridi[*], Amina Zuberi[†], Ali Muhammad Yousafzai[‡], Summia Perveen[§], Misbah Irm[**]

Abstract

Widespread application of Copper (Cu) sulfate ($CuSO_4$) leads to significant health risks to various aquatic organisms. The present study aims to determine the Median Lethal concentration over 96 hours (hrs) period (LC_{50}/96h) and the accumulation of copper sulfate ($CuSO_4.5H_2O$) in tissues across at four aged and developmental stages of Rohu (Labeo rohita) and Mrigal (Cirrhinus mrigala). The determination of LC_{50}/96h values for Cu sulfate was conducted across embryonic and larval, swim-up fry, advanced fry, and fingerling stages for both Labeo rohita and Cirrhinus mrigala. The resulting LC_{50}/96h values were 0.37 ppm, 0.75 ppm, 1.07 ppm, and 1.34 ppm, 0.48 ppm, 0.94 ppm, 1.36 ppm, and 1.52 ppm for Mrigal, respectively. To assess the bioaccumulation of Cu, 1gram samples from the first three developmental stages (Embryonic and larval, Swim-up fry, and Advanced fry) underwent digestion, while at fourth fingerling stage, five tissues (namely gills, liver, kidney, skin, and muscle) were selected and digested. The results indicated a bioaccumulation order of Cu as fingerling > advanced fry > swim-up fry > embryonic and larval stages for both species. Further, results revealed that at the fingerling stage, the bioaccumulation order of Cu in Labeo rohita was observed as gills > liver > skin > kidney > muscle tissues, while in Cirrhinus mrigala, it was liver > gills > kidney > skin > muscle tissues. This study explores the importance of understanding of developmental stage-specific responses to Cu exposure. Moreover, this research provides essential baseline information for establishing permissible Cu levels across developmental stages in freshwater fish species.

Keywords: Embryonic Larval, Swim-up Fry, Advanced Fry, Fingerling, Copper Toxicity, Median Lethal Concentration, Rohu, Mrigal.

Introduction

The aquatic environment is challenged with a major threat posed by potentially hazardous substances, including heavy metals, pesticides,

[*]Corresponding Author: Department of Zoology, Government Post Graduate College, Landi Kotal 24800, Pakistan, azamjan2010@gmail.com

[†]Fisheries & Aquaculture Lab, Department of Zoology, Quaid-i-Azam University, Islamabad 45320, Pakistan, amina.zuberi@qau.edu.pk

[‡]Department of Zoology, Islamia College University, Peshawar 25120, Pakistan, amyousafzai@icp.com

[§]institute of Water Conservancy & Ocean Engineering & Geography Science, Ningbo University, Ningbo 315211, China, summiaperven@outlook.com

[**]Department of Zoology, University of Kotli, Kotli 11100, Azad Jammu & Kashmir, Pakistan, dr.misbahirum@uokajk.edu.pk

and hydrocarbons (Xia et al., 2011; Akan et al., 2012). Heavy metals characterized by their persistence, bio-accumulative nature, and mutagenic properties of metals bring serious consequences on the health of aquatic organisms, particularly fish and associated organisms (Shahjahan et al., 2022; Taslima et al., 2022). Although heavy metals naturally occur in the environment, and various anthropogenic activities such as industrials and domestic waste discharge contribute to elevated concentrations in natural water bodies, which posing potential hazards to aquatic organisms (Sarkar et al., 2016; Ezemonye et al., 2019). Fish frequently encounter highly contaminated water, especially in those areas where wastewater dilution is neglected. Fish serves as a crucial test organism and front-line indicator for detecting suspected aquatic pollutants such as metals (Vieira et al., 2009).

The excessive discharge of heavy metals into the soil and aquatic systems significantly can disrupt the ecological balance of the receiving environment, and impact the diversity of aquatic organisms (Sarkar et al., 2022). Once enter the aquatic environment, heavy metals dissolve and form metallic ions, which can enter into the fish bodies and accumulate in various soft tissues such as liver and kidney (Yaqub et al., 2018; Shukla et al., 2007). Subsequently, these metals are transmitted through the food chain and bio-accumulated in consumer bodies, posing physiological and biochemical abnormalities in fish (Authman 2015; Handy 2003; Afridi et al., 2023), thereby raising serious public health concerns (Jamil et al., 2023).

Copper (Cu) is an essential metal needed for various biological processes, contributing to the maintenance of nervous systems, immune system function, red blood cell formation, and iron metabolism (Gaetke & Chow, 2003). It plays crucial role as a cofactor for the structural and catalytic functions of numerous enzymes, including to tyrosinase, cytochrome c oxidase, lysyl oxidase, dopamine beta-hydroxylase, and Cu-zinc superoxide dismutase (Cu, Zn-SOD) (Uauy et al., 1998). However, exceeding the threshold level of Cu in water bodies can induce considerable toxic effect, elicit biochemical and physiological disruptions (Thangam et al., 2014), and primarily generate free radicals (Valko et al., 2005). Sensitivity to Cu toxicity varies among species, and its developmental stages, and some fish species demonstrating susceptibility while invertebrate species are often highly vulnerable. Toxic effects extend to various organs, but not limited to multifunctional gills, kidneys, and spleens, even impacting those fish species that are relatively more tolerant (Grosell et al., 2005).

Aquatic toxicology plays a crucial role in elucidating the environmental consequences of alarming number of xenobiotics

discharged into aquatic ecosystem (Gross, 2022). Toxicity tests are always used to determine organism sensitivities to a specific toxicant that allow us to regulate the permitted level of that toxicant in aquatic ecosystem. Toxic effect of Cu has been studies on aquatic organism in last decades with greater effect on fish health. Toxicity test in water is necessary to evaluate the toxic effects of toxicant such as heavy metals on fish, and the focus has been confined to the acute toxicity test, where fish mortality is considered as an endpoint (Nekoubin et al., 2012). The Median Lethal Concentration over 96 hours (hrs) period (LC_{50}/9h) commonly serves as a pivotal metrics of toxicity, representing the concentration at which 50% of exposed animals died or killed exposing to a specific toxicant or group of toxicants (Islam et al., 2021).

The susceptibility of organisms to pollutants exhibits variability across species, populations, and life stages (Woltering, 1984). Heavy metals impact the embryonic development of fish, leading to growth retardation, functional impairment, morphological abnormalities, and mortality among the most vulnerable individuals (Boglione et al., 2013). In fish life cycle, embryonic and larval stages are generally considered as the most susceptible to toxicity (Osman et al., 2007; Zhang et al., 2012). Accumulation of heavy metals in fish tissues predominantly depend upon tissues and metal concentrations in water and exposure duration (Turkmen et al., 2005), yet environmental parameters such as temperature, dissolved oxygen, pH, water hardness, salinity, alkalinity, and dissolved organic carbon substantially modulate metal accumulation and toxicity (Eastwood & Couture, 2002; Adhikari et al., 2006). Despite the extensive literature on heavy metal toxicity and reporting of Median LC_{50}/96h at one developmental stage of fish, and there remains a lack of knowledge concerning their effects on the four developmental stages of freshwater species. This study addresses this gap by evaluating Cu toxicity by determining Median LC_{50} and Bioaccumulation of Cu during acute toxicity of cu sulfate at four developmental stages of two freshwater fishes *Labeo rohita* and *Cirrhinus mrigala.*

Material and Methods

Fish Sampling and Preparation of Standard Solution

To collect fish samples induced breeding experiments were accompanied at the Fisheries and Aquaculture research station, Quaid-i-Azam University, Islamabad, utilizing their fish breeding and rearing facilities. Newly fertilized eggs were carefully transported to circular concrete tanks facilitating their incubation under controlled conditions with slow-moving water. Upon yolk sac absorption, fry was shifted to

earthen nursery pond for rearing up to fingerling stages. Four well known developmental stages were selected such as embryonic and larval, swim-up fry (post larval), advanced fry, and fingerling stage of two freshwater fishes. For the preparation of the standard solution, 5.44g analytical grade of Cu sulfate salt (Merck, Germany) representing 1 g Cu was transferred to a one-Liter volumetric flask, and added de-ionized water to achieve the desired concentration up to 1000ppm (1000 mg/L). Shaken well to make a homogeneous solution. Further dilutions ranging from 0.2 to 4.0 ppm were prepared from the initial prepared standard solution. This range of dilutions were selected to encompass a spectrum of concentrations with small increments, facilitating the attainment of accurate results followed the protocols outlined by Afridi et al. (2019).

