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RESEARCH ARTICLE

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Copper and nanocopper toxicity using integrated biomarker response in *Pangasianodon hypophthalmus*

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Abstract

The current study focused on assessing the toxicological effects of copper (Cu) and copper nanoparticles (Cu-NPs) in acute condition on *Pangasianodon hypophthalmus*. The median lethal concentration (LC_{50}) for Cu and Cu-NPs were determined as 8.04 and 3.85 mg L^{-1}, respectively. For the subsequent definitive test, varying concentrations were selected: 7.0, 7.5, 8.0, 8.5, and 9.0 mg L^{-1} for Cu, and 3.0, 3.3, 3.6, 3.9, and 4.2 mg L^{-1} for Cu-NPs. To encompass these concentration levels and assess their toxic effects, biomarkers associated with toxicological studies like oxidative stress, neurotransmission, and cellular metabolism were measured in the liver, kidney, and gill tissues. Notably, during the acute test, the activities of catalase, superoxide dismutase, glutathione-s-transferase, glutathione peroxidase, and lipid peroxide in the liver, gill, and kidney tissues were significantly increased due to exposure to Cu and Cu-NPs. Similarly, acetylcholinesterase activity in the brain was notably inhibited in the presence of Cu and Cu-NPs when compared to the control group. Cellular metabolic stress was greatly influenced by the exposure to Cu and Cu-NPs, evident from the considerable elevation of cortisol, HSP 70, and blood glucose levels in the treated groups. Furthermore, integrated biomarker response, genotoxicity, DNA damage in gill tissue, karyotyping in kidney tissue, and histopathology in gill and liver were investigated, revealing tissue damage attributed to exposure to Cu and Cu-NPs. In conclusion, this study determined that elevated concentrations of essential trace elements, namely Cu and Cu-NPs, induce toxicity and disrupt cellular metabolic activities in fish.

KEYWORDS
acute test, copper nanoparticles, fish, IBR, neurotransmitter, oxidative stress

1 | INTRODUCTION

Metals are significant pollutants in aquatic ecosystems that affect aquatic organisms, including fish. Contamination of aquatic systems has led to major environmental and health issues for both humans and aquatic animals.[1,2] Anthropogenic activities have increased the concentration of metals in natural water, impacting aquatic animals and subsequently human health.[3,4] Inorganic pollutants, such as metals, possess specific properties like dispersal, bioaccumulation, and biomagnification within aquatic food chains.[5,6] Copper (Cu), among various metals, holds a significant presence in the environment. With a global production of approximately 18.7 million metric tons, Cu finds uses in electrical and electronic applications (39%), pipes (30%), and various other sectors (NMIC, 2016). It serves roles in industrial applications, medicine, pesticides,[7] and even functions as an anti-fouling agent in paint.[8] While Cu is essential as a cofactor for over 30 enzymes in living organisms, higher concentrations can prove highly toxic to aquatic animals, including fish.[9,10]

Moreover, Cu is utilized in the form of nano-materials in diverse sectors, further impacting aquatic systems. Excessive use of Cu nanoparticles (Cu-NPs) can intensify contamination in aquatic ecosystems, negatively affecting fish by impeding their growth and development.[11,12] Due to their non-degradability, both Cu and Cu-NPs accumulate in aquatic systems and within fish bodies.[13] This accumulation leads to altered morphology, fatty metamorphosis in liver tissue, hematopoietic tissue destruction, kidney necrosis, gill tissue changes, and elevated cortisol levels in fish.[14,15] In aquatic systems, Cu-NPs dissolve into highly toxic Cu ions for fish.[16,17] Additionally, Cu-NPs aggregate in aquatic systems, negatively impacting fish species due to their high reactivity.[18]

Cu has the capacity to induce mitochondrial dysfunction and alter metabolic energy production in fish.[19,20] Furthermore, Cu can trigger oxidative stress in exposed organisms by excessive reactive oxygen species (ROS) production,[21] which disrupts electron transport and reduces ATP production.[19,20] Key enzymes like Catalase (CAT), Superoxide Dismutase (SOD), Glutathione S-Transferase (GST), and Glutathione Peroxidase (GPx) counterbalance ROS generation, maintaining ATP production during stress.[22,23] Although Cu plays a crucial role in Fenton-like reactions, high concentrations can catalyze ROS production, disrupting the reaction.[24,25] Moreover, Cu binds to negatively charged proteins' SH groups, denaturing enzymes.[26,27] In cold-water fish, Cu exposure triggers Na^+ losses and inhibits Na uptake, directly impacting gill Na^+/K^+-ATPase activities.[28] Cu and Cu-NPs exposure induces stress responses, inhibiting neurotransmitter enzymes in fish. Copper and its nanoparticles lead to elevated adrenaline/cortisol levels, interconnected with increased heat shock protein (HSP 70) levels.[29] Adrenaline/cortisol induces the fish's HSP response.[30] Cu and Cu-NPs accumulate in various fish tissues, including liver, gill, kidney, brain, and muscle. Bioaccumulation may stem from the dissolution nature of Cu and Cu-NPs.[31] Reports suggest that even at low levels, Copper and its nanoparticles can be toxic, evident from altered liver and gill tissue histopathology in fish within contaminated aquatic systems.[31]

Pangasianodon hypophthalmus serves as a vital species for nanoparticle toxicity assessment study.[32,33] Its high growth rate, stress tolerance, and market demand make it ideal for metal and nanoparticle toxicological studies.[34] This study aims to comprehend the escalating threat of metal and nanoparticle toxicity in aquatic environments, particularly their impact on the catfish *P. hypophthalmus*. The study's objectives include comparing the toxicity of Cu and Cu-NPs and evaluating their effects on various biomarkers in *P. hypophthalmus*.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

The study protocol and experimental endpoints were approved by the Research Advisory Committee of ICAR-NIASM. All methods were conducted in compliance with National and International guidelines and regulations. The study adheres to the Animal Research: Reporting of in Vivo Experiments (ARRIVE) guidelines.

2.2 | Experimental animals

The experimental fish used for this investigation was *P. hypophthalmus*. These fish were obtained from Biswas Fish Farm in Kolkata, West Bengal, India, in a healthy condition. The average weight and length of the fish were 9.96 g and 8.53 cm, respectively. During a 40-day acclimation period, the fish were fed a diet containing 30% crude protein. Feeding was cessed 24 h prior to commencing the acute toxicity experiment.

2.3 | Experimental details

In this study, the median lethal concentration (LC_{50}) was determined for copper (Cu) and copper nanoparticles (Cu-NPs) in *P. hypophthalmus* based on the APHA[35] protocol. The experiment was conducted as a static and non-renewable bio-assay to determine the LC_{50} of Cu and Cu-NPs. Copper sulfate and Cu-NPs were used for this bioassay, with a concentration of 100 mg L^{-1} for copper sulfate (HiMedia). Cu-NPs were biologically synthesized using fish gill tissue.[11,12] To determine the LC_{50}, a range-finding test was performed for both Cu and Cu-NPs using concentrations of 5, 10, 15, 20, and 25 mg L^{-1} for Cu, and 2, 4, 6, 8, and 10 mg L^{-1} for Cu-NPs. Each concentration was tested on five fish, and the range-finding test was duplicated. LC_{50} concentrations for the main study were chosen based on these results: Cu (7.0, 7.5, 8.0, 8.5, and 9.0 mg L^{-1}) and Cu-NPs (3.0, 3.3, 3.6, 3.9, and 4.2 mg L^{-1}). The main test was conducted in triplicate, with 12 fish in each replicate. The cumulative mortality was observed at 24, 48, 72, and 96 h of the LC_{50} determining test. The safe application factor of Cu and Cu-NPs was calculated using the method of Hart et al.[36] Water quality parameters were also measured during the experiments (Table S-1). The experimental conditions included a light photoperiod of 13 h and a dark photoperiod of 11 h. Details of the treatments are provided in Table 1.

Presumable harmless concentration $(C) = 48\,h\,LC_{50} \times 0.3/S^2$. where $S = 24\,h\,LC_{50}/48\,h\,LC_{50}$.

The experimental groups were followed in the present study namely, control (No exposure to Cu and Cu-NPs), Cu at 7.0 to 9.0 mg L^{-1} (Cu-7.0, 7.5, 8.0, 8.5 and 9.0 mg L^{-1}) and Cu-NPs at 3.0–4.2 mg L-1 (Cu-NPs- 3.0, 3.3, 3.6, 3.9 and 4.2 mg L^{-1}).

2.4 | Fish tissue extract using green synthesis of Cu-NPs

Gill tissue was thoroughly cleaned with running tap water, followed by distilled water. The cleaned tissues were finely cut and homogenized using a mortar and pestle to obtain a uniform fish tissue extract. The resulting tissue homogenate was centrifuged at 6000*g* at 4°C for 15 min.[33,37] The obtained supernatant was filtered using 0.45 μm pore size filter paper. The filtrate was mixed with a solution of copper sulfate pentahydrate (10 mM) and heated to 100°C for 1 h, maintaining a pH of 11 with 1 M NaOH. The color of the solution changed

TABLE 1 Determination of median lethal concentration (96-LC_{50}) of copper (Cu) and copper nanoparticles (Cu-NPs) to *Pangasianodon hypophthalmus* for a period of 96 h.

Exposure (h)	LC_{50} (mg L^{-1})	95% confidence interval		S value	Safe level (C)	R^2 value	Intercept	Slope
		Lower	Higher					
Copper								
24	10.57	9.34	24.01	1.23	3.52	0.66	−7.93	1.27
48	9.52	8.82	12.72			0.80	−10.67	1.73
72	8.71	8.27	9.84			0.78	−12.6	2.12
96	8.04	7.56	8.59			0.86	−11.2	2.23
Copper nanoparticles								
24	5.03	4.39	9.45	1.33	1.73	0.82	−6.40	2.33
48	4.34	4.04	5.34			0.91	−9.67	3.56
72	4.13	3.85	4.97			0.88	−8.73	3.51
96	3.85	3.60	4.40			0.89	−7.78	3.620

from blue to reddish-brown, indicating the presence of Cu-NPs. The solution was then centrifuged to collect the Cu-NPs using ethanol.[11,12]

2.5 | Characterization of copper nanoparticles

Characterization of Cu-NPs was conducted by measuring the absorption spectrum in the UV–Vis range (300–500 nm) using a Shimadzu UV-1900i spectrophotometer. The particle size and zeta potential of Cu-NPs were determined at 25°C using a Nanoparticle Analyzer (Particle Analyser, Litesizer 500, Antonpaar Austria). The mean size and zeta potential of Cu-NPs were measured as 24.0 nm and −54.7 mV, respectively (Figure 1).

