



## OPEN Ecofriendly synthesis of iron nanoparticles and their role in mitigation of multiple abiotic stresses in fish

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Aquatic animals are frequently exposed to environmental stressors such as elevated temperatures and various inorganic and organic contaminants. The present investigation examines the role of iron nanoparticles (Fe-NPs) in mitigating the combined effects of cadmium (Cd, 1/10th of LC<sub>50</sub> 0.69 mg L<sup>-1</sup>) toxicity and high-temperature stress (34 °C) in *Pangasianodon hypophthalmus* (striped catfish). Four treatments were designed in triplicate: a control group, a group exposed to Cd and high temperature (Cd + T), and two groups exposed to Cd + T but fed with Fe-NPs at 10 and 15 mg kg<sup>-1</sup>, respectively. Fe-NPs were biologically synthesized using fish waste (gill). Experimental diets were prepared by incorporating Fe-NPs at 0, 10, and 15 mg kg<sup>-1</sup>. Dietary supplementation with Fe-NPs significantly improved growth performance under Cd + T stress, including final weight gain %, specific growth rate, protein efficiency ratio, feed conversion ratio, relative feed intake, and daily growth index. Expression levels of growth genes *GH* and *GHR1* in liver tissue were notably upregulated by Fe-NP-fed groups, while *MYST* expression was downregulated. Furthermore, stress and immune-related markers such as cortisol, *HSP70*, *iNOS*, *CYP450*, *Caspase-3a*, and *TNF-α* were significantly reduced in Fe-NP-treated groups compared to Cd + T and control groups, whereas Cd + T exposure alone markedly elevated these markers. Oxidative stress enzymes including catalase (CAT), superoxide dismutase (SOD), and glutathione-S-transferase (GST) in liver and kidney tissues were significantly elevated under Cd + T exposure, but their levels were reduced with dietary Fe-NP supplementation. Additionally, Fe-NPs at 10 and 15 mg kg<sup>-1</sup> enhanced acetylcholine esterase activity in brain tissue compared to control and stressors group (Cd + T). Activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and malate dehydrogenase (MDH) in liver and gill tissues were significantly reduced in Fe-NP-fed groups under stress conditions. Importantly, dietary Fe-NPs also lowered Cd accumulation in various fish tissues. In conclusion, dietary supplementation with Fe-NPs at 10 and 15 mg kg<sup>-1</sup> effectively mitigated the combined stress of Cd toxicity and elevated temperature in *P. hypophthalmus* by modulating gene expression, enhancing physiological performance, and alleviating cellular and metabolic stress.

**Keywords** Iron nanoparticles, Cadmium, Gene regulation, temperature stress, *Pangasianodon hypophthalmus*

### Abbreviations

APHA	American Public Health Association
AOAC	Association of Official Agricultural Chemist
Cd	Cadmium
CMC	Carboxymethyl cellulose
CAS 3a	Caspase 3a
CAT	Catalase
CP	Crude protein

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DGI	Daily growth index
ELISA	Enzyme-linked immunosorbent assay
EE	Ether extract
FCR	Feed conversion ratio
FAO	Food and Agriculture Organization
GPx	Glutathione peroxidase
<i>GHR1</i>	Growth hormone regulator 1
<i>GH</i>	Growth hormone
<i>HSP70</i>	Heat shock protein
T	High temperature
<i>iNOS</i>	Inducible nitric oxide synthase
ICPMS	Inductively Coupled Plasma Mass Spectrometry
Fe-NPs	Iron nanoparticles
Fe	Iron
LC <sub>50</sub>	Lethal concentration
MYST	Myostatin
NFκB	Nuclear factor kappa B
PER	Protein efficiency ratio
RFI	Relative feed intake
RAC	Research Advisory Committee
SGR	Specific growth rate
SOD	Superoxide dismutase
USFDA	United States Food and Drug Administration
TAN	Total ammonia nitrogen
<i>TNFα</i>	Tumor necrosis factor
WHO	World Health Organization

Rising temperatures and pollution are becoming increasingly evident across all ecosystems, including aquatic environments<sup>1</sup>. In the current scenario, abrupt temperature fluctuations driven by climate change, pose significant global threats and challenges. According to IPCC<sup>2</sup>, the global mean temperature is projected to increase by 1.1 °C to 6.4 °C by the year 2100. At the same time, increasing anthropogenic activities are contributing to significant pollution in aquatic ecosystems. Among the various contaminants, heavy metals are of particular concern, with cadmium (Cd) recognized as one of the most toxic to aquatic organisms, especially fish. Even at low concentrations, cadmium exhibits high toxicity and a strong bioaccumulation potential because fish excrete it very slowly, leading to its buildup in multiple tissues<sup>3,4</sup>. Major sources of Cd contamination include industrial discharges from mining, metal plating, plastics, phosphate fertilizers, alloy production, nickel-cadmium batteries, dyes, electrical and electronic industries, as well as corrosion-resistant coatings used in aerospace and marine environments<sup>4,5</sup>. Aquatic organisms are particularly vulnerable to Cd toxicity, especially during sudden exposure events<sup>6</sup>. Cadmium has no known metabolic or physiological function in most organisms, except for its involvement in carbonic anhydrase activity in certain marine algae<sup>7,8</sup>. Moreover, Cd induces neurotoxicity by binding to sulfhydryl groups in enzymes, thereby inhibiting their biological activity and disrupting normal life functions in aquatic animals<sup>9</sup>. In the present study, fish were subjected to combined stress from cadmium and elevated temperature. As poikilothermic animals, fish are highly sensitive to temperature changes, which can drastically alter their physiological functions. Furthermore, the cadmium has a long biological half-life and can be transmitted through water, feed, and environmental exposure. The extent of Cd accumulation in fish tissues depends on several factors, including exposure duration and concentration, fish age and species, dietary mineral and antioxidant content, and overall water quality<sup>10</sup>.

Nutritional approaches are among the most effective strategies to mitigate metal toxicity and high-temperature stress in fish<sup>11–13</sup>. These methods are practical, easily implementable, and highly effective for stress management in aquaculture systems<sup>14,15</sup>. In this study, iron nanoparticles (Fe-NPs) were applied as a nutritional intervention. Iron (Fe) is an essential micronutrient that plays a crucial role in metabolism and physiology, including oxygen transport and cellular respiration in fish<sup>16</sup>. Notably, Fe-NPs have been reported to enhance fish immunity through the NF-κB signaling pathway, involving immune-related genes such as *TNFα* and *Ig*<sup>17,18</sup>. Compared to conventional iron, Fe-NPs offer several advantages, including higher absorption and utilization efficiency due to their nanoscale size, elevated energy levels, increased surface activity, and enhanced ability to penetrate cell membranes<sup>19–21</sup>.

Oxidative stress biomarkers, including superoxide dismutase (SOD), catalase (CAT), and glutathione-S-transferase (GST), serve as critical indicators of oxidative damage arising from an imbalance between reactive oxygen species and antioxidant defenses under multiple stressors such as cadmium exposure and elevated temperature<sup>22</sup>. Stress-responsive genes *HSP70*, *iNOS*, *CYP450*, *Ig*, and *TNFα* are differentially expressed during adverse conditions and contribute to cellular tolerance. Neurotransmitter enzymes also act as sensitive biomarkers for pollution-induced neurotoxicity. Furthermore, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are widely used markers of tissue injury in fish and typically increase under stress. Digestive enzyme activities provide additional insight into the metabolic and overall health status of aquatic organisms. Cadmium accumulation predominantly occurs in the kidney, gills, liver, muscle, and brain tissues of fish. Elevated temperatures further intensify Cd toxicity, as corroborated by the current study<sup>23</sup>. Factors such as pH, salinity, organic matter, and seasonal variation also modulate Cd bioaccumulation across different fish tissues<sup>24–26</sup>. In this study, *Pangasianodon hypophthalmus* was chosen as the experimental species because of its suitability for toxicity assessments, especially under multiple stressors. This species is economically and