Experimental Design at Four Developmental Stages

Depending on fish size different containers were used at each developmental stage of two freshwater fishes. During the embryonic and larval stage, experimental units comprised of ten plastic beakers with a capacity of 6 litre each, equipped with a flow-through system. Subsequently, at this stage, approximately 50 newly fertilized eggs were introduced into each plastic beaker, whereas 50 swim up fry (Post larval) were introduced per plastic beaker per dose, it makes a total of 550 swim up fry per experimental set, and a total of 1650 swim up fry in whole experiments in triplicates. Prior to this, fishes were exposed to de-chlorinated water sourced from a series of overhead aquaria, with controlled concentrations of Cu sulfate as per established protocols (Afridi et al., 2019). For the advanced fry and fingerling stages, each experimental unit comprised of 40-liter glass aquarium (30 x 30 x 60cm) per dose, with triplicate setups were used for both treated and control group of each species of *Labeo rohita* and *Cirrhinus mrigala*, following the method reported by Afridi et al. (2019). To ensure optimal oxygenation, miniature-sized air stones were installed in all aquaria. The treated groups were subjected to different concentrations of Cu sulfate, while the control group remained unexposed. Water within each aquarium was replaced every 24 hrs with de-chlorinated water, with fresh concentrations of Cu sulfate replenished accordingly. The experimental duration spanned 96 hrs (4 days).

Acute Toxicity Test at Four Developmental Stages

Fertilized Eggs Stage

The Median LC_{50} of the fertilized eggs at (Embryonic and larval) of both species *Labeo rohita* and *Cirrhinus mrigala* in response to Cu

toxicity over 96 hrs were determined under controlled laboratory conditions. The experimental unit consisting of ten, each of 6-liter capacity plastic beakers in triplicate fitted with a flow-through system. Each experimental unit, about 50 newly fertilized eggs were randomly transferred in each plastic beakers receiving de-chlorinated water from an arranged overhead series of aquaria having requisite concentrations of Cu sulfate. Fertilized eggs were exposed to water-born Cu toxicity in ten different concentrations in triplicate (0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 mg/L. These concentrations of Cu sulfate were selected based on pre-experimental trials and existing literature, ensuring a comprehensive assessment of the toxicity gradient on these carps.

Swim-up Fry Stage

For the determination of the Median LC_{50}/96h at the Swim-up fry stage, an experimental protocol was executed utilizing a set of 11 plastic beakers for each dose and each with a capacity of 8 Liters. The experimental design comprised triplicate setups, employing a flow-through system of water. Active 50 swim up fry per plastic beaker randomly selected for the experiment, with an average body weight of 0.78±0.091 mg for *Labeo rohita* and 0.85±0.094 mg for *Cirrhinus mrigala*. These swim up fries were evenly distributed among the eleven beakers, while each beaker receiving 50 swim-up fry per concentration per dose. The experimental groups were exposed to different concentrations of Cu, specifically from 0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, to 2.0 mg/L respectively, for a duration of 96 hrs. Throughout the experimental period, the swim-up fry were provided with a diet consisting of 40% larval particulate feed, with particle sizes ranging from 50-100µm.

Advanced Fry Stage

For determination of Median LC_{50}/96h, a total of 480 uniform-sized active advanced fry from both species (with average body weight for *Labeo rohita* 95.70±5.58 mg; *Cirrhinus mrigala* is 143.38±4.40 mg) was taken from reared earthen nursery pond. These fries were subsequently transfers to a laboratory condition and housed separately in circular fiberglass tanks equipped with a flow-through system. After two days acclimatization period, the advance fry was transferred to glass aquaria measuring (30 x 30 x 60cm). The stocking density was set at 2 g/L or 2000 mg/L (equivalent to 10 fries per aquarium). Water heater is used for maintaining a temperature of 26.5°C. Advanced fry from both species were then subjected to different concentrations of waterborne Cu toxicity ranging from 0.0 to 3.2 mg/L. These concentrations were determined based on preliminary trials and existing literature.

Fingerlings Stage

For determination of Median LC_{50}/96h, at fingerling stage similar size of (42.60±7.95 gm for *Labeo rohita* and 40.64±2.39 gm for *Cirrhinus mrigala*) were transferred from earthen ponds to circular fiberglass tanks. Following a 2 days acclimatization period to laboratory conditions, the species were shifted to aquaria (20 aquaria per species) in such a manner that ten fingerlings were introduced per aquarium, with a total of 600 fingerlings in triplicate at a stocking density of 2 grams per liter. After 3 days of acclimatization at a constant temperature of 26.5˚C, the fingerlings were exposed to different concentrations of water born Cu toxicity. The concentrations tested included those previously used for advanced fry, as well as concentrations of 3.4, 3.6, and 3.8 mg/L.

Determination of Median LC_{50}/96h

Median LC_{50} of Cu were used as a toxicant at embryonic and larvae, Swim-up fry (post-larval), advanced fry, and fingerling stages of both species Rohu (*Labeo rohita*) and Mrigal (*Cirrhinus mrigala*) in a laboratory through the semi-static method. Sampling procedures were conducted throughout the LC_{50} experiments over 96 hrs (4 days), with assessment made at 24 hrs intervals at (24, 48, 72, and 96 hrs). Throughout the experiments, dead fish was counted after every 24 hrs and removed from the aquarium to avoid water spoilage. Mortality response of the fish species was taken to be dead when the fish sank to the bottom of the containers and became motionless. Mortality response rate was recorded at the end of the experimental period (4 days).

Toxicological dose-response analysis was performed, involving quantal response (Mortality) induced by Cu toxicity in both species of Rohu and Mrigal at four developmental stages. For determination of LC_{50} Finney's Probit Analysis of Median LC_{50} method, was applied using SPSS (version 21) (Finney 1971). LC_{50}/96h values were calculated based on percentage mortality and its confidence limit of 95%. The mortality rate was determined at the end of the 96 hrs exposure period, and counted from each aquarium and their percentage mortality were expressed in whole number. Both fish species were exposed to different doses of Cu sulfate corresponding to each developmental stage, while control group (0.00ppm) was exempted. To ensure water quality parameters, including temperature (26.5˚C throughout the experiment), dissolved oxygen (DO), calcium carbonate ($CaCO_3$) hardness, and pH) monitoring was performed before and during the bioassay tests.

Cu Bioaccumulation at Four Developmental Stages

Fish sampling and Tissues Digestion

Cu was analyzed across four developmental stages, for this purpose sampling was collected at each developmental stage from both control and treated groups using a plankton net. To remove adhered Cu residue on body surfaces of test species, thoroughly washing body of fish with de-ionized water and at embryonic and larval, and swim-up fry (post-larval) stages whole body sample weighing 0.5 g was collected. At advanced fry stage, whole body samples weighing 1 g was collected for tissue digestion. While at fingerling stage, fish was dissected on clean glass for collecting different tissues namely (gills, liver, kidney, muscle, and skin) of both species of control and treated groups. All of these tissues were stored at -20°C for tissues digestion.