2.6 | Sample preparation for enzymatic analysis

For enzymatic analysis, *P. hypophthalmus* were anesthetized using clove oil at a concentration of 100 μg L^{-1} after 96 h of exposure. Tissues such as liver, gill, kidney, and brain were dissected, thoroughly cleaned, and homogenized in 0.25 M chilled sucrose solution and 1 mM EDTA using a tissue homogenizer (Omni Tissue Master Homogenizer, Kennesaw, GA). The resulting tissue homogenates were centrifuged at 6000×*g* for 20 min at 4°C and stored at −80°C.[11,12]

2.7 | Antioxidant enzymes activity

The activity of superoxide dismutase (SOD; EC 1.15.1.1) was determined using the method described by Misra and Fridovich.[38] The activity of catalase (CAT; EC 1.11.1.6) was determined using the method described by Takahara et al.[39] The activity of glutathione-s-transferase (GST; EC 2.5.1.18) and glutathione peroxidase (GPx; EC 1.11.1.9) was determined using the methods described by Habing et al.[40] and Paglia and Valentine,[41] respectively.

2.8 | Neurotransmitter enzyme activity

The activity of acetylcholine esterase (AChE; EC 3.1.1.7) was measured using the Hestrin method modified by Augustinsson.[42] Briefly, a mixture of phosphate buffer, acetylcholine buffer, and the sample was incubated for 30 min at 37°C. After incubation, alkaline hydroxylamine, HCl, and ferric chloride were added, and readings were taken at 540 nm.

2.9 | Lipid peroxidation

Lipid peroxidation (LPO) was determined using the method of Uchiyama and Mihara.[43] Liver and kidney tissues (0.25 mL) were homogenized with 25 μL of 10 mM butylated hydroxytoluene (BHT) and added to a mixture of 3 mL of 1% phosphoric acid and 1 mL of 0.67% thiobarbituric acid (TBA). The mixture was incubated at 90°C for 45 min, and the final absorbance was measured at 535 nm.

2.10 | Blood glucose

Blood collection was performed on five fish for blood and serum parameter analysis. Prior to collection, the fish were anesthetized using clove oil at a concentration of 100 μL L^{-1}, and blood was sampled from the caudal vein using a syringe containing EDTA as an anticoagulant. Blood glucose was determined using the methods of Nelson[44] and Somoyogi.[45]

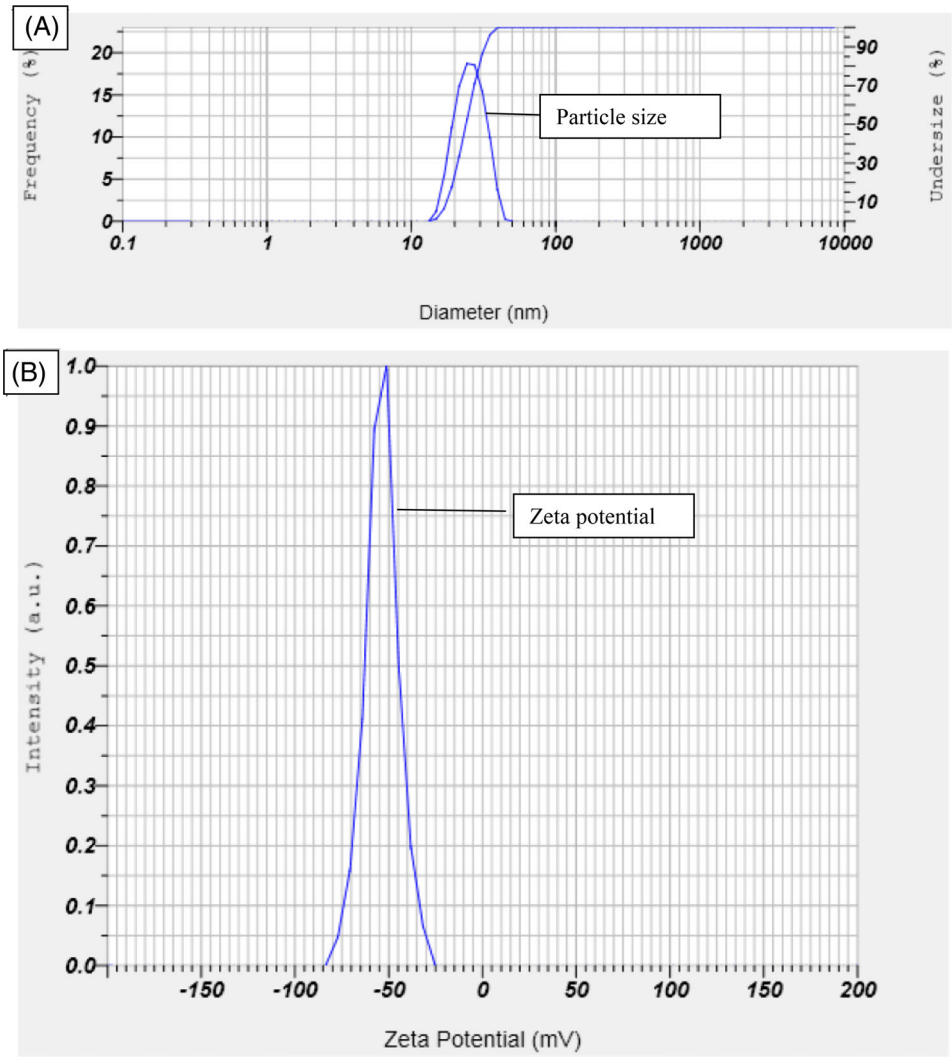

FIGURE 1 (A) Particle size (24 nm) and (B) zeta potential (−54.7 mV) of copper nano-particles (Cu-NPs).

2.11 | Cellular metabolic enzymes

Alanine aminotransferase (ALT; EC 2.6.1.2) and aspartate aminotransferase (AST; EC 2.6.1.1) activities were assessed using the Wootton[46] method. In this approach, the substrates DL-aspartic acid and α-ketoglutarate were employed to measure AST activity, while DL-alanine and α-ketoglutarate were used for ALT activity determination. Specifically, 0.5 mL of substrate was combined with tissue homogenate and incubated for 1 h at 37°C. Subsequently, 2,4-dinitrophenylhydrazine (2,4-DNPH) was added to the solution and left at room temperature for 20 min. The addition of NaOH followed, and absorbance measurements were recorded at 540 nm. Malate dehydrogenase (MDH; EC 1.1.1.37) and lactate dehydrogenase (LDH; EC 1.1.1.27) activities were determined according to the Ochoa[47] method. In this procedure, a phosphate buffer (0.1 M, pH 7.5) was mixed with freshly prepared NADH solution (0.2 mM) and newly prepared sodium pyruvate (0.2 mM), and the absorbance was measured at 340 nm. Notably, for MDH analysis, oxaloacetate was utilized as a substrate in place of sodium pyruvate. The activity of adenosine triphosphatase (ATPase; EC 3.6.1.3) was evaluated using the method outlined by Post and Sen.[48]

2.12 | Total protein, albumin, globulin, A:G ration and respiratory burst activity (NBT)

Serum total protein, albumin, globulin, and the A:G ratio were quantified using a protein estimation kit. Respiratory burst activity was determined through the approach outlined by Secombes,[49] which was adapted by Stasiack and Baumann.[50]

2.13 | Histopathological studies

For histopathological studies, gill and liver tissues were collected from experimental animals, stored in 10% neutral buffered formalin, and prepared as slides using a rotary microtome (7 μm). The slides were stained with hematoxylin and eosin (H&E) following the method described by

Robert.[51] The slides were examined under a microscope (Leica Microsystems Ltd, DM 2000, Heerbrugg, Switzerland) and photographed.

2.14 | Genotoxicity (karyotyping)

Karyotyping in kidney tissue was performed according to a previously established method with minor modifications.[52] Colchicine was injected intramuscularly at a concentration of 0.03 mg mL^{-1}, 4 h prior to the test. Kidney tissue was collected aseptically from three fish per treatment. The tissues were treated with hypotonic solution (KCl) for cell swelling and then fixed with cornoy's fixative. Slides were stained with Giemsa and karyotyping photographs were captured using a fluorescence microscope (Leica Microsystems Ltd, DM 2000, Heerbrugg, Switzerland).

2.15 | Alkaline single-cell gel electrophoresis/ Comet assay

The assessment of DNA damage was conducted using the single-cell gel electrophoresis (SCGE) method (alkaline single-cell gel electrophoresis/comet assay) in gill tissue, employing a three-layer agarose approach as described by Ali and colleagues[53] with slight modifications.[54] To outline the process briefly, gill tissues (50 mg) were initially cleansed with double distilled water, then twice with chilled phosphate-buffered saline (Ca^{2+} Mg^{2+} free), and subsequently transferred to an ice-cold homogenization buffer (20 mM EDTA; 1-X Hanks' balanced salt solution; 10% dimethyl sulfoxide [DMSO], pH 7.0–7.5). These tissues were subsequently cut into small fragments, homogenized to obtain a single-cell suspension, and then centrifuged at 3000 rpm at 4°C for 5 min to yield a cell pellet. This pellet was resuspended in chilled phosphate-buffered saline, readying it for the comet assay. A cell viability test was also performed using the trypan blue exclusion method,[53] which yielded over 86% viable cells. Cleaned glass slides were coated initially with 200 μL of normal agarose (1%), and onto this, 15 μL of the cell suspension (approximately 20 000 cells) mixed with 85 μL of 0.5% low melting point agarose were added. A coverslip was placed on top, and subsequently, the slides were recoated with 100 μL of low melting-point agarose once the coverslips were removed. These slides were then left in a lysing solution at 4°C overnight (comprising 100 mM Na2EDTA, 2.5 M NaCl, 10 mM Tris pH 10 with 10% DMSO, and 1% Triton X-100, added freshly). The slides were later transferred to a horizontal gel

TABLE 2 Effect of comparative toxicity of copper (Cu) and copper nanoparticles (Cu-NPs) on activities of catalase (CAT), superoxide dismutase (SOD) and glutathione-S-transferase (GST) in liver, gill and kidney of *Pangasianodon hypophthalmus* for a period of 96 h.