S. No	Details of the treatments	Notation
1	Control group: Fish fed with control diet	Ctr
2	Fish fed with control diet but concurrently exposed to Cd and high temperature (34 °C)	Cd + T
3	Fish fed with experimental diet of Fe-NPs at 10 mg kg <sup>-1</sup> diet and exposed to Cd + T	Fe-NPs-10 mg kg <sup>-1</sup> + Cd + T
4	Fish fed with experimental diet of Fe-NPs at 15 mg kg <sup>-1</sup> diet and exposed to Cd + T	Fe-NPs-15 mg kg <sup>-1</sup> + Cd + T

**Table 1.** Details of experimental design.

Feed ingredients	Fe-NPs-0 mg kg <sup>-1</sup> diet	Fe-NPs-10 mg kg <sup>-1</sup> diet	Fe-NPs-15 mg kg <sup>-1</sup> diet
Soybean meal <sup>a</sup>	35.5	35.5	35.5
Fish meal <sup>a</sup>	25	25	25
Groundnut meal <sup>a</sup>	10	10	10
Wheat flour <sup>a</sup>	17.47	17.46	17.455
Sunflower oil <sup>a</sup>	4.5	4.5	4.5
Cod liver oil <sup>a</sup>	1.5	1.5	1.5
CMC <sup>b</sup>	2	2	2
Vitamin and mineral mix <sup>c</sup>	2	2	2
Vitamin C <sup>c</sup>	0.03	0.03	0.03
Lecithin <sup>b</sup>	2	2	2
Fe-NPs	0	0.001	0.0015
Proximate composition of the diets			
Crude protein (CP)	35.22 ± 0.28	35.28 ± 0.03	35.05 ± 0.09
Ether extract (EE)	9.29 ± 0.04	9.54 ± 0.12	9.57 ± 0.16
Total carbohydrate (TC)	37.69 ± 0.18	37.19 ± 0.35	36.72 ± 0.45
Organic matter (OM)	90.75 ± 0.04	90.19 ± 0.49	90.29 ± 0.54
Dry matter (DM)	91.44 ± 0.20	91.82 ± 0.43	91.05 ± 0.18
Digestible energy (DE)	356.62 ± 0.82	356.67 ± 1.21	354.05 ± 1.69
Iron (Fe, mg kg <sup>-1</sup> )	2.62 ± 0.13	13.81 ± 0.32	19.48 ± 0.60

**Table 2.** Ingredients composition and proximate analysis of experimental diets (% dry matter) of iron nanoparticles (Fe-NPs), fed to *Pangasianodon hypophthalmus* for 40 days. <sup>a</sup> Procured from local market, <sup>b</sup>Himedia Ltd, Himedia Ltd, <sup>c</sup>SD Fine Chemicals Ltd., India. Digestible energy (DE) (Kcal/100 g) = (% EE × 9) + (% CP × 4) + (TC × 4). Data expressed as mean ± SE, n = 3. Composition of vitamin and mineral mixture (manual prepared mineral-vitamin mixture): vitamin B1:20 mg; vitamin B12 0.6 mcg; vitamin A 55,00,00 IU; vitamin D3 11,00,00 IU; vitamin E 75 mg; K 1,00 mg; nicotinamide 1000 mg; calcium pantothenate 2,50 mg; pyridoxine: 100 mg; I 1,00 mg; Zn 500 mg; Mn: 100 mg; Se: 2 ppm; P 30 g; Ca 50 g; Cu 200 mg; Co 45 mg.

nutritionally important, with desirable taste qualities and strong potential for diversification in aquaculture. Therefore, the objective of this study was to investigate the role of iron nanoparticles (Fe-NPs) in mitigating the combined effects of cadmium toxicity and high-temperature stress in *P. hypophthalmus*.

## Materials and methods

### Experimental animal and design

*Pangasianodon hypophthalmus* (average weight: 45.50 ± 0.40 g; average length: 12.30 ± 0.25 cm) were used as experimental animals. Healthy fish were harvested from the NIASM farm pond. Prior to the experiment, the fish were acclimatized for two weeks in fiber-reinforced plastic (FRP) tanks with a capacity of 500 L. During the acclimatization period, the fish were fed a pelleted diet containing 30% crude protein. After acclimatization, a total of 144 fish were randomly distributed into 12 rectangular plastic tanks (each with a 150 L capacity), with 12 fish per tank. The experiment was arranged in a completely randomized design (CRD), consisting of four treatments with three replicates each. Details of the experimental treatments are provided in Table 1.

Fish were fed diets supplemented with Fe-NPs at 0, 10, and 15 mg kg<sup>-1</sup> twice daily at 9:15 AM and 5:15 PM. Uneaten feed and fecal matter were removed daily by siphoning, and continuous aeration was provided using aerators throughout the experimental period. Water quality parameters were monitored periodically following standard methods described by APHA<sup>27</sup>. To induce cadmium toxicity, a stock solution of cadmium chloride (100 mg L<sup>-1</sup>) was prepared (Hi-Media, Mumbai, Maharashtra, India) as per the method described by Ganeshkar et al.<sup>28</sup>. In this study, the LC<sub>50</sub> value for Cd under high-temperature stress (Cd + T) was determined to be 6.91 mg L<sup>-1</sup>, and 1/10th of this concentration (0.69 mg L<sup>-1</sup>) was used for experimental exposure. Water temperature was maintained at 34 °C using a thermostatic rod heater, as described by Kumar et al.<sup>29</sup>. The experimental diets were formulated as isonitrogenous (35% crude protein) and isocaloric (356.8 ± 1.21 kcal/100 g), as detailed in Table 2. The feed ingredients included fishmeal, groundnut meal, soybean meal, and wheat flour. After heat

processing of the base ingredients, heat-sensitive nutrients such as cod liver oil, lecithin, iron nano-particles (Fe-NPs) and vitamin C were added. A iron free mineral mixture was used for feed formulation. For formulation of the pelleted diet, a manually prepared vitamin and mineral mixture was used. The dough was mixed properly and was pelleted, air dried and kept in a hot air oven at 60 °C until dry and was subsequently stored at 4 °C until required for feeding. The proximate composition of all the experimental diets and fish were analyzed as per the methods of AOAC<sup>30</sup>. Crude protein was measured based on nitrogen content, ether extract (EE) was determined via solvent extraction, and ash content was estimated using a muffle furnace at 550 °C. Carbohydrate content was calculated using the formula:

$$\text{Carbohydrates (\%)} = 100 - (\text{Crude Protein\%} + \text{EE\%} + \text{Ash\%} + \text{Moisture\%}).$$

Gross energy content of the feed was calculated using the Halver method<sup>31</sup>. The feed formulation based on iron nanoparticles (Fe-NPs) were used in our previous study<sup>32</sup>. A detailed composition of the experimental diets is presented in Table 2.

## Iron nanoparticles (Fe-NPs) synthesis using green approach

### Preparation of fish tissue extract

Fe-NPs were synthesized using fish waste, specifically gill tissue. The gill tissues were first cut into small pieces and thoroughly washed with running water to remove blood. The cleaned tissues were then homogenized using a tissue homogenizer (Tissue Master Homogenizer, Kennesaw, GA), and the homogenate was centrifuged at 5,000–6,000 rpm to collect the supernatant. The resulting gill extract was subsequently filtered using Whatman filter paper with a pore size of 0.45  $\mu\text{m}$ <sup>33</sup>.

### Preparation and characterization of iron nanoparticles (Fe-NPs)

Ferrous sulfate (0.4 M) was mixed with the prepared gill extract and stirred at room temperature for 30 min. The mixture was then heated on a magnetic stirrer at 60 °C for 2.5 to 4 h, during which 0.8 M NaOH was added dropwise until a reddish-brown coloration developed, indicating the formation of iron nanoparticles (Fe-NPs). Once the reddish-brown color appeared, the solution was analyzed using a UV-Vis spectrophotometer in the range of 216–600 nm, which revealed a strong absorption peak at 250 nm, confirming the presence of Fe-NPs. The solution was then centrifuged, and the resulting pellet was washed three times with distilled water to remove any impurities. The purified pellet was dried using a concentrator, yielding Fe-NPs in the form of a reddish-brown powder. For characterization, the dried nanoparticles were re-dispersed in Milli-Q water, and their particle size and zeta potential were measured using a particle size analyzer (Litesizer 500, Anton Paar, Austria)<sup>21,32</sup>. The mean particle size and zeta potential were recorded as 130.17 nm and + 53.3 mV, respectively, as shown in Fig. 1A,B.