Tissues Digestion

For tissue digestion, each collected tissue sample was thawed and excess water was removed by blotting with filter paper. Briefly, 0.5 g of whole-body tissue at larval and swim up fry stage, and exactly 1g whole body tissue at advance fry, and at fingerling stage 1g of each tissue was transferred into an oven-dried 50 mL volumetric flask. Now added 5 mL nitric acid (69% HNO_3) and 1 mL of perchloric acid (60% $HCLO_4$) to each flask containing tissues using a pipette following the method outlined by Yousafzai & Shakoori (2006). The flasks were tightly packed and allowed to kept overnight at room temperature. On the next day, additional of 5 mL of HNO_3 and 1 mL of $HClO_3$ were added to each flask, and they were placed on a hot plate at 200 to 250˚C for the digestion, initially indicating by the appearance of brown-red fumes, which later converted into white fumes. Once digestion completed, the samples were allowed to cool, and the volume of the digest was adjusted to 50 mL by the addition of double-distilled water (Afridi et al., 2026). Each digest was then filtered through 0.42 µm filter paper into a 50-mL round bottom flask and the prepared samples were stored at room temperature until further analysis. Cu analysis was conducted using an Atomic Absorption Spectrophotometer (AAS) (Thermo, USA). A standard curve was generated on the spectrophotometer using a standard solution within different concentrations (ppm). The concentration of Cu in different tissues of both species was calculated by comparing the absorbance reading with the standard curve.

Statistical Analysis

Finney's Probit Analysis of LC_{50} Method with a confidence limit of 95% was used for the determination of Median LC_{50}/96h using SPSS (version 21) across four developmental stages. Mortality data were expressed in whole number of percentages. Data were expressed as mean ± SEM. Significant differences ($P < 0.05$) in Cu levels among the control group and experimental groups, one-way ANOVA was conducted, followed by Posthoc Tukey HSD test using an SPSS (version 21). Significance was determined at the $P < 0.05$ level.

Results

Results of present studies are presented in two parts, first part indicates the Median LC_{50}/96h during acute toxicity test of Cu and second part indicates the bioaccumulation of Cu at four developmental stages of two freshwater fishes Rohu (*Labeo rohita*) and Mrigal (*Cirrhinus mrigala*).

Mortality Rate Against Acute Toxicity Test of Cu at Four Developmental Stages of Labeo rohita

The mortality rates of Rohu (*Labeo rohita*) at different four developmental stages viz embryonic and larval, swim-up fry (post larval), advanced fry, and fingerling stages under influence of different concentrations of Cu sulfate were presented in Tables (1 to 4). The percentage mortality was recorded at embryonic and larval, swim-up fry, advanced fry, and fingerling stages. At the embryonic and larval stage, the mortality rate remained 0% at dose of 0.00 ppm under control conditions. However, exposure to increasing concentrations of Cu sulfate led to escalating mortality rates. The observed mortality rates ranged from 6% at 0.2 ppm to 100% at 1.8 ppm are shown in Table 1.

Table 1: Showing dose respondent mortality rate against acute toxicity test of Cu with 96 hrs (24 – 96 hrs) at embryonic and larval stage of Rohu (Labeo rohita). (Average mortality were taken of three triplicates at 24, 48, 72, and 96 hrs).

Concentration of Cu (mgL^{-1})	No. of eggs exposed to Cu	Mortality at 24 hrs	Mortality at 48 hrs	Mortality at 72 hrs	Mortality at 96 hrs	Total dead Fish larvae	Total living Fish larvae	Mortality (%)
Control (0)	50	03	00	00	00	03	47	06
0.2	50	08	02	01	01	18	32	38
0.4	50	11	07	05	03	25	25	50
0.6	50	16	06	04	03	31	29	62
0.8	50	17	09	07	03	36	14	72
1.0	50	19	09	06	05	39	11	78
1.2	50	25	06	07	03	41	09	82
1.4	50	35	07	02	02	46	06	88
1.6	50	42	03	03	00	48	02	92
1.8	50	50	00	00	00	50	00	100

At swim up fry stage, the observed percentage mortality at control were remained at 0.00% at dose of 0.00 ppm, while exposure to Cu concentration led mortality rate ranging from 10% at 0.2 ppm to 100% at 2.4 ppm are shown in Table 2.

Table 2: Showing effect of different concentrations of Cu on mortality rate (%) of swim up fry of Rohu at different time periods (24-96 hrs).

Concentration Of Cu (mgL-1)	No. of swim up fry exposed to Cu	Mortality at 24 hrs	Mortality at 48 hrs	Mortality at 72 hrs	Mortality at 96 hrs	Total dead fish larvae	Total living fish larvae	Mortality (%)
0(Control)	50	00	00	00	00	00	50	00
0.2	50	02	01	00	02	05	45	10
0.4	50	05	03	02	01	11	39	22
0.6	50	07	05	04	03	19	31	38
0.8	50	09	05	07	03	24	26	58
1.0	50	08	06	07	07	28	22	56
1.2	50	12	06	06	07	31	19	62
1.4	50	18	07	07	05	37	13	74
1.6	50	21	09	05	06	41	09	82
1.8	50	26	07	05	06	45	05	90
2.0	50	29	07	06	05	47	03	94
2.2	50	34	09	03	00	46	04	90
2.4	50	47	03	00	00	50	00	100

At the advanced fry stage, mortality rates under control conditions remained at 0.00% at dose of 0.00 ppm. However, exposure to Cu sulfate concentration led to mortality rates ranging from 10% at 0.2 ppm to 100% at 2.8 ppm as shown in Table 3.

Table 3: Showing dose respondent mortality rate (%) against Cu toxicity in acute toxicity bioassays of Cu sulphate with 96 h (24 – 96 hrs) at advanced fry stage of Rohu (Labeo rohita).

Concentration Of Cu (mgL-1)	No. of advance fry exposed to Cu	Mortality at 24 hrs	Mortality at 48 hrs	Mortality at 72 hrs	Mortality at 96 hrs	Total dead fish larvae	Total living fish larvae	Mortality (%)
Control (0)	10	0	0	0	0	0	50	0
0.2	10	0	0	0	0	0	10	0
0.4	10	1	0	0	0	1	9	10
0.6	10	1	1	0	0	2	8	20
0.8	10	2	1	0	0	3	7	30
1.0	10	2	2	0	0	4	6	40
1.2	10	2	2	2	0	6	4	60
1.4	10	3	2	1	1	7	3	70
1.6	10	2	3	0	2	7	3	70
1.8	10	3	2	1	2	8	2	80
2.0	10	2	1	2	3	8	2	80
2.2	10	3	3	1	2	9	1	90
2.4	10	2	3	3	1	9	1	90
2.6	10	4	1	2	1	9	1	90
2.8	10	5	2	1	1	9	1	90
3.0	10	6	3	1	0	10	0	100

At the fingerling stage, the mortality rate was 0.00% at dose of 0.00 ppm under control conditions, while exposure to Cu sulfate resulted in mortality rates ranging from 10% at 0.2 ppm to 100% at 3.6 ppm as shown in Table 4.

Table 4: Showing relationship between the Cu concentration and percentage Mortality rate on Fingerlings of Rohu (Labeo rohita) at different time period (24 – 96) hrs.

Concentration Of Cu (mgL-1)	No. of fingerlings exposed to Cu	Mortality at 24hr	Mortality at 48 hrs	Mortality at 72 hrs	Mortality at 96 hrs	Total dead fish larvae	Total living fish larvae	Mortality (%)
Control (0)	10	0	0	0	0	0	10	0
0.2	10	0	0	0	0	0	10	0
0.4	10	1	0	0	0	0	9	10
0.6	10	2	0	0	0	2	8	20
0.8	10	2	1	0	0	3	7	30
1.0	10	2	1	1	0	3	6	30
1.2	10	2	2	0	0	4	6	40
1.4	10	2	1	1	0	4	6	40
1.6	10	2	2	1	0	5	5	50
1.8	10	3	2	1	1	6	4	60
2.0	10	2	2	2	1	7	3	70
2.2	10	3	2	2	1	8	2	80
2.4	10	3	2	1	1	7	3	70
2.6	10	4	3	1	0	8	2	80
2.8	10	5	3	2	0	10	0	100
3.0	10	6	3	0	0	10	0	100
3.2	10	7	3	0	0	10	0	100
3.4	10	8	2	0	0	10	0	100
3.6	10	10	0	0	0	10	0	100

Mortality Rate Against Acute Toxicity Test of Cu at Four Developmental Stages of Mrigala (Cirrhinus mrigala)

The mortality rates of Mrigal (*Cirrhinus mrigala*) under varying doses of Cu exposure were assessed at four developmental stages at embryonic and larval, swim-up fry, advanced fry, and fingerling stages are summarized in (Tables 5 to 8).