Cu exposure (mg L^{-1})	Catalase (CAT)			Superoxide dismutase (SOD)			Glutathione-S-transferase (GST)		
	Liver	Gill	Kidney	Liver	Gill	Kidney	Liver	Gill	Kidney
Control	12.11^{a} ± 1.36	8.16^{a} ± 1.34	12.45^{a} ± 1.97	58.50^{a} ± 1.16	37.88^{a} ± 1.26	42.43^{a} ± 0.69	0.16^{a} ± 0.02	0.19^{a} ± 0.03	0.17^{a} ± 0.03
Cu-7.0	20.53^{bc} ± 2.55	13.15^{b} ± 0.69	18.01^{b} ± 1.61	77.76^{c} ± 1.60	54.20^{c} ± 1.34	53.81c ± 0.73	0.20^{b} ± 0.05	0.26^{b} ± 0.01	0.31^{b} ± 0.03
Cu-7.5	25.32^{bc} ± 1.30	15.31^{b} ± 1.23	18.67^{b} ± 0.79	77.51^{c} ± 1.14	47.53^{b} ± 1.39	52.78^{c} ± 0.84	0.23^{b} ± 0.01	0.30^{b} ± 0.02	0.33^{b} ± 0.02
Cu-8.0	24.67^{bc} ± 1.60	23.38^{c} ± 3.41	21.41^{bc} ± 2.37	80.46^{cd} ± 2.32	54.84^{c} ± 2.97	52.33^{c} ± 1.14	0.25^{b} ± 0.02	0.38^{c} ± 0.03	0.42^{c} ± 0.01
Cu-8.5	24.42^{bc} ± 1.32	22.76^{c} ± 1.64	30.21^{d} ± 1.05	81.64^{cd} ± 1.46	53.91^{c} ± 2.75	53.99c ± 0.66	0.23^{b} ± 0.03	0.37^{c} ± 0.02	0.59^{d} ± 0.05
Cu-9.0	33.09^{d} ± 2.87	29.81^{d} ± 1.33	29.27^{d} ± 2.25	82.17^{d} ± 1.26	54.78^{c} ± 1.44	55.79c ± 1.75	0.32^{c} ± 0.04	0.50^{d} ± 0.01	0.62^{d} ± 0.04
Cu-NPs-3.0	22.23bc ± 2.13	14.96^{b} ± 2.43	19.41^{b} ± 2.66	67.41^{b} ± 1.12	46.09^{b} ± 2.28	51.75^{c} ± 1.15	0.21^{b} ± 0.01	0.24^{b} ± 0.03	0.30^{b} ± 0.03
Cu-NPs-3.3	22.82^{bc} ± 2.89	13.30^{b} ± 1.69	21.34^{bc} ± 1.49	70.19^{b} ± 1.17	47.92^{b} ± 1.34	44.87^{bc} ± 1.91	0.23^{b} ± 0.02	0.30^{b} ± 0.02	0.30^{b} ± 0.02
Cu-NPs-3.6	31.80^{d} ± 3.12	15.53^{b} ± 1.20	24.57^{c} ± 2.77	70.51^{b} ± 2.08	48.59^{b} ± 2.41	42.89^{a} ± 1.82	0.24^{b} ± 0.03	0.30^{b} ± 0.03	0.38^{bc} ± 0.03
Cu-NPs-3.9	30.21^{d} ± 2.24	20.69^{c} ± 2.22	23.22^{c} ± 2.84	67.49^{b} ± 1.30	52.13^{c} ± 3.26	43.44^{a} ± 1.61	0.25^{b} ± 0.04	0.34^{c} ± 0.01	0.44^{c} ± 0.02
Cu-NPs-4.2	32.13d ± 3.07	22.77^{c} ± 3.43	23.99^{c} ± 3.47	76.27^{c} ± 0.48	48.57^{b} ± 1.70	42.46^{a} ± 1.13	0.31^{c} ± 0.02	0.46^{d} ± 0.05	0.58^{d} ± 0.03
p value	0.0023	0.0041	0.0037	0.020	0.017	0.048	0.0073	0.043	0.0034

Note: Values in the same column with different superscript (a, b, c, d, e) differ significantly ($p < .01$). Data expressed as mean ± SE ($n = 6$). Catalase, SOD, GST: units/mg protein.

TABLE 3 Effect of comparative toxicity of copper (Cu) and copper nanoparticles (Cu-NPs) on glutathione peroxidase (GPx), lipid peroxidation (LPO) and ATPase activities in *Pangasianodon hypophthalmus* for a period of 96 h.

Cu exposure (mg L^{-1})	Glutathione peroxidase			Lipid peroxidation			ATPase	
	Liver	Gill	Kidney	Liver	Gill	Kidney	Liver	Gill
Control	1.74^{a} ± 0.28	1.28^{a} ± 0.29	2.48^{a} ± 0.52	4.50^{a} ± 0.46	3.07^{a} ± 0.24	8.10^{a} ± 0.31	131.61^{e} ± 7.64	126.15^{e} ± 7.82
Cu-7.0	3.22^{b} ± 0.21	3.11^{b} ± 0.32	3.59^{ab} ± 0.34	10.50^{bc} ± 0.37	6.30^{c} ± 0.97	11.21b^{c} ± 0.22	106.85^{d} ± 1.12	97.71^{cd} ± 4.73
Cu-7.5	3.48^{b} ± 0.33	5.91^{de} ± 0.38	4.66^{b} ± 0.28	11.79^{bc} ± 0.64	9.69^{d} ± 0.37	13.24^{c} ± 0.59	98.83^{bc} ± 8.66	81.23^{ab} ± 7.88
Cu-8.0	3.60^{b} ± 0.22	6.25^{e} ± 0.34	5.21^{c} ± 0.19	13.20^{c} ± 0.88	9.59^{d} ± 0.59	14.66^{c} ± 1.21	101.18^{c} ± 9.03	80.07^{ab} ± 7.19
Cu-8.5	4.43^{bc} ± 0.27	6.02^{e} ± 0.28	7.03^{d} ± 0.46	15.26^{d} ± 2.69	10.50^{d} ± 1.60	16.06^{d} ± 0.37	102.06^{c} ± 14.57	72.58^{a} ± 8.77
Cu-9.0	5.12^{c} ± 0.24	6.76^{e} ± 0.29	7.91^{d} ± 0.65	12.59^{d} ± 0.57	12.58^{e} ± 0.56	18.76^{e} ± 0.50	104.09^{cd} ± 3.98	82.74^{ab} ± 7.12
Cu-NPs-3.0	3.01^{b} ± 0.26	3.16^{b} ± 0.32	3.77^{ab} ± 0.31	8.95^{b} ± 0.31	4.67^{b} ± 0.49	9.40^{b} ± 0.42	83.76^{a} ± 5.88	95.40^{c} ± 4.15
Cu-NPs-3.3	2.96^{b} ± 0.44	3.88^{b} ± 0.52	3.84^{ab} ± 0.34	10.70^{bc} ± 0.27	7.12^{c} ± 0.76	10.87^{bc} ± 0.56	95.73^{bc} ± 5.71	102.0^{d} ± 10.14
Cu-NPs-3.6	3.62^{b} ± 0.47	3.40^{b} ± 0.31	4.58^{b} ± 0.26	11.96^{bc} ± 0.34	9.69^{d} ± 0.59	9.12^{b} ± 0.64	90.14^{b} ± 4.14	95.70^{c} ± 9.28
Cu-NPs-3.9	3.92^{b} ± 0.12	3.69^{b} ± 0.23	5.46^{c} ± 0.39	13.11^{c} ± 0.40	11.01^{de} ± 0.83	10.95^{bc} ± 0.72	95.39^{bc} ± 6.43	93.36^{c} ± 4.47
Cu-NPs-4.2	4.07^{bc} ± 0.56	4.78^{c} ± 0.22	5.68^{c} ± 0.44	13.45^{c} ± 0.67	10.63^{d} ± 0.53	15.31^{cd} ± 0.96	101.68^{c} ± 2.44	95.72^{c} ± 2.86
p value	.0064	.0021	.0036	.0017	.0028	.0047	.005	.0022

Note: Values in the same column with different superscript (a, b, c, d, e, f) differ significantly ($p < .01$). Data expressed as mean ± SE ($n = 6$).
Abbreviations: ATPase, µg phosphorus released/min/mg protein; GST, units/mg protein; LPO, *n* mole TBARS formed/h/mg protein.

electrophoresis unit with electrophoresis buffer (1 M Na_2EDTA, 300 mM NaOH, and 0.2% DMSO, pH >13.5) for a 20-min electrophoresis at 4°C, utilizing 15 V and 300 mA. Following electrophoresis, the slides underwent a triple wash in neutralizing buffer with 0.4 M tris buffer (pH 7.5). To visualize DNA damage, the slides were stained with 75 µL of ethidium bromide (20 µg ml^{-1}) for 5 min. Subsequently, the slides were examined using a Fluorescent Microscope (Leica Microsystems Ltd, DM 2000, Heerbrugg, Switzerland), and the captured images were analyzed using an image analysis system called Open Comet. The selected parameter for quantifying DNA damage was percent tail DNA (i.e., % tail DNA = 100% head DNA) as determined by the software.

2.16 | Integrated biomarker response

This study encompassed the determination of several stress biomarkers, including the bioaccumulation and concentration of Cu in different fish tissues and water samples. The obtained results were subjected to an integrated biomarker response (IBR) assessment, as outlined by Beliaeff & Burgeot[55] and adapted by Sanchez et al.[56] The calculation of IBR was conducted under various conditions of Cr exposure, encompassing varying pH levels and elevated temperatures. Additionally, an unexposed group (Control group) for Cu exposure was incorporated for each biomarker. To begin the analysis, the data underwent log-transformation (Yi), following which the overall mean (µ) and standard deviation (s) were computed. Subsequently, the Yi values were standardized employing the formula: $Z_i = (Y_i - \mu)/s$, and this yielded the determination of A values as the difference between Zi and Z0. The IBR value was then derived by summing up the contributions from all biomarkers across the different exposure groups.

2.17 | Sample preparation for copper analysis

Tissues were collected during the sampling process to estimate the concentration of Cu in both the tissues and water samples. For this purpose, 0.1–0.2 g of tissues were subjected to digestion using 5:1 ratio of HNO_3, 69% (Himedia Laboratory Pvt. Ltd., Mumbai, India) and H_2O_2 in a Microwave Digestion System (Microwave Reaction System, Multiwave PRO, Anton Paar GmbH, Austria, Europe). The digested tissues and water samples were subsequently filtered using Whatman papers (with a pore size of 45 µm) and then adjusted to a final volume of 50 mL using double-distilled water (DDW). Correspondingly, 10 mL of exposure water was collected for the analysis of Cu. The water samples were acidified with 100 µL of HNO_3, and both the tissue and

TABLE 4 Effect of comparative toxicity of copper (Cu) and copper nanoparticles (Cu-NPs) on alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase and malate dehydrogenase (LDH) activities in *Pangasianodon hypophthalmus* for a period of 96 h.