## Tissue homogenate preparation and blood collection

Under aseptic conditions, tissues including the gill, intestine, muscle, brain, liver, and kidney of *P. hypophthalmus* were collected from fish anesthetized with clove oil (50  $\mu\text{L L}^{-1}$ ). The tissues were homogenized using an Omni Tissue Master Homogenizer (Kennesaw, GA, USA) in a cold 5% sucrose solution (w/v; 0.25 M) containing 1 mM EDTA for subsequent enzyme analysis. Muscle (*MYST*) and liver tissues were immediately snap-frozen in liquid nitrogen for gene expression analysis of *HSP70*, *iNOS*, *TNF $\alpha$* , *GHR1*, *Ig*, *GH*, *CYP450*, and *Cas 3a*. The homogenates were centrifuged at 5,000  $\times$  g for 15 min at 4 °C using a refrigerated centrifuge (Eppendorf AG, 5430R, Hamburg, Germany), and the resulting supernatants were stored at -20 °C for further biochemical analysis. Additionally, the blood was collected from the caudal vein for serum, from nine fish from each treatment without anticoagulant and allowed to clot for 2 h followed by collection of serum with a micropipette and stored at -22 °C until use.

## Protein estimation

Protein content in different tissues (gill, intestine, muscle, brain, liver, and kidney) was determined following the method of Lowry et al.<sup>34</sup>, using freshly prepared 1 N Folin-Ciocalteu reagent and alkaline  $\text{CuSO}_4$ . For this, 0.1 mL of tissue homogenate was treated with 1 mL of 10% trichloroacetic acid (TCA) to precipitate proteins. The mixture was centrifuged at 5,000 rpm for 20 min, after which the supernatant was discarded. This residue was dissolved in 0.5 mL of 0.1 N NaOH, and 0.1 mL of the resulting solution was used for analysis. The absorbance was recorded at 660 nm.

## Analysis of physiological indices

### Growth performance

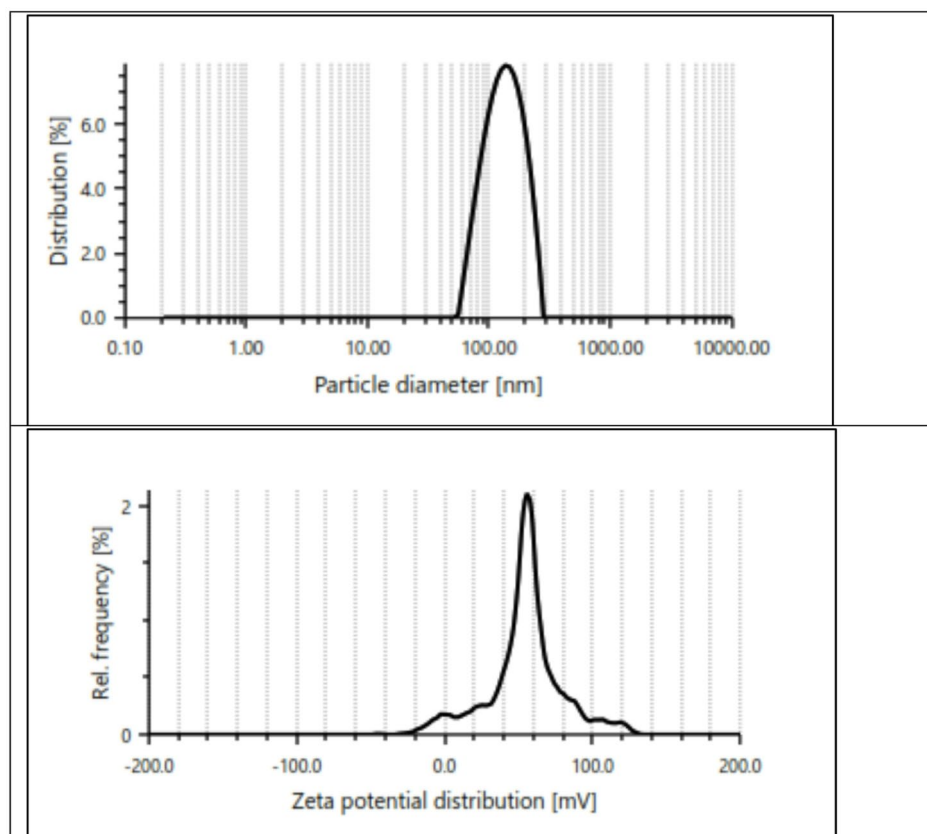
The growth performance of the fish was evaluated using our laboratory's standardized methodologies, including weight gain percentage, specific growth rate (SGR), protein efficiency ratio (PER), feed conversion ratio (FCR), daily growth index (DGI), and relative feed intake (RFI). Weight measurements were recorded at 20-day intervals<sup>28,35</sup>.

$$\text{SGR} = 100 (\ln \text{FBW} - \ln \text{IBW}) / \text{number of days}$$

$$\text{FCR} = \text{Total dry feed intake (g)} / \text{Wet weight gain (g)}.$$

$$\text{Weight gain (\%)} = \text{Final body weight (FBW)} - \text{Initial body weight (IBW)} / \text{Initial body weight (IBW)}$$

$$\text{Relative feed intake, (FI) (\%/d)} = 100$$



**Fig. 1.** (A,B) Size of iron nanoparticles (Fe-NPs): 130 nm B. Zeta potential 53.7 mV.

$$\text{PER} = \text{Total wet weight gain (g)} / \text{crude protein intake (g)}.$$

$$\text{PER} = \text{Total wet weight gain (g)} / \text{crude protein intake (g)}.$$

$$\text{Daily growth index, DGI (\%)} = (\text{FBW}^{1/3} - \text{IBW}^{1/3}) / \text{days} \times 100$$

## Biochemical and gene expression analysis

### Cortisol

Cortisol levels in the serum were measured using a commercially available ELISA kit (Cortisol EIA kit, catalogue no. 500360, Cayman Chemicals, USA). The assay was conducted following the instructions provided with the kit. An ELISA plate reader from Biotek India Pvt. Ltd. was used to perform the measurements, ensuring accurate detection of cortisol levels in the serum samples.

### Antioxidant enzyme activities

**Catalase assay (EC 1.11.1.6)** Catalase (CAT) activity in liver and kidney tissues was determined following the method of Takahara et al.<sup>36</sup>. The reaction mixture contained 1.0 mL of freshly prepared hydrogen peroxide solution (Cat. No. 1.93.007.0521, Merck Life Science, Vikhroli, Mumbai, India), 50  $\mu\text{L}$  of tissue homogenate and 2.45 mL of 50 mM phosphate buffer (pH 7). After mixing, the decrease in absorbance at 240 nm was recorded for three minutes to measure catalase activity.

**Superoxide dismutase assay (EC 1.15.1.1)** Superoxide dismutase (SOD) activity in liver and kidney tissues was analyzed following the procedure developed by Misra and Fridovich<sup>37</sup>. The reaction mixture was prepared by combining 0.5 mL of freshly prepared epinephrine (Cat. No. 1236970, Merck Life Science, Vikhroli, Mumbai, India), 1.5 mL of phosphate buffer, and 50  $\mu\text{L}$  of tissue homogenate. The mixture was thoroughly mixed, and the absorbance was measured immediately at 480 nm for three minutes using a UV spectrophotometer (Shimadzu (Asia Pacific) Pte Ltd, Cintech IV, Science Park I, Singapore-118264).

**Glutathione-S-transferase assay (EC 2.5.1.18)** Glutathione S-transferase (GST) activity in liver and kidney tissues was determined using a spectrophotometric method, with S-2,4-dinitrophenyl glutathione (CDNB) (Cat. No. S101915, Merck Life Science, Vikhroli, Mumbai, India) as the substrate, following the protocol established by Habing et al.<sup>38</sup>. The reaction involves the formation of an adduct between glutathione (GSH) and S-2,4-dinitrophenyl glutathione in the presence of CDNB. The increase in absorbance at 340 nm, relative to a blank, was used to quantify the production of this adduct, indicating GST activity.