Table 5: Correlation between the Cu concentrations and the per aquaria mortality rate with time (24 –96 hrs) of Mrigal (Cirrhnus mrigala) at embryonic and Larval stage.

Concentration of Cu (mgL-1)	No. of eggs exposed to Cu	Mortality at 24 hrs	Mortality at 48 hrs	Mortality at 72 hrs	Mortality at 96 hrs	Total dead fish larvae	Total living Fish larvae	Mortality (%)
Control (0)	50	02	00	00	00	02	48	04
0.2	50	6	2	1	0	14	36	28
0.4	50	8	5	3	1	17	23	34
0.6	50	13	6	3	2	24	26	48
0.8	50	16	7	6	5	34	16	68
1.0	50	21	6	4	5	36	14	72
1.2	50	27	6	7	3	43	6	86
1.4	50	35	7	2	2	46	4	92
1.6	50	41	6	1	0	48	2	96
1.8	50	50	1	0	0	49	1	98

At the control group, mortality remained at 0% at dose of 0.00 ppm. However, with increasing Cu doses ranging from 0.2 to 2.0 ppm, mortality rates escalated, reaching 100% at the highest dose as shown in Table 5 above. At swim up fry stage, observed percentage mortality at control was remained at dose of 0.00ppm, and exposure to Cu led to a dose-dependent responses in mortality rates. Mortality rates ranged from 0% at 0.2 ppm to 100% at 2.6 ppm as shown in Table 6.

Table 6: Showing the effect of different concentration of Cu on Mortality rate (%) of Swim up fry of Mrigal (Cirrhnus mrigala) at different time period (24 – 96 hrs).

Concentration of Cu (mgL-1)	No. of swim up fry exposed to Cu	Mortality at 24hr	Mortality at 48 hrs	Mortality at 72 hrs	Mortality at 96 hrs	Total dead Fish larvae	Total living fish larvae	Mortality (%)
Control (0)	50	0	0	0	0	0	50	0
0.2	50	1	1	1	0	2	48	4
0.4	50	3	1	3	0	5	45	10
0.6	50	5	3	3	0	9	39	18
0.8	50	7	4	3	3	17	33	34
1.0	50	9	5	6	5	25	25	50
1.2	50	13	7	7	4	31	19	62
1.4	50	14	9	8	5	36	13	72
1.6	50	16	11	6	6	39	9	78
1.8	50	22	9	6	7	42	5	84
2.0	50	23	11	7	7	48	3	98
2.2	50	31	9	6	0	46	4	92
2.4	50	35	7	6	0	48	2	96
2.6	50	48	2	0	0	50	0	100

Table 7: Showing dose respondent mortality rate (%) against Cu toxicity in acute toxicity bioassays of Cu sulphate with 96 h (24 – 96 hrs) at advanced of Mrigal (Cirrhnus mrigala).

Concentration of Cu (mgL-1)	No. of advance fry exposed to Cu	Mortality at 24hr	Mortality at 48 hrs	Mortality at 72 hrs	Mortality at 96 hrs	Total dead fish larvae	Total living fish larvae	Mortality (%)
Control (0)	10	0	0	0	0	0	10	0
0.2	10	0	0	0	0	0	10	0
0.4	10	0	0	0	0	0	10	0
0.6	10	1	0	0	0	1	9	10
0.8	10	1	1	0	0	2	8	20
1.0	10	2	1	0	0	3	7	30
1.2	10	2	1	1	0	4	6	40
1.4	10	3	1	1	0	5	5	50
1.6	10	2	1	1	2	6	4	60
1.8	10	2	2	1	1	6	4	60
2.0	10	2	1	2	2	7	3	70
2.2	10	3	3	1	1	8	2	80
2.4	10	2	3	3	0	8	2	80
2.6	10	4	2	1	2	9	1	90
2.8	10	4	3	1	1	9	1	90
3.0	10	5	3	2	0	10	0	100

At advanced fry stage, Mortality at the control group remained 0.00% at dose of 0.00 ppm. However, exposure to Cu resulted in mortality rates escalating from 0% to 100% as the concentration increased from 0.2 to 3.0 ppm as shown in Table 7 above. At the fingerlings stage the percentage mortality rate at the control condition was (0.00%) at dose of 0.00 ppm. Nevertheless, exposure to increasing Cu concentrations lead to a gradual rise in mortality, with rates ranging from 0% to 100% as concentrations ranged from 0.2 to 3.8 ppm shown in Table 8.

Table 8: Correlation between the Cu concentration and the Mortality rate on time periods of (24 h – 96 hrs) on Fingerlings of Mrigal (Cirrhnus mrigala).

Concentration of Cu (mgL-1)	Fingerlings exposed to Cu	Mortality at 24hr	Mortality at 48 hrs	Mortality at 72 hrs	Mortality at 96 hrs	Total dead fish larvae	Total living fish larvae	Mortality (%)
Control (0)	10	0	0	0	0	0	10	0
0.2	10	0	0	0	0	0	10	0
0.4	10	0	0	0	0	0	10	0
0.6	10	1	0	0	0	1	9	10
0.8	10	1	1	0	0	2	8	20
1.0	10	2	1	0	0	3	7	30
1.2	10	2	1	0	0	3	7	30
1.4	10	2	2	0	0	4	6	40
1.6	10	2	1	1	0	4	6	40
1.8	10	2	2	1	0	5	5	50
2.0	10	2	2	1	1	6	4	60
2.2	10	2	2	2	1	7	3	70
2.4	10	3	2	2	1	8	2	80
2.6	10	4	3	1	0	8	2	80
2.8	10	5	3	1	0	9	1	90
3.0	10	5	3	1	0	9	1	90
3.2	10	6	3	1	0	10	0	100
3.4	10	7	3	0	0	10	0	100
3.6	10	8	2	0	0	10	0	100
3.8	10	10	0	0	0	10	0	100

Median LC_{50}/96h Values at Four Developmental Stages of Rohu (Labeo rohita) and Mrigal (Cirrhinus mrigala)

Median LC_{50}/96h values were determined to assess the LC50 causing 50% mortality of the test animal within a 96 hrs period at four developmental stages viz embryonic and larval, swim-up fry, advanced fry, and fingerling stage of Rohu and Mrigal For Rohu, the LC_{50}/96h values were found to be 0.37 ppm at (Embryonic and larval), 0.75 ppm at (Swim-up fry), 1.07 ppm at (Advanced fry), and 1.34 ppm at (Fingerling).

For Mrigal, the corresponding LC_{50}/96h values were 0.48 ppm, 0.94 ppm, 1.36 ppm, and 1.52 ppm, at embryonic and larval, swim-up fry, advanced fry, and fingerling stage respectively are presented in Table 9. The Median LC_{50}/96h values of each species at four developmental stages were obtained through analysing the mortalities data presented in (Tables

1, 2, 3, and 4) for Rohu and presented in (Tables 5, 6, 7, and 8) for Mrigal through the SPSS probit test.

Table 9: Showing values of LC50/96h (50% Mortality in ppm) of acute toxicity bioassays tests of Cu at four developmental stages viz embryonic and larval, swim-up fry, advance fry, and fingerling stages of two freshwater fishes Rohu (Labeo rohita) and Mrigal (Cirrhnus mrigala).