Cu exposure (mg L^{-1})	Alanine amino transferase (ALT)		Aspartate amino transferase (AST)		Lactate dehydrogenase (LDH)		Malate dehydrogenase (LDH)	
	Liver	Gill	Liver	Gill	Liver	Gill	Liver	Gill
Control	$11.55^{a} \pm 1.03$	$7.41^{a} \pm 0.88$	$13.61^{a} \pm 0.74$	$8.46^{a} \pm 0.99$	$2.55^{a} \pm 0.06$	$1.54^{a} \pm 0.27$	$0.77^{a} \pm 0.06$	$0.92^{a} \pm 0.04$
Cu-7.0	$16.21^{b} \pm 0.89$	$12.59^{b} \pm 1.52$	$20.12^{b} \pm 1.63$	$13.98^{b} \pm 1.04$	$3.86^{b} \pm 0.15$	$2.63^{b} \pm 0.52$	$1.05^{b} \pm 0.02$	$1.43^{c} \pm 0.09$
Cu-7.5	$18.52b^{c} \pm 0.98$	$16.49^{c} \pm 1.50$	$21.64^{b} \pm 1.18$	$14.92^{b} \pm 1.17$	$4.11^{b} \pm 0.19$	$3.16^{b} \pm 0.35$	$1.05^{b} \pm 0.03$	$1.38^{c} \pm 0.08$
Cu-8.0	$20.06c \pm 0.44$	$17.75^{c} \pm 1.21$	$22.56^{bc} \pm 0.98$	$16.59^{bc} \pm 0.65$	$4.12^{b} \pm 0.34$	$3.53^{b} \pm 0.39$	$0.99^{b} \pm 0.04$	$1.38^{c} \pm 0.07$
Cu-8.5	$21.41^{cd} \pm 1.16$	$17.02^{c} \pm 0.87$	$25.38^{c} \pm 1.31$	$16.72^{bc} \pm 1.12$	$4.32^{b} \pm 0.18$	$3.43^{b} \pm 0.27$	$1.05^{b} \pm 0.01$	$1.66^{c} \pm 0.08$
Cu-9.0	$23.40^{d} \pm 0.84$	$20.32^{d} \pm 0.94$	$28.09^{d} \pm 0.67$	$21.82^{c} \pm 2.19$	$5.10^{c} \pm 0.22$	$4.07^{c} \pm 0.45$	$1.36^{c} \pm 0.05$	$1.76^{d} \pm 0.04$
Cu-NPs-3.0	$18.23^{bc} \pm 0.75$	$11.44^{b} \pm 1.51$	$20.29^{b} \pm 1.64$	$12.97^{b} \pm 1.35$	$3.25^{b} \pm 0.18$	$3.21^{b} \pm 0.41$	$1.05^{b} \pm 0.08$	$1.10^{b} \pm 0.08$
Cu-NPs-3.3	$18.14^{bc} \pm 2.06$	$12.64^{b} \pm 1.81$	$21.40^{b} \pm 2.44$	$14.77^{b} \pm 2.43$	$4.10^{b} \pm 0.20$	$3.15^{b} \pm 0.26$	$1.10^{b} \pm 0.02$	$1.18^{b} \pm 0.03$
Cu-NPs-3.6	$19.70^{c} \pm 1.52$	$17.85^{c} \pm 1.92$	$20.40^{b} \pm 2.09$	$17.42^{bc} \pm 2.06$	$4.28^{b} \pm 0.23$	$3.30^{b} \pm 0.25$	$1.17^{b} \pm 0.04$	$1.57^{c} \pm 0.10$
Cu-NPs-3.9	$22.65^{d} \pm 1.91$	$18.03^{cd} \pm 2.83$	$23.97^{bc} \pm 1.29$	$18.74^{bc} \pm 1.83$	$4.27^{b} \pm 0.31$	$3.62^{b} \pm 0.13$	$1.25^{bc} \pm 0.03$	$1.55^{c} \pm 0.04$
Cu-NPs-4.2	$22.91^{d} \pm 1.41$	$21.63^{d} \pm 0.49$	$28.65^{d} \pm 1.24$	$19.69^{c} \pm 3.99$	$5.24^{c} \pm 0.11$	$3.74^{b} \pm 0.06$	$1.52^{c} \pm 0.05$	$2.03^{e} \pm 0.02$
P-value	0.017	0.023	0.0056	0.0043	0.0059	0.0063	0.0054	0.0045

Note: Values in the same column with different superscript differ significantly ($p < .01$). Data expressed as mean ± SE ($n = 6$).
Abbreviations: ALT, nmole of sodium pyruvate formed/mg protein/min at 37°C; AST, nmole oxaloacetate released/min/mgproteinat 37°C; LDH and MDH, units/min/mg protein at 37°C.

water samples were then subjected to analysis using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Agilent 7700 series, Agilent Technologies, USA). Calibration curves were generated using multi-element standard solutions of 10 μg mL^{-1}. Only calibration curves with R^2 values exceeding 0.999 were deemed acceptable for the purpose of concentration calculations, in accordance with previous studies.[57,58]

2.18 | Statistics

Probit analysis was computed utilizing SPSS modules (SPSS version 16, SPSS Inc., Chicago, IL, USA) to determine the lethal concentrations of both Cu and Cu-NPs. Concerning the experiment on lethal effects, the collected data underwent assessments for normality and homogeneity of variance through the utilization of Shapiro–Wilk's and Levene's tests, respectively. Subsequently, a one-way ANOVA (Analysis of Variance) accompanied by Tukey's multiple range test (presented in Tables 2–5) and Duncan's multiple range test (DMRT) were employed to ascertain statistically significant differences, considering a threshold of $p < .05$. The resulting data were then presented in the form of means ± standard errors.

3 | RESULTS AND DISCUSSION

3.1 | Lethal concentration (LC_{50}) of Cu and Cu-NPs in *Pangasianodon hypophthalmus*

The lethal toxicity of copper (Cu) and copper nanoparticles (Cu-NPs) on *P. hypophthalmus* is presented in Table 1. The lethal concentration (LC_{50}) of Cu after exposure periods of 24, 48, 72, and 96 h was found to be 10.57, 9.52, 8.71, and 8.04 mg L^{-1}, respectively. Similarly, the LC_{50} of Cu-NPs was recorded as 5.03, 4.34, 4.13, and 3.85 mg L^{-1} at 24, 48, 72, and 96 h, respectively (Table 1). Results revealed that Cu-NPs are more toxic than $CuSO_4$ at the 96-hour LC_{50}.

The present study underscores the toxic effects of copper (Cu) and copper nanoparticles (Cu-NPs) on fish. Specifically, lethal concentrations (LC_{50}) of Cu and Cu-NPs were determined to be 8.04 and 3.85 mg L^{-1}, respectively, after 96 h of exposure. However, the

TABLE 5 Effect of comparative toxicity of copper (Cu) and copper nanoparticles (Cu-NPs) on total protein, albumin, globulin, A:G ratio and NBT in *Pangasianodon hypophthalmus* for a period of 96 h.

Cu exposure (mg L^{-1})	Total protein	Albumin	Globulin	A:G ratio	NBT
Control	$0.87^{e} \pm 0.03$	$0.35^{c} \pm 0.02$	$0.52^{d} \pm 0.02$	$0.67^{b} \pm 0.01$	$0.53^{b} \pm 0.01$
Cu-7.0	$0.72^{d} \pm 0.04$	$0.28^{b} \pm 0.01$	$0.45^{c} \pm 0.04$	$0.63^{b} \pm 0.06$	$0.51^{b} \pm 0.00$
Cu-7.5	$0.58^{c} \pm 0.02$	$0.28^{b} \pm 0.02$	$0.30^{b} \pm 0.04$	$0.98^{d} \pm 0.24$	$0.50^{b} \pm 0.00$
Cu-8.0	$0.55^{bc} \pm 0.03$	$0.22^{ab} \pm 0.02$	$0.33^{b} \pm 0.02$	$0.70^{b} \pm 0.13$	$0.49^{b} \pm 0.01$
Cu-8.5	$0.53^{b} \pm 0.04$	$0.22^{ab} \pm 0.03$	$0.32^{b} \pm 0.06$	$0.78^{b} \pm 0.27$	$0.48^{b} \pm 0.02$
Cu-9.0	$0.41^{a} \pm 0.03$	$0.19^{a} \pm 0.01$	$0.22^{a} \pm 0.05$	$1.01^{d} \pm 0.39$	$0.44^{a} \pm 0.03$
Cu-NPs-3.0	$0.60^{c} \pm 0.05$	$0.27^{b} \pm 0.01$	$0.33^{b} \pm 0.04$	$0.84^{c} \pm 0.07$	$0.51^{b} \pm 0.00$
Cu-NPs-3.3	$0.56^{bc} \pm 0.05$	$0.19^{a} \pm 0.01$	$0.37^{bc} \pm 0.05$	$0.55^{a} \pm 0.10$	$0.50^{b} \pm 0.02$
Cu-NPs-3.6	$0.46^{b} \pm 0.03$	$0.16^{a} \pm 0.02$	$0.30^{b} \pm 0.02$	$0.54^{a} \pm 0.02$	$0.49^{b} \pm 0.01$
Cu-NPs-3.9	$0.47^{b} \pm 0.02$	$0.20^{a} \pm 0.01$	$0.26^{ab} \pm 0.01$	$0.78^{b} \pm 0.07$	$0.45^{ab} \pm 0.03$
Cu-NPs-4.2	$0.51^{b} \pm 0.03$	$0.17^{a} \pm 0.02$	$0.34^{b} \pm 0.04$	$0.52^{b} \pm 0.11$	$0.41^{a} \pm 0.01$
p value	0.0033	0.0047	0.0019	0.011	0.0017

results indicate that Cu and Cu-NPs induced toxicity in *P. hypophthalmus* at concentrations of 7.0 to 9.0 mg L^{-1} and 3.0 to 4.2 mg L^{-1}, respectively. Cu-NPs exhibited greater toxicity compared to Cu, attributed to nanoparticle properties such as faster dissolution and a larger surface area, facilitating interaction with solvent molecules. Furthermore, dissolution plays a significant role in the oxidation of Cu-NPs,[11,18,59] leading to the release of Cu ions and consequently increasing toxicity by 40-fold compared to Cu.[60,61] It is also noteworthy that the production, use, and eventual release of nanoparticles into aquatic systems pose unexpected hazards to aquatic organisms, including fish.[62] The excessive intake of Cu within fish does not necessarily lead to its accumulation in different organs of the fish body; instead, it may bind with cations and negatively affect cellular metabolism. The entry of Cu through fish gills obstructs Na^{+} efflux and affects Na^{+}/K^{+}-ATPase activity in fish.[63,64] Cu is also an essential trace element for living organisms, including fish, participating in various biological processes as a co-factor for enzymes such as cytochrome oxidase, superoxide dismutase, lysyl oxidase, dopamine hydroxylase, and tyrosinase.[65] Nevertheless, the demarcation between the beneficial and toxic nature of Cu and Cu-NPs remains narrow. Although the present findings provide the first report on the toxicity of Cu and Cu-NPs on *P. hypophthalmus*, the data recorded in this study could be utilized for formulating fish feed based on Cu and Cu-NPs.