### Neurotransmitter enzyme activities

Acetylcholinesterase (EC 3.1.1.7) activity in brain tissue was assessed using a modified Hestrin method as described by Augustinsson<sup>39</sup>. In this procedure, 0.2 mL of the sample was mixed with 1 mL each of acetylcholine buffer and phosphate buffer, and the mixture was incubated at 37 °C for 30 min. Following incubation, alkaline hydroxylamine, HCl, and ferric chloride (Himedia, Cat. No. GRM 1379, Thane, Mumbai, India) were added. The enzymatic activity was determined by measuring the absorbance at 540 nm.

### Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and malate dehydrogenase (MDH)

AST (E.C.2.6.1.1) and ALT (E.C.2.6.1.2) activities in liver and gill tissues were determined following the method described by Wootton<sup>40</sup>. The reaction mixture consisted of sodium hydroxide (1 N), potassium dihydrogen phosphate, dipotassium hydrogen phosphate, and 2,4-dinitrophenyl hydrazine. For the ALT assay,  $\alpha$ -ketoglutarate and DL-alanine were used as substrates, while  $\alpha$ -ketoglutarate and DL-aspartic acid were used in the AST assay. The tissue homogenate and substrate were mixed and incubated at 37 °C for 1 h, after which DNPH was added and the absorbance at 540 nm was measured. LDH activity in liver and gill tissues was evaluated using the Wroblewski and Ladue<sup>41</sup> method. A reaction mixture was prepared by adding sodium dihydrogen phosphate (0.1 M) and disodium hydrogen phosphate to a phosphate buffer, followed by the addition of freshly prepared NADH and sodium pyruvate. After incubating the mixture with the sample/enzyme extract for 20 min, absorbance was measured at 320 nm. Similarly, MDH activity was assessed following Ochoa's<sup>42</sup> method, with the reaction mixture remaining the same but substituting sodium pyruvate with oxaloacetate as the substrate.

### RNA isolation and quantification and cDNA synthesis and quantitative PCR

Total RNA was extracted from the liver tissue of *P. hypophthalmus* using the TRIzol method (Catalogue no. 15596018; Invitrogen™ Life Technologies, Carlsbad, CA, USA). Liver samples were homogenized in liquid nitrogen, followed by phase separation with chloroform. For each treatment, nine fish were used; samples from three fish within a replicate were pooled, yielding three biological replicates for RNA extraction. RNA was separated by centrifugation, dissolved in nuclease-free water, and stored at – 80 °C. Integrity was verified on a 1% agarose gel, and concentration was determined using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). For cDNA synthesis, residual DNA was removed with DNase I, and first-strand cDNA was generated using the RevertAid First Strand cDNA Synthesis Kit (Catalog no. K1622, Thermo Fisher Scientific Baltics UAB, Lithuania). Each reaction contained 100 ng RNA in 12  $\mu$ l with 15 pmol oligo(dT) primers, incubated at 65 °C for 5 min and chilled on ice. Subsequently, 1  $\mu$ l RiboLock RNase inhibitor (20 U/ $\mu$ l), 2  $\mu$ l 10 mM dNTP mix, and 1  $\mu$ l reverse transcriptase were added. The mixture was centrifuged briefly, incubated at 60 °C for 42 min, then at 70 °C for 5 min, and stored at – 20 °C.  $\beta$ -actin served as the reference gene to confirm cDNA synthesis. Quantitative real-time PCR was performed on a QuantStudio 5 system (Applied Biosystems, Thermo Fisher Scientific) using SYBR Green (Catalog no. A25742, Bio-Rad, Lithuania) and gene-specific primers. Cycling conditions included initial denaturation at 95 °C for 10 min, followed by 39 cycles of 95 °C for 15 s and 60 °C for 1 min, following Pfaffl<sup>43</sup>. Primer sequences are provided in Table 3.

Gene	Primer sequence (5' – 3')	Accession number	PCR Efficiency
<i>HSP 70</i>	F- CTCCTCTAAACCCCGAGTC R- CCACCAGCACGTTAAACACA	XM_026934573.2	1.486
<i>iNOS</i>	F-ACACCACGGAGTGTGTTTCGT R-GGATGCATGGGACGTTGCTG	XM_026931613.2	1.493
<i>TNF<math>\alpha</math></i>	F-TGGAGTTCTGCTTGCCGTGG R-GCAGCCTTTGCAGTCTCGGA	XM_026942329.2	1.498
<i>Ghr1</i>	FTATTGGCTACAGCTCGCCGC R-AATCACCCCGACTGTGCTGC	XM_034306157.1	1.5650
<i>Ig</i>	F- GGCCAGTAATCGTACCTCCA R- CTTCTGAAGTCCCCACTGA	XM_026923540.2	1.499
<i>MYST</i>	F-GGGAAAGACCTGGCCGTGAC R-TCGAGCCGGATTCTCGTCT	XM_026910492.2	1.496
<i>GH</i>	F-CCCAGCAAGAACCTCGGCAA R-GCGGAGCCAGAGATCGTTC	GQ859589.1	1.673
<i>CYP P450</i>	F-GATTGGCATCCGTGCGTGC R-GATGTGGCTGGGACGAGCA	NC_047599.1	1.686
<i>Cas 3a</i>	F-CGGCATGAACCAGCGCAAC R-TCCACCGCACCATCTGTCCC	NC_047622.1	1.487
$\beta$ -Actin	F-CAGCAAGCAGGAGTACGATG R-TGTGTGGTGTGTGGTTGTTTG	XM_031749543.1	1.787

**Table 3.** Details of primer for relative quantitative real-time PCR. *HSP*: Heat shock protein; *iNOS*: Nitric oxide synthase; *TNF $\alpha$* : Tumor necrosis factor; *TLR*: Toll like receptor; *Ghr*: Growth hormone receptor; *IL*: Interleukin; *Ig*: Immunoglobulin; *MYST*: myostatin; *SMT*: Somatostatin; *CYP P450*: Cytochrome P450; *Cas 3a* and *GH*: Growth hormone.

## Cadmium analysis in fish tissues and experimental water

Cadmium concentrations in water and fish tissues (liver, muscle, gill, brain, and kidney) were analyzed across all treatment groups. Water samples were filtered through 0.45 µm membranes and acidified with 100 µl of concentrated HNO<sub>3</sub> (69%, Himedia Laboratory Pvt. Ltd., Mumbai, India). Tissue samples were digested in a microwave reaction system (Multiwave PRO, Anton Paar GmbH, Austria) using a 5:1 mixture of HNO<sub>3</sub> and H<sub>2</sub>O<sub>2</sub>. After digestion, the solutions were cooled, filtered through 0.45 µm Whatman paper, and diluted to a final volume of 50 ml. Cadmium levels were then quantified using Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Agilent 7700 series, Agilent Technologies, USA) following the protocol of Kumar et al.<sup>44,45</sup>.

## Statistics

Data analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 16 software. To assess the normality of the data, the Shapiro-Wilk test was employed, while the Levene test was used to examine the homogeneity of variances. A One-Way Analysis of Variance (ANOVA) was conducted, followed by Duncan's multiple range test for post-hoc comparisons. The significance threshold for all tests was set at  $p < 0.05$ .

## Results and discussion

### Growth performance

In the present investigation, the growth performance of fish was evaluated based on final weight gain percentage, feed conversion ratio (FCR), specific growth rate (SGR), protein efficiency ratio (PER), daily growth index (DGI), and relative feed intake (RFI). The fish were exposed to concurrently with cadmium toxicity and high-temperature stress for 40 days. Fish were fed either a control diet or Fe-NPs-supplemented diet to reduce the impact of Cd+T stress. The results indicated that supplementation of Fe-NPs at 10 and 15 mg kg<sup>-1</sup> in the diet with the stressors (Cd+T) significantly ( $p=0.0024$ ) enhanced weight gain percentage, SGR ( $p=0.001$ ), PER ( $p=0.0015$ ), DGI ( $p=0.001$ ), and RFI ( $p=0.012$ ) compared to the control group and the group treated with Cd+T stress alone. Moreover, the group exposed to both Cd+T stress and fed the control diet exhibited the lowest growth performance. Additionally, the FCR was significantly ( $p=0.0014$ ) reduced with Fe-NPs supplementation at 10 and 15 mg kg<sup>-1</sup> in the diet under stress conditions (Table 4).