	Values of LC50/96 hrs (ppm) at four developmental stages			
Fish species	Embryonic and larval	Swim-up fry	Advance fry	Fingerling
Labeo rohita	0.37	0.75	1.07	1.34
Cirrhnus mrigala	0.48	0.94	1.36	1.52

Bioaccumulation of Cu During Acute Toxicity Test at Four Developmental Stages of Rohu (Labeo rohita)

Cu was analyzed in the whole-body tissues of first three developmental stages such as embryonic and larval, swim up (post larval) and advance fry as shown in Table 10, while at fourth stage (fingerling) five specific tissues are selected as shown in Table 11. Result indicates that there is a significant increase ($P < 0.05$) in Cu concentration observed after 96 hrs (4th day) of exposure during the embryonic and larval stages in contrast to the control group as shown in Table 10. Similarly, at the swim-up fry stage, Cu levels were significantly elevated ($P < 0.05$) at 72 hrs and 96 hrs compared to both the control group and the initial 24 hrs (1st day) of exposure period as shown in Table 10. Moreover, during the advanced fry stage, Cu concentration remained significantly higher ($P < 0.05$) throughout the 1st, 2nd, 3rd, and 4th days of exposure when compared to the control group as shown in Table 10. Furthermore, the data revealed a significant increase ($P < 0.05$) in Cu bioaccumulation within the treated group as compared to the control group of first three developmental stages as shown in Table 10.

Table 10: Showing age and developmental stage specific bioaccumulation of Cu during acute toxicity of Cu sulfate (LC50/96hr) at embryonic and larval, swim-up fry, and advance fry stages of Rohu (Labeo rohita).

Developmental stage		Cu-exposure (hrs)			
	Control	24	48	72	96
Embryonic & larval	0.35± 0.11b	1.06± 0.26ab	1.27± 0.087ab	1.37± 0.54ab	2.54± 0.52a
Swim up fry	2.96± 0.99b	2.99± 0.28b	4.04± 0.37ab	5.27± 0.29a	5.34± 2.22a
Advanced fry	5.49± 0.48b	6.89± 1.39a	8.20± 1.85a	6.70± 1.75a	9.80± 0.78a

Data are represented by Means ± SE; n= number of the sample (n=6); Means followed by different letters within the row are significantly different ($P < 0.05$). (ANOVA followed by Tukey HSD test). (24 hrs = 1st day, 48 hrs = 2nd day, 72 hrs = 3rd day, 96 hrs = 4th day).

The tissues specific bioaccumulation of Cu during acute toxicity of Cu sulfate at fingerling stage of Rohu (Labeo rohita) are summarized in

Table 11. Significantly higher Cu concentrations ($P < 0.05$) were observed in all examined tissues of the treated groups compared to the control group. In gills, Cu levels were significantly elevated ($P < 0.05$) after 96 hrs (4^{th} day) of exposure compared to both the control and the 48 hrs exposure period. No significant variation in Cu content was observed among treated groups in liver tissue ($P > 0.05$). In kidney tissue, Cu concentrations were significantly higher ($P < 0.05$) throughout the 1st, 2^{nd}, 3^{rd}, and 4^{th} days of Cu exposure compared to the control group. Muscle tissue exhibited significantly higher Cu levels ($P < 0.05$) on the 4^{th} day of exposure compared to the control group. Similarly, skin tissue displayed significantly elevated Cu concentrations ($P < 0.05$) on the 4^{th} day of exposure compared to the control group as shown in Table 11.

Table 11: Showing tissues specific bioaccumulation of Cu during acute toxicity of Cu sulfate (LC50/96hr) at fingerling stage of Rohu (Labeo rohita).

		Cu-exposure (hrs)			
Tissues	Control	24	48	72	96
Gills	3.54±0.71b	4.22±0.37ab	3.26±0.32b	5.05±0.46ab	7.30±1.36a
Liver	3.50±0.25a	4.71±0.55a	4.01±0.59a	6.45±3.66a	7.27±2.37a
Kidney	2.23±0.26b	2.72±0.64a	4.00±1.74a	4.39±2.21a	3.57±0.66a
Muscle	0.42±0.17b	0.82±0.16ab	0.65±0.10b	0.83±0.07ab	1.31±0.06a
Skin	1.58±0.15b	1.69±0.48b	2.30±0.73ab	3.46±0.20ab	4.47±0.71a

Data are represented by Means ± SE; n= number of the sample (n=6); Means followed by different letters within the row are significantly different ($P < 0.05$; Tukey HSD test).

Bioaccumulation of Cu During Acute Toxicity Test at Four Developmental Stages of Mrigal (Cirrhinus mrigala)

Bioaccumulation of Cu of first three developmental such as embryonic and larval, swim up and advance fry of Mrigal are shown in Table 12. While at fingerling stage (tissues specific) are presented in Table 13. The age and developmental stage-specific bioaccumulation of Cu during acute toxicity of Cu sulfate (LC_{50}/96 hrs) were investigated in Mrigal at four life stages, including embryonic and larval, swim-up fry, advanced fry, and fingerling stages. Significantly higher levels of Cu ($P < 0.05$) were observed in the Cu-exposed groups compared to the control group during the acute toxicity test. Specifically, at the embryonic and larval stages, Cu levels were significantly higher on the 4^{th} day of exposure compared to the control and earlier exposure days as shown in Table 12. Similarly, at the swim-up fry stage, Cu levels were significantly elevated on the 4^{th} day of exposure compared to earlier exposure days as shown in Table 12. At the advanced fry stage, Cu accumulation was significantly highest on the 2^{nd} and 3^{rd} days of exposure, while the lowest levels were recorded in the control group as shown in Table 12.

Furthermore, the accumulation of Cu in various fish tissues during acute doses of Cu sulfate exposure at the fingerling stage of Mrigal are summarized in Table 13. In all tissues examined, Cu levels were

significantly higher ($P < 0.05$) in the Cu-exposed groups compared to the control group during the acute toxicity test. Specifically, in gill tissue significantly higher Cu levels were observed on the 4th day of exposure. In liver tissue, significantly elevated Cu levels were observed throughout the four days of exposure compared to the control group. Similarly, in kidney tissue, significantly higher Cu levels were recorded on the 4th day compared to earlier exposure days. In muscle tissue, significantly higher Cu levels were observed on the 3rd day of exposure compared to the control and other exposure days. Lastly, in skin tissue, significantly elevated Cu levels were observed on the 4th day compared to earlier exposure days are summarized in Table 13.

Table 12: Bioaccumulation of Cu during acute toxicity of Cu sulphate (LC50/96h) at embryonic and larval, swim-up fry, and advance fry stages of Mrigal (Cirrhnus mrigala).

		Cu-exposure (hrs)			
Developmental stage	Control	24	48	72	96
Embryonic and larval	0.2910± 0.13 b	0.840± 0.06ab	0.7633± 0.06ab	0.9133± 0.28ab	1.2700± 0.075a
Post-larval	2.1733± 0.18ab	1.3400± 0.44b	2.293± 0.25ab	2.460± 0.12ab	3.1633± 0.20a
Advanced fry	4.3767± 0.51c	6.030± 0.42bc	8.4167± 0.56a	8.4733± 0.54a	7.9533± 0.32ab

Data are represented by Means ± SE; n= number of the sample (n=6); Means followed by different letters within the row are significantly different ($P < 0.05$). (ANOVA followed by Tukey HSD test).

Table 13: Showing tissues specific bioaccumulation of Cu during acute toxicity of Cu sulfate (LC50/96hr) at fingerling stage of Mrigal (Cirrinhus mrigala).

		Hrs of Cu Exposure			
Tissues	Control	24	48	72	96
Gills	3.6667± 0.49c	4.0867± 0.17bc	4.3667± 0.50bc	5.7133± 0.34ab	6.7267± 0.19a
Liver	4.3000± 0.32b	4.7200± 0.71a	4.9967± 0.95a	6.5600± 0.76a	6.8133± 0.90a
Kidney	3.0533± 0.19b	4.420± 0.40ab	4.6233± 0.73ab	4.6133± 0.45ab	5.6633± 0.40a
Muscle	1.7667± 0.19ab	1.4567± 0.055b	2.5200± 0.20ab	2.590± 0.37a	2.4733± 0.22ab
Skin	2.5333± 0.39b	2.1233± 0.54b	2.4633± 0.22b	2.4133± 0.12b	4.2767± 0.52a

Data are represented by Means ± SE; n= number of the sample (n=6); Means followed by different letters within the row are significantly different ($P < 0.05$; Tukey HSD test).