3.2 | Cu and Cu-NPs induce oxidative stress during acute toxicity test

Cu and Cu-NPs exposure significantly impacted the activities of CAT, SOD, GST, and GPx after 96 h of exposure in *P. hypophthalmus*, as depicted in Tables 2 and 3. Notably, there was a substantial rise in CAT (liver, $p = .023$; gill, $p = .0041$; kidney, $p = .0037$), SOD (liver, $p = .020$; gill, $p = .017$; kidney, $p = .025$), GST (liver, $p = .0073$; gill, $p = .043$; kidney, $p = .0034$), and GPx (liver, $p = .0064$; gill, $p = .0021$; kidney, $p = .0036$) activities in the liver, gill, and kidney tissues of Cu and Cu-NPs exposed groups compared to the unexposed group. The CAT activities in the liver, kidney, and gill showed increases of 69%–173%, 61%–265%, and 44%–135%, respectively, in the Cu exposed groups compared to the control group. Similarly, CAT activities increased by 83%–165%, 83%–178%, and 55%–92% in the Cu-NPs exposed group compared to the control group. Moreover, in the liver, gill, and kidney, SOD activities increased in the Cu exposed group (7.0–9.0 mg L^{-1}) by 32%–40%, 43%–44%, 26%–31%, and in the Cu-NPs exposed group by 15%–30%, 21%–28%, and 21–0%, respectively. Notably, GST activities in the liver, gill, and kidney exhibited variations of 29%–99%, 40%–169%, and 76%–254%, respectively, in the Cu exposed group. In comparison, GST activities showed variations of 58%–94%, 26%–148%, and 69%–232% following Cu-NPs and Cu exposures in liver, gill, and kidney tissues, respectively. For GPx, the activities were higher by 89%–194%, 143%–429%, and 45%–219%, respectively, due to Cu exposure, and by 73%–134%, 147%–274%, and 52%–129% due to Cu-NPs exposure in liver, gill, and kidney tissues. Furthermore, LPO levels in the liver ($p = .0017$), gill ($p = .0028$), and kidney ($p = .0047$) were significantly elevated ($p < .01$) in *P. hypophthalmus* exposed to Cu and Cu-NPs (Table 3). Specifically, LPO levels varied by 133%–179%, 105%–310%, and 38%–131% in the exposure group of 7.0–9.0 mg L^{-1} Cu, and by 98%–198%, 52%–246%, and 16%–88% in the 3.0–4.2 mg L^{-1} Cu-NPs exposure group across respective tissues.

In this investigation, exposure to Cu (7.0–9.0 mg L^{-1}) and Cu-NPs (3.0–4.2 mg L^{-1}) triggered oxidative stress in *P. hypophthalmus*, as evidenced by elevated activities of CAT, SOD, GST, and GPx in liver, gill, and kidney tissues. The modulation of CAT activities due to Cu and Cu-NPs exposure could elevated from protein carbonylation and altered protein function through lipid peroxidation products.[66] Similarly, increased SOD activity post exposure to Cu and Cu-NPs suggests activation of the antioxidant defense mechanism, likely

against ROS production.[66] This notion aligns with previous work demonstrating ROS overproduction in *Mytilus edulis* exposed to Cu and Cu-NPs, resulting in oxidation of metabolic cellular components like lipids and proteins.[67] The study underscores that Cu and Cu-NPs induce physiological toxicity via catalytic ROS production, directly correlating with their toxic nature at higher concentrations.[68] The heightened LPO results further indicate oxidative lipid damage in liver, gill, and kidney, potentially arising from insufficient production of antioxidants to counter tissue damage. Oxidative stress stands as a prime indicator for toxicity assessment, readily tracked through enzymes such as CAT, SOD, GST, and GPx.[69] Earlier research on metals and metal nanoparticles, such as Pb, Zn, Zn-NPs, Se, and Se-NPs, have similarly displayed significant oxidative stress escalation in biological systems upon metal/metal NPs exposure.[32,70,71] In this study, the results establish that Cu-NPs induce more potent oxidative stress than Cu, potentially attributed to the smaller size of the material compared to Cu, resulting in heightened ROS production.[72] Additionally, GPx functions to mitigate oxidative damage by converting lipid hydroperoxides to alcohols, more efficiently eliminated by organisms.[73,74] It also plays a pivotal role in intracellular protection against toxic materials such as Cu, Cu-NPs, Zn, Zn-NPs, Se, and Se-NPs.[32,75] Furthermore, GPx acts as an enzymatic defense against hydrogen peroxide (H_2O_2), conjugating with GSH to catalyze the reaction between glutathione and H_2O_2, leading to the formation of glutathione disulfide (GSSG).[41,76]

3.3 | Acetylcholinesterase and lipid peroxidation on Cu and Cu-NPs exposure to *Pangasianodon hypophthalmus*

The effects of Cu and Cu-NPs exposure on acetylcholinesterase activities (AChE) in the brain and lipid peroxidation (LPO) in liver, gill, and kidney tissues of *P. hypophthalmus* were assessed. Notably, brain AChE activities were significantly inhibited ($p = .0039$) in fish exposed to Cu and Cu-NPs during the acute toxicity test (Figure 2A). Brain AChE activities were inhibited by a significant margin of 27%–43% and 25%–40% in the Cu and Cu-NPs exposure groups, respectively, in comparison to the control group.

Acetylcholine esterase (AChE) is a critical neurotransmitter enzyme, and its inhibition due to Cu and Cu-NPs exposure underscores their capacity to disrupt neuronal signaling molecules.[11,77,78] Furthermore, Cu is recognized for its ability to bind with thiol groups within AChE enzymes (Hultberg et al.). AChE serves as a prime indicator for investigating the mechanisms of toxic materials, including metals,[79,80] in aquatic animals. The inhibition of AChE could be attributed to the binding of Cu or Cu-NPs to functional protein groups such as imidazole, sulfhydryl, and carboxyl groups.[79] This binding may augment the hydrolysis of the neurotransmitter acetylcholine, consequently reducing the activation of nicotinic and muscarinic receptors subsequent to Cu and Cu-NPs exposure. Additionally, AChE's competitive interaction with calcium for cellular absorption sites could be at play.[81]

3.4 | ATPase during Cu and Cu-NPs exposure to *Pangasianodon hypophthalmus*

ATPase production in the liver ($p = .0018$) and gill ($p = .022$) exhibited significant inhibition ($p < .01$) in the groups exposed to Cu and Cu-NPs compared to the control group (Table 3). The inhibition of ATPase amounted to 18%–20% in the liver and 22%–24% in the gill tissues of organisms exposed to concentrations of 7.0 and 9.0 mg L^{-1}, respectively. Similarly, the ATPase measurements in the liver and gill of the group exposed to concentrations of 3.0 and 4.2 mg L^{-1} of Cu-NPs indicated inhibitions of 36%–24% and 25%–24%, respectively.

The present study highlights a substantial decline in ATPase levels within liver and gill tissues following exposure to Cu and Cu-NPs. The reduction in ATPase activities in these tissues could potentially be attributed to lipid-rich membranes enhancing vulnerability to protein loss, thus inducing significant shifts in the metabolic functions of membrane activity.[82,83] Furthermore, the decline in ATPase activities might also stem from heightened usage or disrupted oxidative phosphorylation, leading to a decrease or inhibition in ATPase activity.[84] It is widely recognized that Cu contributes to the inhibition of gill respiration and ion regulation.[85]

3.5 | Stress biomarker during exposure to Cu and Cu-NPs exposure to *Pangasianodon hypophthalmus*

Cortisol, blood glucose, and HSP 70 levels in the liver and gill tissues of *P. hypophthalmus* exhibited significant increases ($p < .01$) upon exposure to Cu and Cu-NPs (Figure 2B–D). The concentration of cortisol was notably elevated ($p = .0073$) in the group exposed to 4.2 mg L^{-1} Cu-NPs, followed by 3.9 mg L^{-1} Cu-NPs and 9.0 mg L^{-1} Cu, in comparison to the control group and other exposure groups. Cortisol levels exhibited variations of 10%–39% and 19%–46% in organisms exposed to 7.0 and 9.0 mg L^{-1} of Cu, and 3.0 and 4.2 mg L^{-1} of Cu-NPs, respectively, as compared to the control group (Figure 2B). Furthermore, notably higher concentrations of HSP 70 were observed in the liver ($p = .0024$) and gill ($p = .0031$) of fish exposed to 9.0 mg L^{-1} of Cu, when compared to the control group and other Cu and Cu-NPs exposure groups. In the liver, the HSP 70 concentration varied from 63 to 103%, and from 15% to 76% in Cu and Cu-NPs exposed groups, respectively, in comparison to the control group. Similarly, in the gill, HSP 70 concentrations were elevated by 49%–131% and 32%–92% in Cu and Cu-NPs exposed groups, respectively (Figure 2C). Moreover, blood glucose levels ($p = .0052$) were significantly higher ($p < .01$) in the group exposed to 9.0 mg L^{-1} Cu, compared to the control group and other Cu and Cu-NPs exposure groups. Blood glucose levels varied by 15%–46% and 10 to 24% in the 7.0 and 9.0 mg L^{-1} Cu exposure groups, and 3.0 and 4.2 mg L^{-1} Cu-NPs exposure groups, respectively, compared to the control group (Figure 2D).

Cortisol, HSP 70, and blood glucose were utilized as biochemical indicators of stress responses. The heightened cortisol levels resulting from Cu and Cu-NPs exposure promote increased transport of

chloride cell proliferation, consequently boosting ATPase activities, as observed in this study.[86] This cortisol enhancement leads to raised Na^+ concentration, Na^+/K^+-ATPase activity, and osmolarity. This temporary response serves as a protective mechanism against Cu and Cu-NPs toxicity,[87] ultimately culminating in a disruption of ion and osmoregulation.[88] The synthesis of HSP 70 is triggered by cellular protein machinery damage. Exposure to Cu and Cu-NPs results in enhanced HSP 70 productions due to proteotoxicity,[89] which in turn might counterbalance the damage in liver and gill tissues of *P. hypophthalmus*. The heightened blood glucose levels following Cu and Cu-NPs exposure point towards a disruption in carbohydrate metabolism within fish. This elevation in blood glucose levels is accompanied by the secretion of catecholamines and glucocorticoids.[90] Furthermore, the catecholamines may convert into glycogen upon Cu exposure, stimulating gluconeogenesis and glycogenolysis. This elevated glucose level also serves as an additional energy source to restore impaired osmotic balance.[91]

3.6 | Protein metabolic and glycolytic enzymes during Cu and Cu-NPs exposure to *Pangasianodon hypophthalmus*

Protein metabolic enzymes, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST), displayed a substantial increase ($p < .01$) in both the liver and gill tissues of fish exposed to Cu and Cu-NPs, when compared to the control group (Table 4). ALT activity in the liver ($p = .017$) and gill ($p = .023$) exhibited variations ranging from 40% to 102% and 74% to 169% in response to Cu exposure at concentrations of 7.0 and 9.0 mg L^{-1}, and from 57% to 98% and 54% to 191% in the Cu-NPs exposure group. Similarly, AST activities ranged from 47% to 107% and 65 to 157% in fish exposed to Cu, and from 49% to 110% and 52% to 132% in response to Cu-NPs exposure at concentrations of 3.0 and 4.2 mg L^{-1}. Furthermore, LDH and MDH activities demonstrated significant increases ($p < .01$) in the liver and gill tissues following exposure to Cu and Cu-NPs, compared to the control group (Table 4). LDH activities exhibited increments from 51% to 100% and 70 to 163% in the liver ($p = .0059$) and gill ($p = .0063$) tissues of fish exposed to Cu concentrations of 7.0 and 9.0 mg L^{-1}, respectively. In the same manner, LDH activities increased by 27% to 105% and 107% to 142% in the gill and liver of fish exposed to Cu-NPs, compared to the control group. Similarly, MDH activity showed increments of 36 to 76% and 54 to 91% in the liver ($p = .0054$) and gill ($p = .0045$) of fish exposed to Cu concentrations of 7.0 and 9.0 mg L^{-1}. Finally, MDH activity increased by 36% to 97% and 19% to 120% in fish exposed to Cu-NPs concentrations of 3.0 and 4.2 mg L^{-1}.