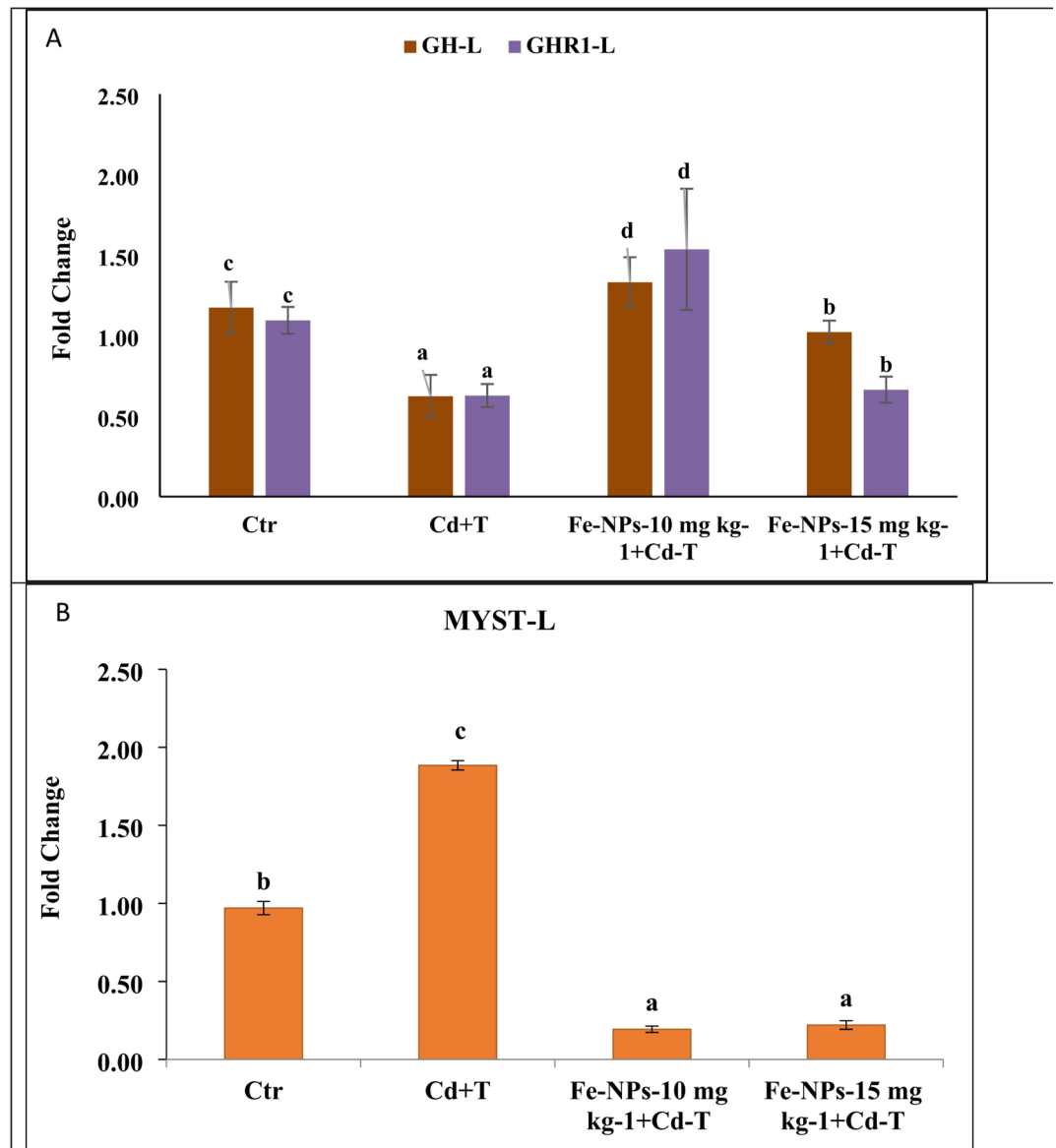
In the present investigation, Fe-NPs were incorporated into the diet at 10 and 15 mg kg<sup>-1</sup> to evaluate their effectiveness in reducing the adverse effects of low-dose cadmium exposure combined with high-temperature stress (34 °C). The results demonstrated substantial improvements in weight gain, FCR, PER, SGR, RFI, and DGI in *P. hypophthalmus* subjected to Cd + T stress for 40 days following Fe-NP supplementation. The growth-promoting effects of Fe-NPs are likely attributed to their role in maintaining iron homeostasis within the organism, thereby enhancing growth performance under combined Cd + T stress<sup>21,46</sup>. Furthermore, the smaller particle size of Fe-NPs may contribute to their efficient utilization when incorporated into the diet<sup>47-50</sup>. The higher bioavailability and improved absorption of Fe-NPs further support their positive influence on growth in fish, both under control and stressful conditions<sup>21,32</sup>. In contrast, concurrent exposure to Cd + T resulted in reduced growth and feed utilization. However, Fe-NPs supply the essential nutrients through the bloodstream, providing energy and acting as enzyme cofactors that enhance nutrient breakdown and absorption<sup>51</sup>. The supplementation of Fe-NPs at 10 and 15 mg kg<sup>-1</sup> also improved the daily growth index and relative feed intake, underscoring their role in supporting growth attributes.

### Effect of Fe-NPs on growth performing genes (*GH*, *GHR1* and *MYST*)

The expression of growth-promoting genes, such as *GH* and *GHR1* in liver tissue and *MYST* in muscle tissue of *P. hypophthalmus* reared under concurrent exposure to cadmium and high-temperature stress for 40 days, is presented in Fig. 2A,B. The results showed that *GH* ( $p=0.0016$ ) and *GHR1* ( $p=0.0021$ ) were significantly upregulated by dietary Fe-NPs at 10 mg kg<sup>-1</sup> in *P. hypophthalmus* under Cd+T stress, compared to the control and stressor groups. In contrast, *MYST* was substantially ( $p=0.0017$ ) downregulated by dietary Fe-NPs at both 10 and 15 mg kg<sup>-1</sup> in fish exposed to Cd+T stress, relative to the control and stressor groups. Additionally, the group exposed to Cd and high temperature and fed with the control diet exhibited significantly downregulated

Diet	Iron nanoparticles diets (Fe-NPs)			
	Control	Cd + T	Fe-NPs-10 mg kg <sup>-1</sup>	Fe-NPs-15 mg kg <sup>-1</sup>
Weight gain %	49.38 <sup>b</sup> ± 0.55	26.45 <sup>a</sup> ± 2.05	60.55 <sup>c</sup> ± 1.37	66.53 <sup>d</sup> ± 1.55
FCR	2.74 <sup>b</sup> ± 0.04	4.77 <sup>c</sup> ± 0.30	2.38 <sup>ab</sup> ± 0.03	2.19 <sup>a</sup> ± 0.04
SGR	0.45 <sup>b</sup> ± 0.01	0.26 <sup>a</sup> ± 0.02	0.53 <sup>c</sup> ± 0.01	0.57 <sup>c</sup> ± 0.01
PER	1.05 <sup>b</sup> ± 0.02	0.66 <sup>a</sup> ± 0.04	1.30 <sup>c</sup> ± 0.01	1.42 <sup>d</sup> ± 0.05
DGI	0.77 <sup>b</sup> ± 0.01	0.44 <sup>a</sup> ± 0.03	0.90 <sup>c</sup> ± 0.02	0.98 <sup>d</sup> ± 0.01
RFI	135.24 <sup>b</sup> ± 1.64	124.87 <sup>a</sup> ± 1.59	143.97 <sup>c</sup> ± 1.35	145.60 <sup>c</sup> ± 0.94

**Table 4.** Effect of dietary Fe-NPs on growth performance (Weight gain %, FCR, SGR, PER, and RFI) of *Pangasianodon hypophthalmus* reared under control or cadmium and high temperature for 40 days. Values in the same row with different superscript (a, b, c, d) differ significantly. Data expressed as Mean ± SE ( $n=3$ ). FCR feed conversion ratio, SGR specific growth rate, PER protein efficiency ratio, RFI relative feed intake.