Discussions

Acute toxicity studies can help to evaluate pollution by providing reliable estimates of safe concentration, from which water quality criteria can be derived (Paruruckumani et al., 2015). Median LC_{50}/96h have been utilized to assess the LC50 causing 50% mortality in the test animal within a 96-hrs period and in best manner evaluate the susceptibility and survival potential of test organisms.

The current study was done for first time to investigate the age and developmental stage-specific toxicity response of Cu for two freshwater

fishes of Rohu and Mrigal. The Median LC_{50}/96h values during acute toxicity test of Cu were determined at four developmental stages such as embryonic and larval, post-larval, advanced fry, and fingerling stages as follows as 0.37, 0.75, 1.07, and 1.42 ppm for *Labeo rohita* and follows as 0.48, 0.94, 1.36 and 1.53 ppm, for *Cirrinhus mrigala*, respectively. These LC_{50}/96h values are comparatively low, which clearly indicating the higher toxicity of Cu towards both freshwater fishes. This observation is consistent with findings by Gharedaashi et al. (2013), who reported that Cu is more toxic than lead to Caspian Sea kutum with LC_{50} values of 2.31 ppm for Cu and 268 ppm for lead over a 96 hrs exposure period. In addition, Nekoubin et al. (2012) observed Cu toxicity toward Caspian Roach (*Rutilus rutilus caspicus*) supporting our results. The resulted LC_{50}/96h values for both fish species (*Labeo rohita* vs *Cirrinhus mrigala*), followed the order: at embryonic and larval stage is (0.37 vs 0.48ppm) < swim-up fry is (0.75 vs 0.94 ppm) < advance fry is (1.07 vs 1.36ppm) < fingerling is (1.34 vs 1.52 ppm) respectively. Consequently, it can be concluded from our results that the susceptibility at four developmental stages of Rohu and Mrigal to Cu follows the order as larval < swim-up fry < advanced fry < fingerling stage. It is evident from the results of this study that Rohu is more sensitive toward Cu toxicity than Mrigal. Shah & Altindu (2005) reported that some toxicants are harmful at low concentration, but it may be less toxic to some other organisms at the higher concentration. From the results of this study, it is concluded that that Median LC_{50}/96h of Cu used as toxicant are age and developmental stage-specific.

Cu is an essential mineral element crucial for the normal physiological function and growth of organisms, including fish. However, Cu is frequently discharged into aquatic environments as a by-product of mining processes, metal finishing industries, and the use of Cu-based products in agriculture, such as fertilizers, pesticides, fungicides, herbicides, algaecides, antibacterial, antiviral agents in fish culture ponds, and molluscicides (De Oliveira-Filho et al., 2004). As a result of its widespread use, the concentration of Cu in aquatic ecosystems has increased (Eisler, 2000). Increased concentrations of Cu can induce oxidative damage through the generation of aberrant free radicals, which in turn can adversely affect essential biological macromolecules, including lipids, proteins, and nucleic acids (Panigrahi, 2014). Cu is recognized as one of the most toxic metals to fish, exerting detrimental effects on various physiological parameters such as blood parameters, growth, behavior, enzyme activity, and reproduction (Abou El-Naga et al., 2005).

The present study demonstrate the sensitivities of two freshwater fishes toward Cu toxicity across at four developmental stages. The

sensitivity of organisms to pollutants is known to vary among species, and life stages as early life stages of fishes are more sensitive in comparison to later (adult) stages (Sloman & McNeil, 2012). It is widely recognized that the embryonic and larval stages represent the most sensitive periods in the entire life cycle of fish in terms of toxicity (Osman et al., 2007; Zhang et al., 2012). Previous studies examining the impact of heavy metals on developing fish, particularly embryos and larvae have consistently reported high incidences of mortality, altered body shape, delay hatching, and various anatomical anomalies (Jezierska et al., 2009b). In this study, it is observed that the embryonic and larval stage is indeed the most affected one by Cu toxicity.

This study assessed the sensitivity of fertilized eggs and larvae to fingerlings stage of Rohu and Mrigal toward Cu toxicity. Results indicate that fertilized eggs of both species exhibited more sensitivity toward Cu toxicity, while Rohu shown more susceptibility compared to Mrigal. Additionally, early aged developmental stages such as fertilized eggs and larvae, showed greater sensitivity in contrast to higher aged such as fingerlings of both species (Sloman & McNeil, 2012). This variation in sensitivity between early life stages and adults may be attributed to factors such as differences in surface area, underdeveloped homeostatic mechanisms for detoxification, weaker immune systems, and increased uptake of toxicants from the environment (Petersen & Kristensen, 1998). The results of present studies revealed that the Median LC_{50}/96h values are species, age and developmental stage specific. In this study particularly 50% mortality occurred at lower doses in earlier developmental stages compared to later stages. This suggested the higher toxicity of Cu, particularly even at lower concentrations. Hedayati et al. (2016) reported that metal which is highly toxic to a fish species at low concentrations may be less or even non-toxic to other species at the same or even higher concentrations. However, it is acknowledged that toxicity levels may vary depending on the test species, age, feeding habits, sex, toxicant composition, and experimental conditions (Latif et al., 2013; Witeska et al., 1995). Furthermore, Cu analyses demonstrated aged and developmental stage-specific bioaccumulation patterns in both freshwater fishes Rohu and Mrigal. Cu concentration increased with advancing developmental stages in response of their mortality, and results showing with highest accumulation of Cu in fingerlings. Rohu exhibited higher sensitivity to Cu toxicity compared to Mrigal across all developmental stages studied.

Various factors influencing metal toxicity and bioaccumulation including metal concentration, mode of metal uptake, environmental factors as well as intrinsic factors such as fish age and feeding habits

(Vosyliene & Jankaite, 2006; Farombi et al., 2007; Vinodhini & Narayanan, 2008). Ambedkar & Muniyan, (2011) previously reported a higher concentration of Cu in the liver tissue of *Saurida undosquamis* from the Kollidam River in India. Similarly, George et al. (2013), detected Cu in *Oreochromis niloticus*, *Schilbe mystus* and *Tilapia zillii* wherein the highest concentration was observed in *Oreochromis niloticus*, and with *Schilbe mystus* exhibiting the lowest concentration. In the present study, Cu is analyzed at tissues at fingerling stage. It is observed that in Rohu, Cu accumulated predominantly in the gills, followed by the liver, skin, kidney, and muscle, whereas in Mrigal, the order of accumulation was liver > gills > kidney > skin > muscle. Yousafzai & Shakoori (2006), reported similar finding to this that heavy metals accumulation is greater in liver and kidney as compared to muscles and skin.

In this study the highest accumulation observed in the gills and liver of Rohu, and in the liver of Mrigal. This observation aligns with the results reported by Vinodhini and Narayanan (2008), who demonstrated organ-specific accumulation of metals, where lead and cadmium exhibited higher accumulation in the kidney and gills compared to chromium and nickel. Notably, the liver and kidney are recognized as key organs involved in heavy metal detoxification (Vinodhini & Narayanan 2008). Furthermore, study is consistent with previous research of (Yousafzai & Shakoori, 2006; Has-Schon et al., 2008; Yousafzai et al., 2012). This study suggests tissue-specific bioaccumulation of metals, greater accumulation observed in the liver and kidney compared to muscle and skin tissues. This phenomenon may be attributed to the presence of metal-binding proteins, such as metallothione in the liver, which can significantly accumulate higher metal contents compared to muscle tissues (Ploetz et al., 2007; Uysal et al., 2009). Additionally, the distribution of metals across different tissues is influenced by the nature and type of metal (Jezierska & Witeska, 2001). This study reports for the first time that the Median LC_{50}/96h of Cu and bioaccumulation exhibit specificity concerning age and developmental stage specific pattern in these two freshwater fishes. Consequently, it is suggested for the implementation of pre-treatment measures for industrial effluent before discharge into freshwater ecosystems to mitigate potential adverse effects on fish.