ALT and AST, as pivotal and sensitive enzymes, serve as biomarkers in ecotoxicology studies, providing early indicators of detrimental changes resulting from organic pollutants[80] and inorganic pollutants[92] in aquatic ecosystems.[93] In this study, the heightened ALT and AST activities observed in the gill and liver tissues after Cu and Cu-NPs exposure indicate injuries to vital organs, substantiated by histopathological findings revealing tissue damage, particularly in the liver. These observations align with our prior studies on lead (Pb) exposure,[71] arsenic (As) exposure,[94] and zinc and Zn-NPs

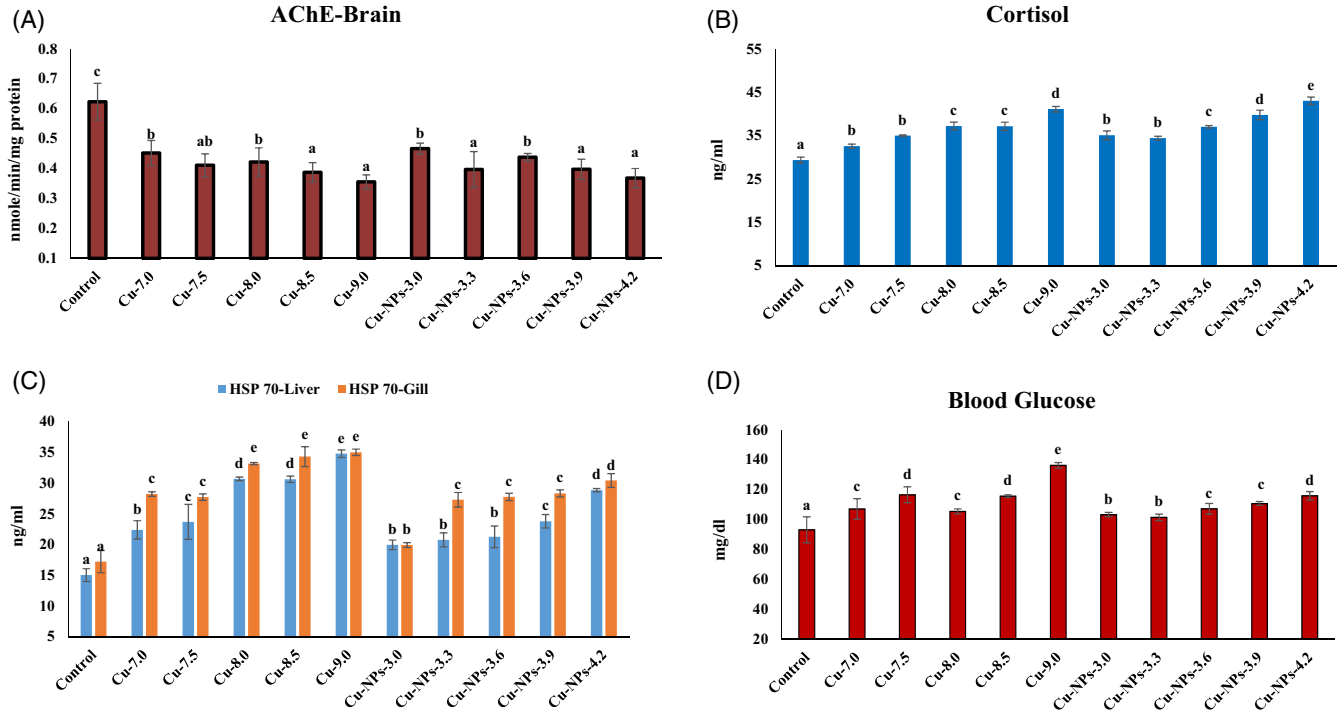

FIGURE 2 (A–D) Effect of comparative toxicity of copper (Cu) and copper nanoparticles (Cu-NPs) on acetylcholine esterase (AChE, $p = .0039$), cortisol (0.0073), HSP 70 in liver ($p = .0024$) and gill ($p = .0031$) and blood glucose in *Pangasianodon hypophthalmus* for a period of 96 h. Data expressed as mean ± SE (AChE, $n = 6$, cortisol, HSP 70 and Blood glucose: $n - 3$).

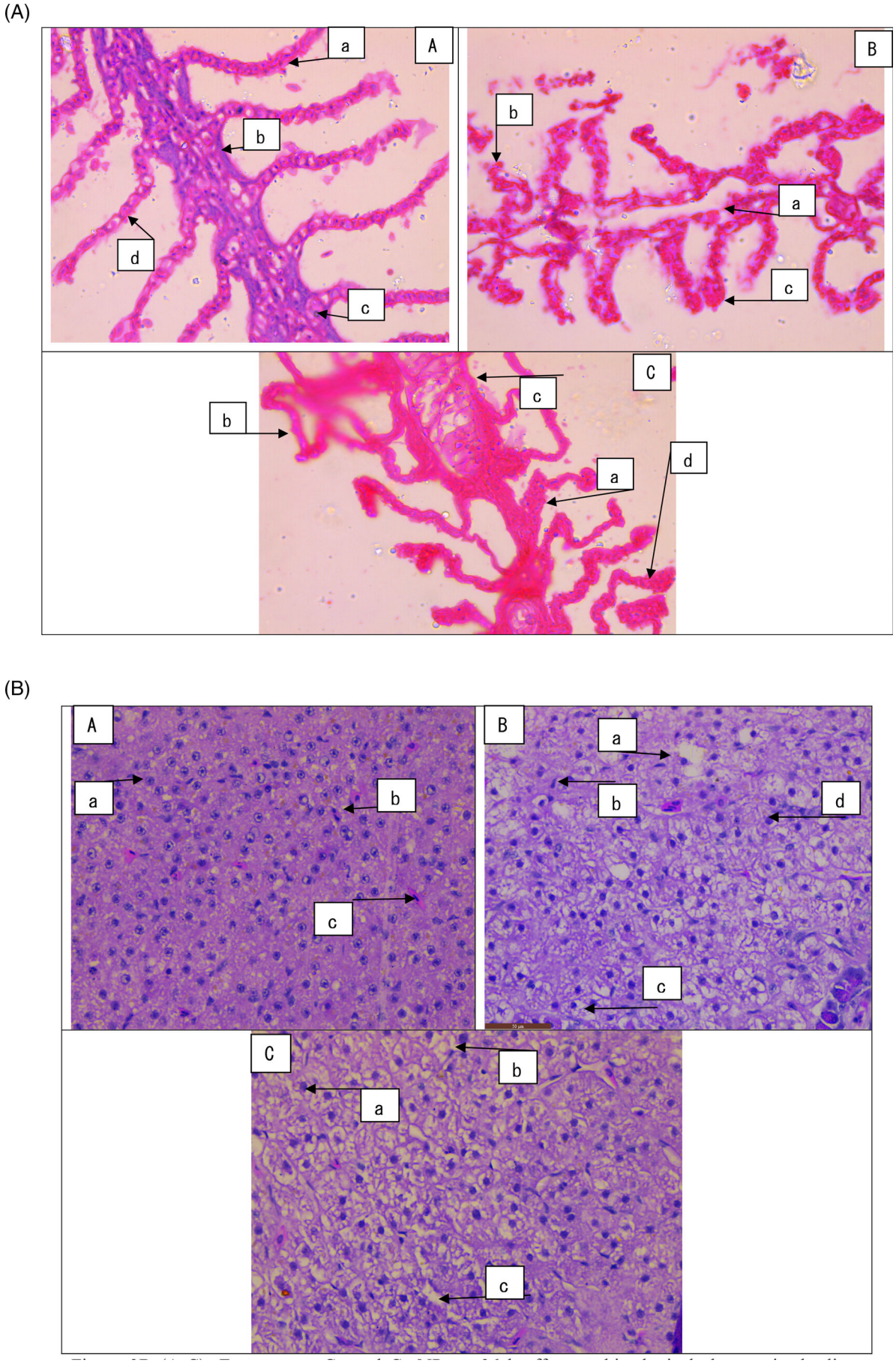

FIGURE 3 Legend on next page.

exposure,[70] where elevated ALT and AST activities were also linked to hepatocellular damage or degradation.[95,96] Furthermore, during the acute exposure to Cu and Cu-NPs, LDH and MDH activities experienced significant enhancement. Typically, tissues necessitate oxygen for their metabolic processes to function properly and prevent the buildup of metabolites. However, during exposure to Cu and Cu-NPs, induced stress and the development of anaerobic conditions led to the production of lactate, which in turn amplified LDH and MDH activities. LDH primarily functions to convert lactic acid into pyruvic acid, thus providing cellular energy.[80,97] An additional reason for elevated LDH and MDH levels during Cu and Cu-NPs exposure could be the alteration of oxidative metabolism in mitochondria due to hypoxic conditions and impairment of the oxygen-binding capacity of the respiratory pigment.[98]

3.7 | Immunological status (total protein, albumin, globulin, A: G ratio and respiratory activity, NBT)

Total protein, albumin, globulin, albumin-to-globulin ratio (A:G ratio), and respiratory activity (NBT) exhibited significant alterations in *P. hypophthalmus* following exposure to Cu and Cu-NPs. Total protein ($p = .0033$), albumin ($p = .0047$), globulin ($p = .0019$), A:G ratio ($p = .011$), and NBT ($p = .0017$) were notably decreased ($p < .01$), while the A:G ratio was significantly increased in fish exposed to Cu (7.0 and 9.0 mg L^{-1}) and Cu-NPs (3.0 and 4.2 mg L^{-1}) for 96 h, in comparison to the control group (Table 5).

Total protein, albumin, globulin, A:G ratio, and NBT are recognized as immune indices that contribute to the enhancement of fish immunity. In this study, total protein, albumin, globulin, and NBT levels significantly decreased ($p < .01$), while the A:G ratio significantly increased after exposure to Cu (7.0–9.0 mg L^{-1}) and Cu-NPs (3.0–4.2 mg L^{-1}). This could potentially lead to a reduction in fish immunity as a result of exposure to Cu and Cu-NPs. Copper plays an important role in protein biosynthesis,[99] which aids in enhancing the immune indices of fish.

3.8 | Histopathological study

The results demonstrate that exposure to Cu and Cu-NPs induced alterations in the gill and liver histology of *P. hypophthalmus* (Figures 3A,B). In the unexposed group (control), gill histology exhibited a normal structure. However, fish exposed to Cu at 9.0 mg L^{-1} displayed changes in gill tissues, including the loss of primary and secondary gill lamellae, along with the clubbing of secondary gill lamellae. Furthermore, fish exposed to Cu-NPs at 4.2 mg L^{-1} exhibited complete loss of primary lamellae, clubbing of secondary gill lamellae, and hypertrophy of lamellar epithelium. Similarly, the histopathology of liver tissues revealed nuclear degeneration, distortion of hepatic cells, lymphocyte infiltration, necrosis, and pyknotic nuclei in response to Cu exposure at 9.0 mg L^{-1}. On the other hand, exposure to Cu-NPs at 4.2 mg L^{-1} led to the presence of abnormal nuclei, lipid vacuoles, immature nuclei, and hepatic tissues displaying focal necrosis.