**Fig. 2.** (A,B) Potential role of Fe-NPs diets on gene expression of *GH*, *GHR1* and *MYST* in liver tissue in fish reared under control or cadmium and high temperature stress for 40 days. Within endpoints and groups, bars with different superscripts differ significantly (a–d). Data expressed as Mean  $\pm$  SE ( $n=6$ ).

*GH* and *GHR1* genes, while the *MYST* gene was upregulated in *P. hypophthalmus* throughout 40-day experimental period.

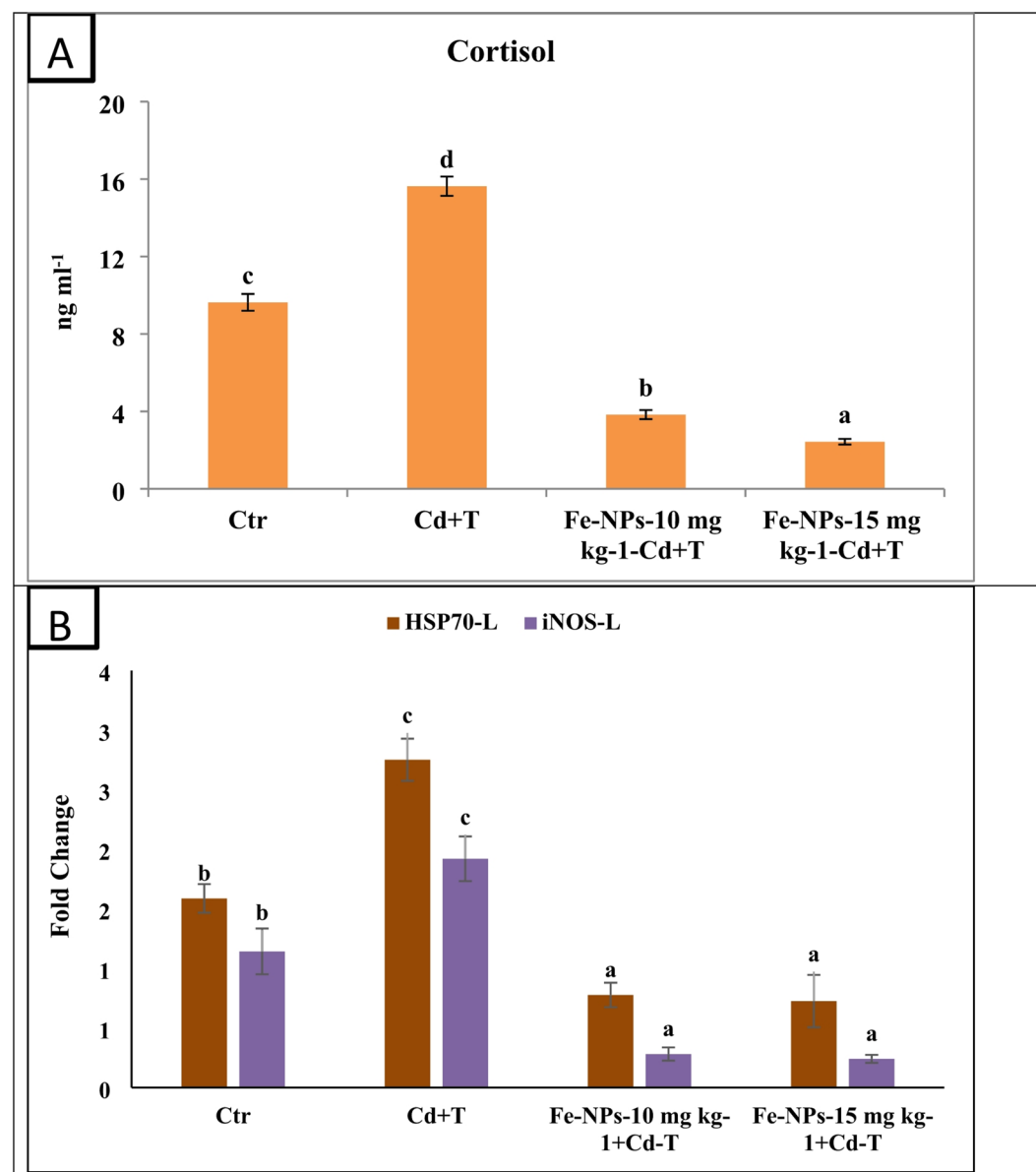
The regulation of *GH*, *GHR1*, and *MYST* genes was significantly downregulated in fish exposed to Cd + T stress and fed with the control diet, indicating that the combined exposure to cadmium and high temperature negatively impacts the gene regulatory mechanisms associated with growth enhancement. However, supplementation with Fe-NPs at 10 and 15 mg kg<sup>-1</sup> diet resulted in the upregulation of *GH* and *GHR1* gene expression, while *MYST* expression was downregulated. This effect could be attributed to the role of Fe-NPs in reducing myoblast activity, which promotes terminal differentiation and muscle fiber enlargement<sup>52</sup>. Additionally, Fe-NPs may facilitate the binding of *GH* to its receptor in liver cells, stimulating the release and synthesis of *IGF*, further enhancing growth. Fe-NPs also play a crucial role in the secretion and regulation of *IGF*, which directly influences growth performance via the central nervous system of fish, whether reared under normal or stressed conditions<sup>53,54</sup>. Ghrelin, which regulates GH release from the pituitary gland, is also essential in this process. It stimulates the growth hormone-releasing hormone and somatostatin axis, leading to increased *IGF* production in the liver<sup>55</sup>.

#### Effect of Fe-NPs on cortisol, *HSP 70* and *iNOS*

Cortisol is the primary stress hormone, and its levels increase during stress conditions. In this study, cortisol levels were significantly elevated ( $p=0.0015$ ) in the group exposed to concurrent Cd + T stress and fed with the control diet. However, dietary supplementation with Fe-NPs at 15 mg kg<sup>-1</sup> resulted in a significant reduction in

cortisol levels, followed by a reduction in the group fed with Fe-NPs at 10 mg kg<sup>-1</sup>, compared to the control and Cd + T groups (Fig. 3A). Additionally, the gene expression of *HSP 70* and *iNOS* in liver tissue of *P. hypophthalmus* reared under control and combined Cd + T stress for 40 days is presented in Fig. 3B. Gene expression of *HSP 70* ( $p=0.0013$ ) and *iNOS* ( $p=0.001$ ) was significantly downregulated by dietary Fe-NPs at 10 and 15 mg kg<sup>-1</sup> in the presence of stressors, compared to the control and Cd + T groups. In contrast, upregulation of *HSP 70* and *iNOS* was observed in the group exposed to Cd + T stress and fed the control diet, compared to both the control and Fe-NPs supplemented groups.