Conclusion

The present study represents the investigation into the determination of Median LC_{50}/96h values of Cu across at four developmental stages such as embryonic and larval, post-larval, advanced Fry, and fingerling in two commercially important freshwater fish species, Rohu (*Labeo rohita*) and Mrigal (*Cirrhinus mrigala*). The LC_{50}/96h values

obtained for Rohu are recorded at 0.37 ppm, 0.75 ppm, 1.07 ppm, and 1.42 ppm whereas for Mrigal, corresponding values are 0.48 ppm, 0.94 ppm, 1.36 ppm, and 1.53 ppm at embryonic and larval, post-larval, advanced fry, and fingerling across the four developmental stages respectively. The results suggest differential susceptibility of the two freshwater fish species to Cu toxicity across developmental stages, with the order of susceptibility being Larval < Swim-up fry < Advanced fry < Fingerling stages. Furthermore, results indicate bioaccumulation order of Cu across the four developmental stages, with Fingerling stage exhibiting the highest bioaccumulation followed by advanced fry, swim-up fry, and embryonic and larval stages for both Rohu and Mrigal. At the Fingerling stage, Cu bioaccumulation demonstrated tissue specificity, with the order of accumulation being gills > liver > skin > kidney > muscle tissues for Rohu, and liver > gills > kidney > skin > muscle tissues for Mrigal.

Acknowledgments: We would like to thank Mr. Muhammad Akram, Senior Scientific Officer, Pakistan Council of Scientific and Industrial Research (PCSIR). We would like to thanks for Dr. Muhammad Shahab Chairman of Animal Sciences, and Dr. Aziz Ullah Kakar assistant professor Department of Animal science Quaid-i-azam University Islamabad, Pakistan for facilitating and providing guidance during the sample analysis.

Funding Declaration: For this project, funds were provided by Islamia College University, Peshawar, and the Higher Education Commission (HEC) Islamabad Pakistan.

Author's Contributions: The study's conception and design, plan, experimental work, laboratory work, data collection, and first drafts of the manuscript were done by Azam Jan Afridi. Summia Perveen and Misbah Irm did data validation, statistical analysis and interpretation. Final drafts were edited and reviewed by Amina Zuberi, and Ali Muhammad. The final manuscript was edited, revised and improved by Azam Jan Afridi. All authors read and approved the final manuscript.

Declaration of competing interest: All the authors have no financial or any other conflict of interest.

References

Abou El-Naga, E. H., El-Moselhy, K. M., & Hamed, M. A. (2005). Toxicity of cadmium and copper and their effect on some

biochemical parameters of marine fish *Mugil seheli*. Egyptian journal of aquatic research, 31(2), 60-71.

Adhikari, S., Ghosh, L., & Ayyappan, S. (2006). Combined effects of water pH and alkalinity on the accumulation of lead, cadmium and chromium to *Labeo rohita* (Hamilton). International Journal of Environmental Science & Technology, 3, 289-296.

Afridi A.J, Zuberi A, Yousafzai A. M, Kamran M, Ullah S (2019) Hemp (Marijuana) reverted copper-induced toxic effects on the essential fatty acid profle of *Labeo rohita* and *Cirrhinus mrigala*. Mol Biol Rep 46:391–401.

Afridi, A. J., Zuberi, A., Yousafzai, A. M., & Kamran, M. (2023). Therapeutic Role of Hemp (Cannabis sativa) Against Copper-Induced Toxicity in *Labeo rohita* and *Cirrhinus mrigal*a. *Biological Trace Element Research*, 1-12.

Afridi, A. J., Zuberi, A., Yousafzai, A. M., Saeed, K., HASEEB, A., & HASSAN, Z. (2026). Analysis of proximate composition, fatty acids profile, and mineral elements of hemp (*Cannabis sativa*). Pak. J. Bot, 58, 1.

Akan, J. C., Abbagambo, M. T., Chellube, Z. M., & Abdulrahman, F. I. (2012). Assessment of pollutants in water and sediment samples in Lake Chad, Baga, North Eastern Nigeria. *Journal of Environmental Protection* 3(11), 1428.

Ambedkar, G., and Muniyan, M., (2011). Bioaccumulation of some Heavy Metals in the selected five freshwater fish from Kollidam River, Tamilnadu, India. *Adv. Appl. Sci. Res.*, *2* (5), 221-225.

Authman, M.M., (2015). Use of Fish as Bio-Indicator of the Effects of Heavy Metals Pollution. *Journal of Aquaculture Research and development*6:1–13

Boglione, C., Gisbert, E., Gavaia, P., E Witten, P., Moren, M., Fontagné, S., and Koumoundouros, G., (2013). Skeletal anomalies in reared European fish larvae and juveniles. Part 2: main typologies, occurrences and causative factors. Review in Aquaculture5 (s1).

De Oliveira-Filho, E. C., Lopes, R. M., & Paumgartten, F. J. R. (2004). Comparative study on the susceptibility of freshwater species to copper-based pesticides. Chemosphere, 56 (4), 369-374.

Eastwood, S., & Couture, P. (2002). Seasonal variations in condition and liver metal concentrations of yellow perch (*Perca flavescens*) from a metal-contaminated environment. Aquatic Toxicology, 58(1-2), 43-56.

Eisler, R., (2000). Handbook of Chemical Risk Assessment: Health Hazards to Humans, Plants, and Animals, Three Volume Set (Vol. 1). CRC press.

Ezemonye, L.I.,Adebayo, P.O.,Enuneku, A.A, Tongo, I.,Ogbomida, E. (2019). Potential Health Risk Consequences of Heavy Metal Concentrations in Surface Water, Shrimp (*Macrobrachium macrobrachion*) and Fish (*Brycinus longipinnis*) from Benin River, Nigeria. *Toxicol. Rep.6*: 1–9.

Farombi EO, Adelowo OA, Ajimoko YR (2007). Bioaccumulation of oxidative stress and heavy metal levels as indicators of environmental pollution in African Catfish (*Clarias gariepinus*) from Nigeria Ogun River. Int. J. Environ. Public Health 4(2):158-165.

Finney, D.J., (1971). Probit Analysis. 3 (ed) London, rd New York, Cambridge University Press, pp: 333.

Gaetke, L. M., & Chow, C. K. (2003). Copper toxicity, oxidative stress, and antioxidant nutrients. *Toxicology*, *189*(1-2):147-163.

George, U., Asuquo, F., Idung, J., & Andem, A. (2013). Bioaccumulation of heavy metals in three fresh water fishes caught from Cross River system. Eur. J. Exp. Biol, 3(3), 576-582.

Gharedaashi, E., Imanpour, M. R., & Taghizadeh, V. (2013). Determination of median lethal concentration (LC50) of copper sulfate and lead nitrate and effects on behavior in Caspian Sea kutum (*Rutilus frisiikutum*). *J. of Toxic and Environ Health Sci.*, *5*(1): 12-16.

Grosell, M., Wood, C. M., Wilson, R. W., Bury, N. R., Hogstrand, C., Rankin, C., & Jensen, F. B. (2005). Bicarbonate secretion plays a role in chloride and water absorption of the European flounder intestine. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 288(4): 936-946.

Gross, E. M. (2022). Aquatic chemical ecology meets ecotoxicology. Aquatic Ecology, 56 (2), 493-511.

Handy, R. D. (2003). Chronic effects of copper exposure versus endocrine toxicity: two sides of the same toxicological process? *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 135(1):25-38.

Has-Schon, E., Bogut, I., Rajković, V., Bogut, S., Čačić, M., & Horvatić, J. (2008). Heavy metal distribution in tissues of six fish species included in human diet, inhabiting freshwaters of the Nature Park "Hutovo Blato"(Bosnia and Herzegovina). *Archives of environmental contamination and toxicology*, 54(1): 75-83.

Hedayati, A., Safahieh, A., Savari, A., andMarammazi, J. G., (2016). Detection of Acute Toxicity of Mercury Chloride in Yellowfin Sea Bream (*Acanthopagrus latus*). *Bio sci Biotechnol Res Asia, 7*(1): 65-73.

Islam, M. A., Amin, S. N., Brown, C. L., Juraimi, A. S., Uddin, M. K., & Arshad, A. (2021). Determination of median lethal concentration (LC50) for endosulfan, heptachlor and dieldrin pesticides to African catfish, *Clarias gariepinus* and their impact on its behavioral patterns and histopathological responses. Toxics, 9(12), 340.