The gills play a vital role in respiration, osmoregulation, and excretion, constantly coming into contact with the water's surface and filtering out toxic substances.[97,100] In this study, both Cu and Cu-NPs exposure in *P. hypophthalmus* led to significant alterations in the gill and liver tissues. The gill tissues displayed changes such as loss of primary and secondary gill lamellae and clubbing of secondary gill lamellae due to Cu exposure. Additionally, exposure to Cu-NPs at 4.2 mg L^{-1} resulted in the complete loss of primary lamellae, clubbing of secondary gill lamellae, and hypertrophy of lamellar epithelium. Similar results were observed in our previous study involving exposure of fish to inorganic and organic contaminants.[97] Furthermore, the liver, responsible for detoxifying toxic substances, displayed alterations characterized by abnormal nuclei, lipid vacuoles, immature nuclei, and focal necrosis in response to exposure to Cu at 9.0 mg L^{-1}. The formation of large vacuoles resulting from Cu and Cu-NPs exposure could potentially be attributed to the inhibition of protein synthesis, energy depletion, microtubule disaggregation, or shifts in substrate utilization.[101] Generally, the gills are continuously in direct contact with the water pollutants which induces the excessive secretion of mucous from the goblet cells.[102] Although, gill edema could be resulting of toxicity of Cu and Cu-NPs which inhibition of ionic transport across the gill epithelium by the branchial Na+/K+ ATPase, which will, in turn, leads to disruption of the osmotic regulation and then osmotic imbalance occurs.[103] The exposure to Cu-NPs might induce osmoregulatory failure, negatively affect the counter current gaseous exchange mechanisms, and decrease the uptake of dissolved oxygen from the water, which will subsequently lead to asphyxiation, respiratory failure, and death.[104] Cu-NP also responsible for vacuolation of the hepatocytes could be due to linked to bioaccumulation of triglycerides in the hepatocytes and change the size and shape of the nuclei, as well as focal necrosis.[104]

FIGURE 3 (A) (A–C) Exposure to copper (Cu) and copper nanoparticles (Cu-NPs) at 96 h effect on histological changes in the gill tissues of *Pangasianodon hypophthalamus*. (A) Control group: (a) Normal primary lamellae; (b) secondary gill lamellae; (c) chloride and (d) pillar cells. (B) Exposure to Cu at 9.0 mg L^{-1} showed complete (a) loss of primary and (b) secondary gill lamella, (c) clubbing of secondary gill lamella. (C) Exposure to Cu-NPs at 4.2 mg L^{-1} showed (a) complete loss of primary lamella, (b) clubbing of secondary gill lamella, (c) hypertrophy of lamellar epithelium and (d) swelling of secondary gill lamella. B (A–C) Exposure to Cu and Cu-NPs at 96 h affect on histological changes in the liver tissues of *Pangasianodon hypophthalamus*. (A) Control group: (a) normal hepatic cell; (b) normal kuffer cell; (c) sinusoid cell. (B) Exposure to Cu at 9.0 mg L^{-1} showed (a) degenerative changes, (b) distortion of hepatic cell, (c) necrosis, (d) pyknotic nuclei. (C) Exposure to Cu-NP at 4.2 mg L^{-1} showed (a) abnormal nuclei, (b) lipid vacuoles, and (c) hepatic tissues showing focal necrosis.

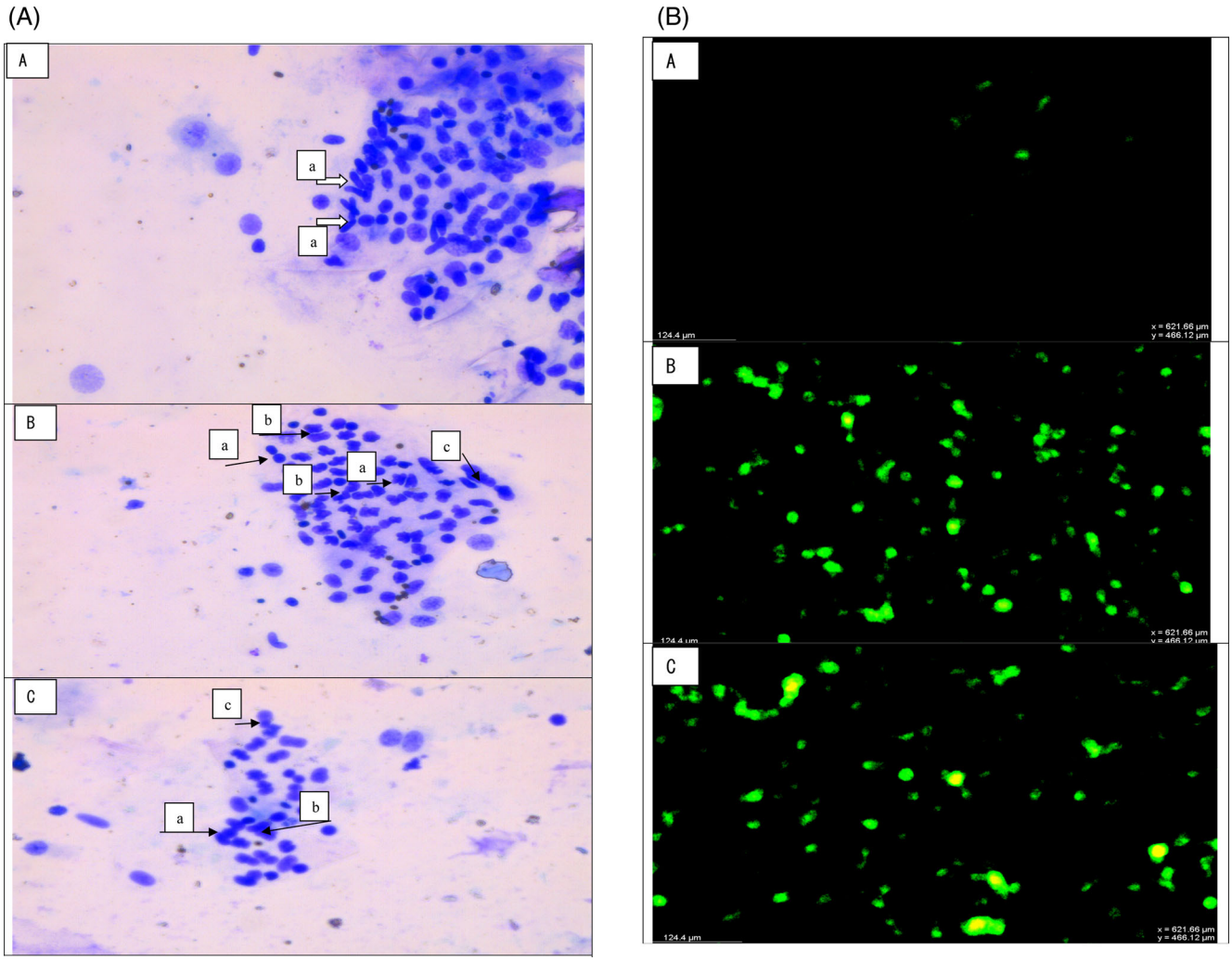

FIGURE 4 (A) (A–C) Chromosome structure altered with exposure to Cu (B) and Cu-NPs (C) during 96 h in kidney tissue of *Pangasianodon hypophthalmus*. (A) a, normal structure of chromosome. (B) a, shorten arm; b, end-to-end joining; and c, condensed chromosome. (C) a, shorten arm; b, end-to-end joining; and c, condensed chromosome. (B) (A–C) DNA damage in gill tissues of *Pangasianodon hypophthalmus* reared under (A) control (B) Cu exposed and (C) Cu-NPs exposed during 96 h.

3.9 | Genotoxicity of kidney tissues with exposure to Cu and Cu-NPs

A genotoxicity test was conducted to assess the genotoxic effects of Cu and Cu-NPs on *P. hypophthalmus* (Figure 4A(A–C)). In the control group, normal chromosome structures were observed in the kidney tissues of *P. hypophthalmus*. In contrast, exposure to Cu and Cu-NPs led to abnormal chromosome structures, including shortened arms, end-to-end joining, and condensed chromosomes. Additionally, DNA damage in gill tissues was determined using the single-cell gel electrophoresis/comet assay, and the results are presented in Table 6 and Figure 4B(A–C). Parameters such as comet area, comet DNA, head DNA, head DNA (%), tail area, tail DNA, and tail DNA (%) were evaluated. The results revealed that tail DNA (%) ranged from 93.52% to 95.52% in the Cu-exposed groups (7.0 and 9.0 mg L^{-1}) and from 68.58% to 87.24% in the Cu-NPs exposure group.

The genotoxicity study utilizing karyotyping serves as robust evidence of the nature of the toxic effects of chemicals, as demonstrated in the present study. Exposure to Cu and Cu-NPs led to chromosome aberrations in fish, potentially arising from the inhibition of cell division, DNA repair, and protein synthesis, as previously reported by Chandra and Khuda-Bukhsh.[105] This study illustrated the toxic effects of Cu and Cu-NPs, particularly at higher concentrations, which induced apoptosis in the form of DNA damage within gill tissues. The observed DNA damage might indicate that cells tolerate such damage and become resistant to cell death at lower concentrations of Cu and Cu-NPs. These substances can induce genotoxic effects through both direct mechanisms (affecting DNA structure or repair systems) and indirect mechanisms (intermediate molecule induction).[106] Due to the smaller size of Cu-NPs, they can readily access the nucleus and interact with genetic material, leading to DNA damage.[107] Compared to other metals, Cu may directly interact with DNA or even with DNA

TABLE 6 Determination of DNA damage in gill tissue of *Pangasianodon hypophthalmus* reared in control or copper (Cu) or copper nanoparticles (Cu-NPs) exposed during 96 h.

Treatments	Comet area	Comet DNA	Head area	Head DNA	Head DNA %	Tail area	Tail DNA	Tail DNA %)
Ctr	589.0 ± 4.78	64088.0 ± 15.85	502.0 ± 5.19	60286.0 ± 28.45	94.07 ± 2.78	87.0 ± 2.01	3802.0 ± 3.67	5.93 ± 0.72
Cu-7.0	686.85 ± 11.56	60243.85 ± 16.59	38.92 ± 5.38	3753.38 ± 6.74	6.48 ± 1.13	647.92 ± 3.74	56490.46 ± 26.78	93.52 ± 1.03
Cu-9.0	786.0 ± 4.92	76864.33 ± 4.78	28.89 ± 1.11	3617.56 ± 9.12	4.22 ± 0.89	757.11 ± 11.78	73246.78 ± 14.18	95.78 ± 2.49
Cu-NPs-3.0	716.18 ± 5.87	61457.27 ± 22.48	201.55 ± 1.43	22734.36 ± 21.36	31.42 ± 1.08	514.64 ± 4.12	38722.91 ± 11.12	68.58 ± 5.12
Cu-NPs-4.2	459.0 ± 6.18	32289.0 ± 19.17	79.0 ± 3.15	4121.0 ± 8.19	12.76 ± 1.21	380.0 ± 3.79	28168.0 ± 22.04	87.24 ± 4.11

Note: Data expressed as mean ± SE ($n = 3$).