The overexpression of *HSP70* and *iNOS* gene expression under Cd + T stress may be attributed to the activation of heat shock promoters by heat shock factors (HSF), triggered by high temperature and cadmium toxicity<sup>56</sup>. Moreover, both stressors can reduce the methylation levels at the HSF binding motif in the *HSP70* promoter, resulting in its hypomethylation and subsequent overexpression<sup>57</sup>. The *iNOS* gene, on the other hand, is an early inflammatory marker and plays a central role in initiating cellular inflammatory responses<sup>58</sup>. Its expression helps regulate cellular metabolism during concurrent exposure to Cd + T. Elevated cortisol levels under Cd + T stress conditions may be due to disruptions in the interrenal cells of the kidney within the pituitary-adrenal axis, impairing corticosteroidogenesis and leading to excessive cortisol production<sup>59</sup>. However, dietary supplementation with Fe-NPs at 10 and 15 mg kg<sup>-1</sup> significantly downregulated *HSP70* expression, likely by protecting against proteasomal degradation, preventing protein aggregation, aiding the degradation

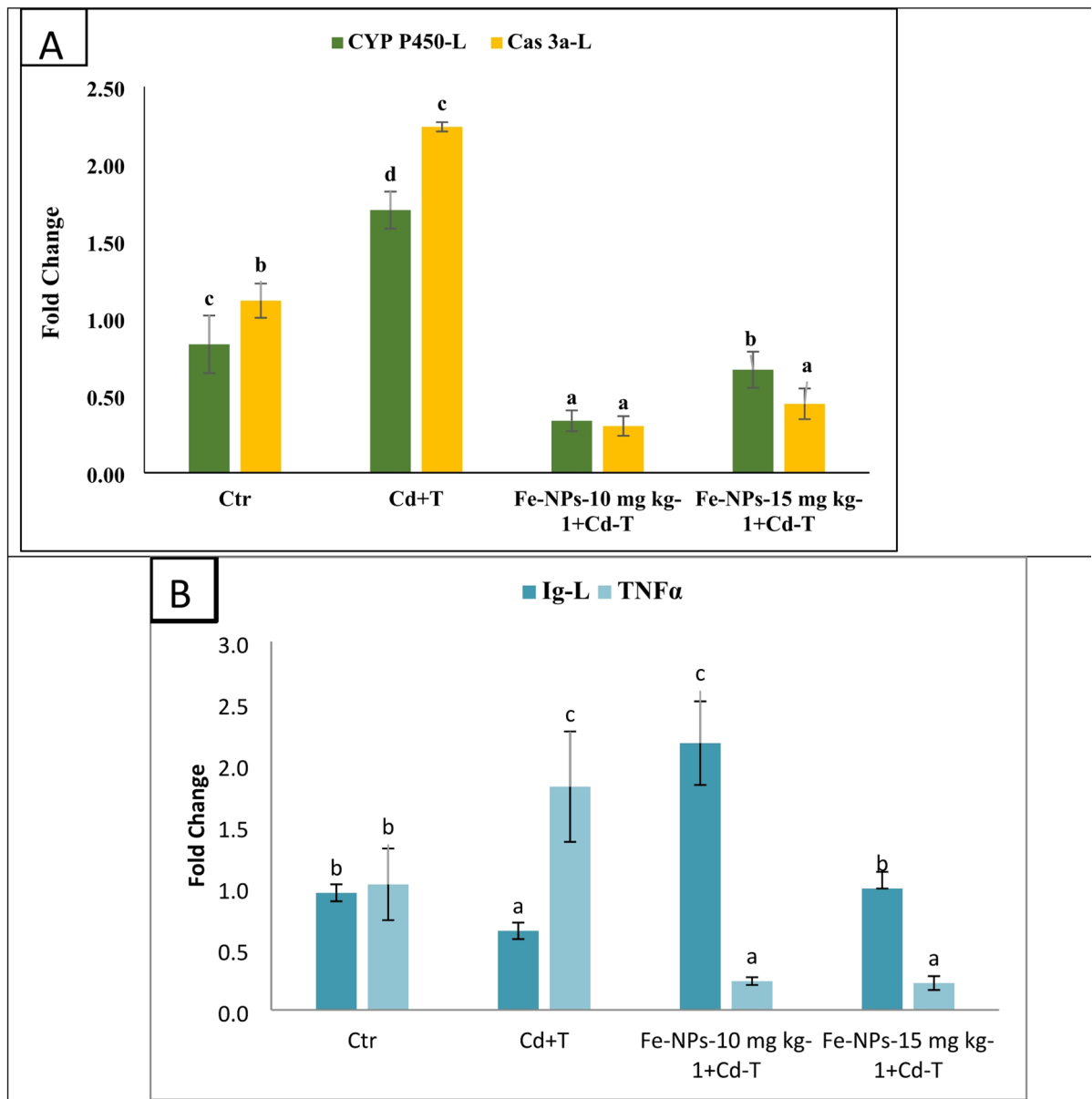


**Fig. 3.** (A,B) Potential role of Fe-NPs diets on cortisol and gene expressions of *HSP 70* and *iNOS* in liver tissue in fish reared under control or cadmium and high temperature stress for 40 days. Within endpoints and groups, bars with different superscripts differ significantly (a–d). Data expressed as Mean  $\pm$  SE ( $n=6$ ).

of denatured proteins, and supporting the correct folding of misfolded proteins<sup>60,61</sup>. Furthermore, Fe-NPs may influence *HSP* transcription factor activity by reducing heat shock element binding, thereby decreasing *HSP70* expression<sup>62,63</sup>. A strong interrelationship exists between iron metabolism and *iNOS* gene expression. Iron nanoparticles have been shown to reduce oxidative stress by preventing the accumulation of reactive oxygen species via a NOS-dependent mechanism<sup>64</sup>. Additionally, iron can promote cellular hypertrophy by upregulating anti-apoptotic proteins such as Bcl-2 and survivin, as well as hypertrophic agents like endothelin-1 (ET-1) and leukemia inhibitory factor (LIF), all of which contribute to reduced H<sub>2</sub>O<sub>2</sub>-induced necrosis<sup>65,66</sup>.

#### Effect of Fe-NPs on *CYP 450*, *Cas 3a*, *Ig* and *TNF $\alpha$*

The expression patterns of *CYP450*, *Caspase 3a* (*Cas 3a*), Immunoglobulin (*Ig*), and Tumor Necrosis Factor-alpha (*TNF $\alpha$* ) genes in the liver of *Pangasianodon hypophthalmus* reared under control conditions and concurrent exposure to cadmium and high temperature (Cd+T) stress for 40 days are presented in Fig. 4A,B. The gene expression of *CYP450* was significantly downregulated ( $p=0.0011$ ) with dietary supplementation of Fe-NPs at 10 mg kg<sup>-1</sup>, followed by 15 mg kg<sup>-1</sup>, under stress conditions when compared to the control and Cd+T groups. Similarly, *Cas 3a* expression was also markedly reduced ( $p=0.0025$ ) in the groups supplemented with Fe-NPs at 10 and 15 mg kg<sup>-1</sup> compared to the control and stress groups (Fig. 4A). Furthermore, *Ig* gene expression was significantly upregulated ( $p=0.001$ ) by Fe-NPs at 10 mg kg<sup>-1</sup> diet when compared to other treatment groups.



**Fig. 4.** (A,B) Potential role of Fe-NPs diets on gene expressions of *CYP 450*, *CAS 3a*, *Ig* and *TNF $\alpha$*  in liver tissue in fish reared under control or cadmium and high temperature stress for 40 days. Within endpoints and groups, bars with different superscripts differ significantly (a–d). Data expressed as Mean  $\pm$  SE ( $n=6$ ).

Meanwhile, *TNF $\alpha$*  expression was strongly downregulated ( $p=0.00016$ ) in fish fed Fe-NPs at 10 and 15 mg kg<sup>-1</sup> diet under Cd + T stress compared to the control and stress-exposed groups. These findings clearly indicate that concurrent exposure to cadmium and elevated temperature significantly alters the regulation of genes associated with xenobiotic metabolism, apoptosis, immune response, and inflammation in *P. hypophthalmus*, while dietary Fe-NPs mitigate these effects in a dose-dependent manner.

In this study, concurrent exposure to cadmium (Cd) and elevated temperature (34 °C) stress led to a marked upregulation of *CYP450* gene expression, likely due to the activation of oxidative stress-related kinases and the consequent generation of reactive oxygen species (ROS), which in turn triggered apoptosis and elevated inflammatory cytokine levels<sup>67</sup>. The increased transcription of *CYP450* under Cd + T stress also suggests its involvement in Cd detoxification processes, during which excessive ROS are produced, intensifying oxidative stress<sup>68</sup>. However, dietary supplementation with Fe-NPs significantly downregulated *CYP450* expression, indicating their role in modulating oxidative damage induced by Cd + T stress<sup>69</sup>. Cd exposure also disrupts cellular homeostasis by promoting ROS accumulation, which affects intracellular Ca<sup>2+</sup> mobilization, leads to mitochondrial membrane depolarization, and causes the release of cytochrome c into the cytoplasm, ultimately activating caspase-3 and initiating apoptosis<sup>70</sup>. Notably, Fe-NPs appear to mitigate these effects through the involvement of protein disulfide isomerase (PDI), a multifunctional enzyme capable of regulating apoptosis via caspase pathways<sup>71</sup>, and by binding transition metals such as copper and zinc at active thiol sites<sup>72</sup>. Cd-induced stress also activates death receptor pathways, particularly via *TNF $\alpha$* , enhancing its expression and contributing to liver inflammation and programmed cell death in fish<sup>73,74</sup>. Conversely, dietary Fe-NPs suppressed *TNF $\alpha$*  expression, potentially through their regulatory influence on ferritin levels within macrophages<sup>75</sup>, thereby exerting anti-inflammatory effects. Additionally, Cd + T stress significantly reduced total immunoglobulin (Ig) gene expression, whereas Fe-NP supplementation was able to restore Ig expression levels, suggesting an immunoprotective role under combined metal and thermal stress.