Jamil Emon, F., Rohani, M. F., Sumaiya, N., Tuj Jannat, M. F., Akter, Y., Shahjahan, M., & Goh, K. W. (2023). Bioaccumulation and Bioremediation of Heavy Metals in Fishes—A Review. *Toxics*, *11*(6), 510.

Jezierska, B., & Witeska, M. (2001). Summary of metal-induced disturbances in fish organism. *Metal Toxicity to Fish. Wydawnictvo Akademii Podlaskej, Siedlce*, 214-243.

Jezierska, B., Sarnowski, P., Witeska, M., Ługowska, K., (2009b). Disturbances of early development of fish caused by heavy metals (a review). Electron J. Ichthyol., 5(2):76–96.

Latif A., Ali M., Sayyed A.H., Iqbal F., Usman K., Rauf M. and Kaoser R., (2013).Effect of copper sulphate and lead nitrate, administered alone or in combination, on the histology of liver and kidney of *Labeo rohita*, Pak. J. Zool., 45(4), 913-920.

Nekoubin, H., Gharedaashi, E., Hatefi, S., Sudagar, M., Shahriari, R., &Asgharimoghadam, A. (2012). Determination of LC50 of copper sulfate and lead (II) nitrate and behavioral responses of grass carp (*Ctenopharyngodon idella*). *Walailak Journal of Science and Technology (WJST)*, *9*(4): 333-340.

Osman, A. G., Mekkawy, I. A., Verreth, J., &Kirschbaum, F. (2007). Effects of lead nitrate on the activity of metabolic enzymes during early developmental stages of the African catfish, *Clarias gariepinus* (Burchell, 1822). *Fish Physiology and Biochemistry*, *33*(1), 1-13.

Panigrahi, A., (2014). Heavy metal toxicity. *EVERYMAN'S SCIENCE.*, 418.

Paruruckumani, P. S., Maharajan, A., Seema, P., &Ganapiriya, V. (2015). Acute toxicity bioassay of copper on the juveniles of Asian sea bass, *Lates calcarifer* (Bloch). International Journal of Fisheries and Aquatic Studies 2015; 3(2): 337-342.

Petersen, G. I. and Kristensen, P. (1998): Bioaccumulation of lipophilic substances in fish early life stages. Environmental Toxicology and Chemistry 17: 1385-1395.

Ploetz, D. M., Fitts, B. E., Rice, T. M., (2007). Differential accumulation of heavy metals in muscles and liver of a marine fish (King Mackerel, *Scomberomorus cavalla*, Cuvier) from the Northern

Gulf of Mexico, USA. Bull. Environ. Contam. Toxicol. 78: 134-137.

Sarkar, M.M.; Rohani, M.F.; Hossain, M.A.R.; Shahjahan, M. (2022). Evaluation of Heavy Metal Contamination in Some Selected Commercial Fish Feeds Used in Bangladesh. *Biol. Trace Elem. Res.* 200: 844–854

Sarkar, T.; Alam, M.M.; Parvin, N.; Fardous, Z.; Chowdhury, A.Z.; Hossain, S.; Haque, M.E.; Biswas, N. (2016). Assessment of Heavy Metals Contamination and Human Health Risk in Shrimp Collected from Different Farms and Rivers at Khulna-Satkhira Region, Bangladesh. *Toxicol. Rep.* 3: 346–350.

Shah S.L. and Altindu A., (2005). Effects of heavy metal accumulation on the 96hr LC50 values in tench, Tincatinca L., 1758. Turk. J. vet. Anim. Sci., 29: 139-144.

Shahjahan, M.; Taslima, K.; Rahman, M.S.; Al-Emran, M.; Alam, S.I.; Faggio, C. (2022). Effects of Heavy Metals on Fish Physiology—A Review. *Chemosphere*, *300*, 134519

Shukla V, Dhankhar M, Prakash J, Sastry KV (2007). Bioaccumulation of Zn, Cu and cd in *Channa punctatus*. J Environ Biol 28(2):395.

Sloman, K. A., & McNeil, P. L. (2012). Using physiology and behaviour to understand the responses of fish early life stages to toxicants. Journal of fish biology, 81(7), 2175-2198.

Taslima, K.; Al-Emran, M.; Rahman, M.S.; Hasan, J.; Ferdous, Z.; Rohani, M.F.; Shahjahan, M. (2022). Impacts of Heavy Metals on Early Development, Growth and Reproduction of Fish—A Review. *Toxicol. Rep. 9:* 858–868.

Thangam, Y., Jayaprakash, S., & Perumayee, M. (2014). Effect of copper toxicity on hematological parameters to fresh water fish *Cyprinus carpio* (common carp). Journal of Environmental Science, Toxicology and Food Technology, 8(9), 50-60.

Turkmen, A., Turkmen, M., Tepe, Y., & Akyurt, I. (2005). Heavy metals in three commercially valuable fish species from Iskenderun Bay, Northern East Mediterranean Sea, Turkey. Food chemistry, 91(1), 167-172.

Uauy, R., Olivares, M., & Gonzalez, M. (1998). Essentiality of copper in humans. *The American journal of clinical nutrition*, *67*(5): S952-S959.

Uysal, K., Kose, E., Bulbul, M., Donmez, M., Erdogan, Y., Koyun, M., Omeroglu, A.,Ozmal, F.,(2009). The comparison of heavy metal accumulation ratios of some fish species in Enne Dame Lake (Kutahya Turkey). Environ. Monit. Assess. 157: 355–362.

Valko, M. M. H. C. M., Morris, H., & Cronin, M. T. D. (2005). Metals, toxicity and oxidative stress. Current medicinal chemistry, 12(10), 1161-1208.

Vieira, L. R., Gravato, C., Soares, A. M. V. M., Morgado, F., &Guilhermino, L. (2009). Acute effects of copper and mercury on the estuarine fish *Pomatoschistus microps*: linking biomarkers to behaviour. *Chemosphere*, *76*(10), 1416-1427.

Vinodhini, R., Narayanan, M., (2008). Bioaccumulation of heavy metals in organs of fresh water fish Cyprinus carpio (Common carp). Int. J. Environ. Sci. Technol., 5(2):179-182.

Vosyliene MZ, Janakaite A (2006). Effect of heavy metal model mixture on rainbow trout biological parameters. Ekologia 4:12-14.

Witeska, M., Jezierska, B., & Chaber, J. (1995). The influence of cadmium on common carp embryos and larvae. *Aquaculture*, *129*(1): 129-132.

Woltering, D. M. (1984): The Growth-Response in Fish Chronic and Early Life Stage Toxicity Tests - a Critical-Review. Aquatic Toxicology 5: 1-21.

Xia, J., Zhang, Y. Y., Zhan, C., & Ye, A. Z. (2011). Water quality management in China: the case of the Huai River Basin. *International Journal of Water Resources Development*, *27*(1): 167-180.

Yaqub, A., Anjum, M. K., Bari, S., Ayub, M., Kamran, M., Malkani, N., (2018) Evaluation of Cd (II) and Cr (VI) accumulation in Thaila fsh *Gibelion catla* (formerly *Catla catla*) collected from industrially polluted water. Pak J Zool 33:88–9.

Yousafzai, A. M., Siraj, M., Habib, A., & Chivers, D. P. (2012). Bioaccumulation of heavy metals in common carp: implications for human health. *Pak J of Zool*, *44*(2).

Yousafzai, A.M., A.R. Shakoori. 2006. Bioaccumulation of chromium, nickel, lead, copper, and zinc in the skin of *Tor putitora*as an indicator of the presence of the heavy metal load in River Kabul Pakistan. Pak J. of. Zool.38: 341-346

Zhang, H., Cao, H., Meng, Y., Jin, G., Zhu, M., 2012. The toxicity of cadmium (Cd2?) towards embryos and pro-larva of Soldatov's catfish (*Silurus soldatovi*). *Ecotoxicol Environ Saf*., 80:258–265.