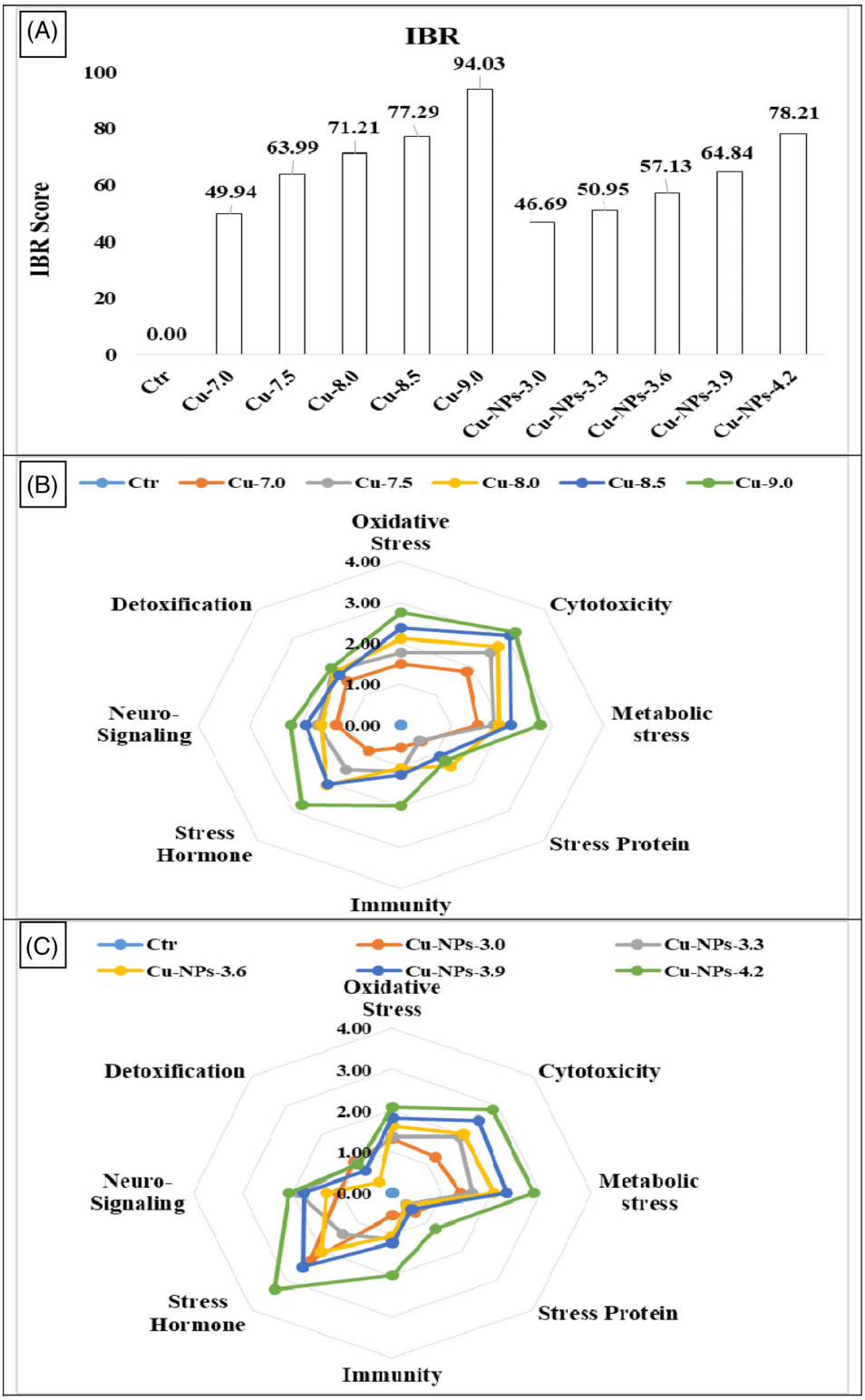

FIGURE 5 (A–C): (A) Integrated biomarkers response (IBR) score; (B,C) IBR score for oxidative stress, cytotoxicity, metabolic stress, stress protein, immunity, stress hormone, neuro-signaling and detoxification in exposure to Cu and Cu-NPs in *Pangasianodon hypophthalmus*.

repair enzymes, thereby inducing DNA damage.[108] The results also suggested that, different apoptosis pathways are triggered by Cu and Cu-NPs at different time points of the exposure period, as the increase in transcripts was sequential, instead of simultaneous. also suggesting the existence of crosstalk interactions between them.[109]

3.10 | Integrated biomarkers response

The results of the integrated biomarker response (IBR) assessment in *P. hypophthalmus* exposed to various concentrations of Cu and Cu-NPs over a 96-hour period are presented in Figure 5A–C. The IBR

TABLE 7 Determination of copper (Cu) and copper nanoparticles (Cu-NPs) concentration in different fish tissues (mg kg^{-1}) and experimental water (μl L^{-1}) at different time interval.

	Water (μg L^{-1})				Tissues (mg L^{-1})				
Cu exposure (mg L^{-1})	24 h	48 h	72 h	96 h	Muscle	Gill	Liver	Kidney	Brain
Control	10.34	11.49	10.27	11.23	1.14	1.69	1.91	1.83	0.45
Cu-7.0	2424.30	2005.09	1772.07	1465.09	2.04	3.46	3.35	3.97	0.85
Cu-7.5	3128.29	2978.47	2405.86	2133.21	2.07	3.81	3.55	4.16	1.00
Cu-8.0	3352.36	2700.53	2593.73	2326.58	2.26	4.26	3.51	3.72	0.97
Cu-8.5	3588.95	3290.76	2653.06	2358.00	2.03	3.72	3.70	3.93	0.84
Cu-9.0	3538.49	3136.46	2860.43	2354.07	2.06	4.36	3.88	4.49	0.69
Cu-NPs-3.0	1206.73	1137.12	968.65	814.02	1.25	2.96	2.77	3.16	0.86
Cu-NPs-3.3	1272.82	1005.19	996.88	778.17	1.05	2.99	2.81	3.22	0.82
Cu-NPs-3.6	1175.04	980.32	953.32	724.35	1.06	2.24	3.38	3.65	0.52
Cu-NPs-3.9	1100.37	959.15	956.63	810.90	1.16	2.77	2.95	3.03	0.69
Cu-NPs-4.2	1324.15	983.40	975.51	804.10	1.42	2.52	3.07	3.42	0.87

Note: Data expressed as mean ± SE ($n = 3$).

scores for Cu and Cu-NPs exposure groups are depicted in star plots. The findings reveal that as the exposure concentration of Cu and Cu-NPs increases, the IBR scores also increase, as visually represented in the star plot where the index becomes more robust. To simplify the interpretation of results and account for the number of biomarkers, the mean score was calculated for each physiological function, as depicted in Figure 5B,C. The outcomes further illustrate the upward trend of IBR scores with increasing concentrations of Cu and Cu-NPs in *P. hypophthalmus*.

The diverse biomarkers were categorized into distinct physiological functions, encompassing oxidative stress, cytotoxicity, neurosignaling, metabolic stress, stress proteins, stress hormones, immunity, and detoxification. These physiological functions collectively serve as reliable biomarkers in aquatic animals, including fish.[54,110] Neurosignaling emerges as a potent biomarker for contamination in aquatic ecosystems, with other biomarkers also contributing to the suite of pollution indicators.

3.11 | Cu and Cu-NPs concentration in water and bioaccumulation in fish tissues

Concentrations of Cu and Cu-NPs in the water were assessed at 24, 48, 72, and 96 h, and the bioaccumulation was determined in muscle, gill, liver, kidney, and brain tissues after 96 h of exposure, as outlined in Table 7. In the control group, the water concentration of Cu was measured at 10.34 μg L^{-1}. However, the concentrations of Cu in the exposed groups ranged from 2424 to 3538 μg L^{-1}. For the Cu-NPs exposed group, Cu-NPs bio-concentration ranged from 1206 to 1324 μg L^{-1} at 24 h. Similarly, at 48, 72, and 96 h, the concentrations of Cu and Cu-NPs in the water ranged from 2005 to 3136, 1772 to 2860, 1465 to 2354, and 1137 to 983, 968 to 975, 814 to 804 μg L^{-1}, respectively, for the exposure concentration of 7.0–9.0 mg L^{-1}.

During the acute toxicity experiment, the concentrations of Cu and Cu-NPs were monitored in the experimental water at 24, 48, 72, and 96 h. The concentration of Cu was highest at 24 h (3538 μg L^{-1}), after which it gradually decreased from 24 to 96 h. This reduction might be attributed to the accumulation of Cu within different tissues of the fish over time. Similarly, the bioaccumulation of Cu varied among tissues, with the following sequence of Cu tissue-distribution observed: kidney > liver > gill > brain > muscle tissues. The kidney and liver tissues exhibited the highest Cu accumulation, possibly due to their detoxification functions.[70,73,111] The liver, as an enzymatic site for the degradation of toxic compounds, plays a pivotal role in this context.[112] Copper's significance in enhancing red blood cells (RBC), boosting the immune system, and maintaining nerve cells in aquatic animals such as fish is noteworthy, with a defined desirable dose limit.

4 | CONCLUSION

The present study has revealed the toxic effects of both inorganic copper (Cu) and nano-copper (Cu-NPs) at elevated concentrations on *P. hypophthalmus*. The median lethal concentration (LC50) for Cu and Cu-NPs was determined as 8.04 and 3.85 mg L^{-1}, respectively, indicating that Cu-NPs exhibit higher toxicity compared to inorganic Cu. Oxidative stress enzymes, such as CAT, SOD, GST, and GPx, as well as LPO in the liver, gills, and kidneys, showed elevated levels at higher concentrations of Cu (9.0 mg L^{-1}) and Cu-NPs (4.2 mg L^{-1}). Protein metabolic enzymes, including ALT, AST, and carbohydrate metabolic enzymes such as LDH and MDH, also increased at higher concentrations of Cu (9.0 mg L^{-1}) and Cu-NPs (4.2 mg L^{-1}). Furthermore, both forms of copper induced significant biochemical and

metabolic disruptions, leading to tissue damage in the liver and gill tissues of the fish after a 96-hr exposure period. These findings highlight the environmental implications of Cu and Cu-NPs when introduced into aquatic ecosystems. Given the crucial role of copper in metabolic regulation among aquatic animals, this study could serve as a foundational reference for the development of feed formulations containing Cu and Cu-NPs for fish. Moreover, the results of this research can contribute to the establishment of policies regulating the release of Cu and Cu-NPs into aquatic environments.

AUTHOR CONTRIBUTIONS

Neeraj Kumar: Conceptualization, Data Curation, Data validation, Performed the experiment, Writing – Original Draft Preparation, Writing – Review & Editing. Eric Gismondi: Analysis of integrated biomarker response (IBR). Kotha Sammi Reddy: Language editing and correction.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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