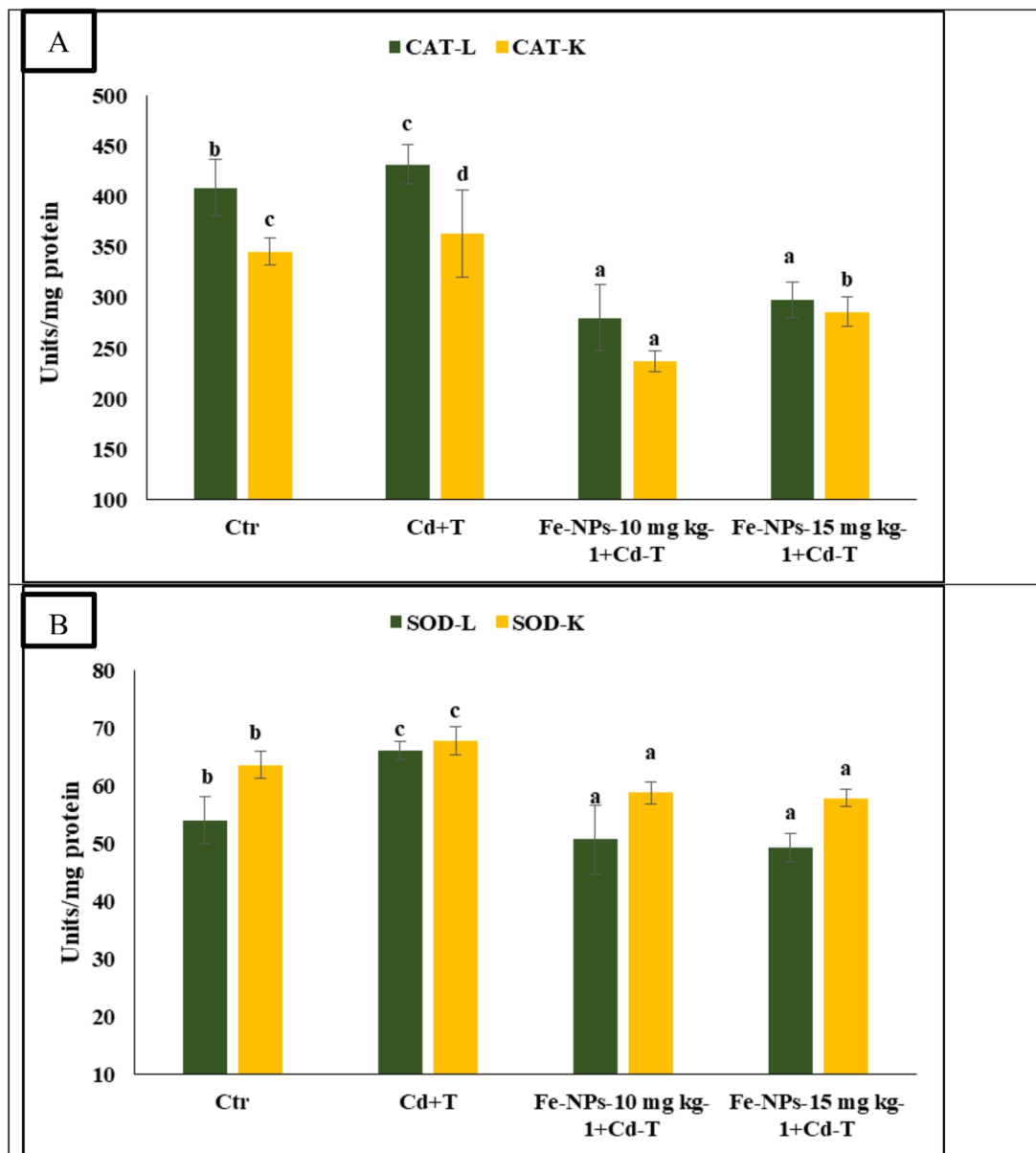
### Effect of Fe-NPs on catalase, SOD, GST and AChE

The activities of catalase (CAT), superoxide dismutase (SOD), and glutathione S-transferase (GST) in liver and kidney tissues, along with acetylcholinesterase (AChE) activity in brain tissue of *Pangasianodon hypophthalmus* reared under control conditions and concurrent exposure to cadmium and high temperature (Cd + T) for 40 days, are presented in Figs. 5 and 6A,B. CAT activity in both liver ( $p=0.001$ ) and kidney ( $p=0.001$ ) tissues was significantly elevated in the Cd + T group fed the control diet, compared to both the control and Fe-NP-supplemented groups. Interestingly, dietary supplementation with Fe-NPs at 10 mg kg<sup>-1</sup> diet, followed by 15 mg kg<sup>-1</sup>, markedly reduced CAT activity in these tissues. Similarly, SOD activity in the liver and kidney was substantially lowered in groups supplemented with Fe-NPs at both 10 and 15 mg kg<sup>-1</sup> diets when compared to the control and Cd + T groups (Fig. 5A,B). Furthermore, GST activity in the liver ( $p=0.0011$ ) and kidney ( $p=0.012$ ) was significantly reduced in the group supplemented with 10 mg kg<sup>-1</sup> Fe-NPs relative to other groups. In contrast, concurrent exposure to Cd + T stress in fish fed the control diet significantly elevated the activities of CAT, SOD, and GST in both liver and kidney tissues compared to the Fe-NP-supplemented and control groups. Regarding neurological effects, AChE activity in brain tissue was significantly inhibited ( $p=0.00015$ ) by combined Cd and high-temperature stress. However, supplementation with Fe-NPs at 10 mg kg<sup>-1</sup>, followed by 15 mg kg<sup>-1</sup>, restored AChE activity in stressed fish, showing higher levels compared to both the control and Cd + T only groups (Fig. 6A,B).

Concurrent exposure to cadmium and elevated temperature (Cd + T) triggered oxidative stress in *P. hypophthalmus*, as evidenced by elevated activities of antioxidant enzymes such as CAT, SOD, and GST. This response indicates an overproduction of reactive oxygen species (ROS), which can lead to cellular damage<sup>76,77</sup>. Under such stress, the fish initiate protective mechanisms to neutralize superoxide (O<sub>2</sub><sup>-</sup>) radicals; however, these mechanisms can be overwhelmed. Supplementation with Fe-NPs significantly modulated this response by reducing the activities of CAT, SOD, and GST, suggesting a protective role in oxidative stress mitigation<sup>21,32</sup>. Fe-NPs exhibit multiple biological functions, including serving as components of the antioxidant defense system. They are likely to enhance the activity of enzymes like SOD and GPx, thereby preventing excessive free radical generation and lipid peroxidation<sup>78,79</sup>. Catalase, a heme-containing enzyme, works synergistically with SOD to neutralize cellular ROS and improve the antioxidant capacity of fish cells<sup>80</sup>. Additionally, AChE activity, which was markedly inhibited by Cd + T exposure, reflects oxidative damage to critical cellular structures such as the endoplasmic reticulum, plasma membrane, and Golgi apparatus<sup>81</sup>. Interestingly, Fe-NPs were able to restore AChE activity under combined stress conditions, likely by preserving neuronal integrity and limiting oxidative damage<sup>21</sup>.

### Effect of Fe-NPs on ALT, AST, LDH and MDH

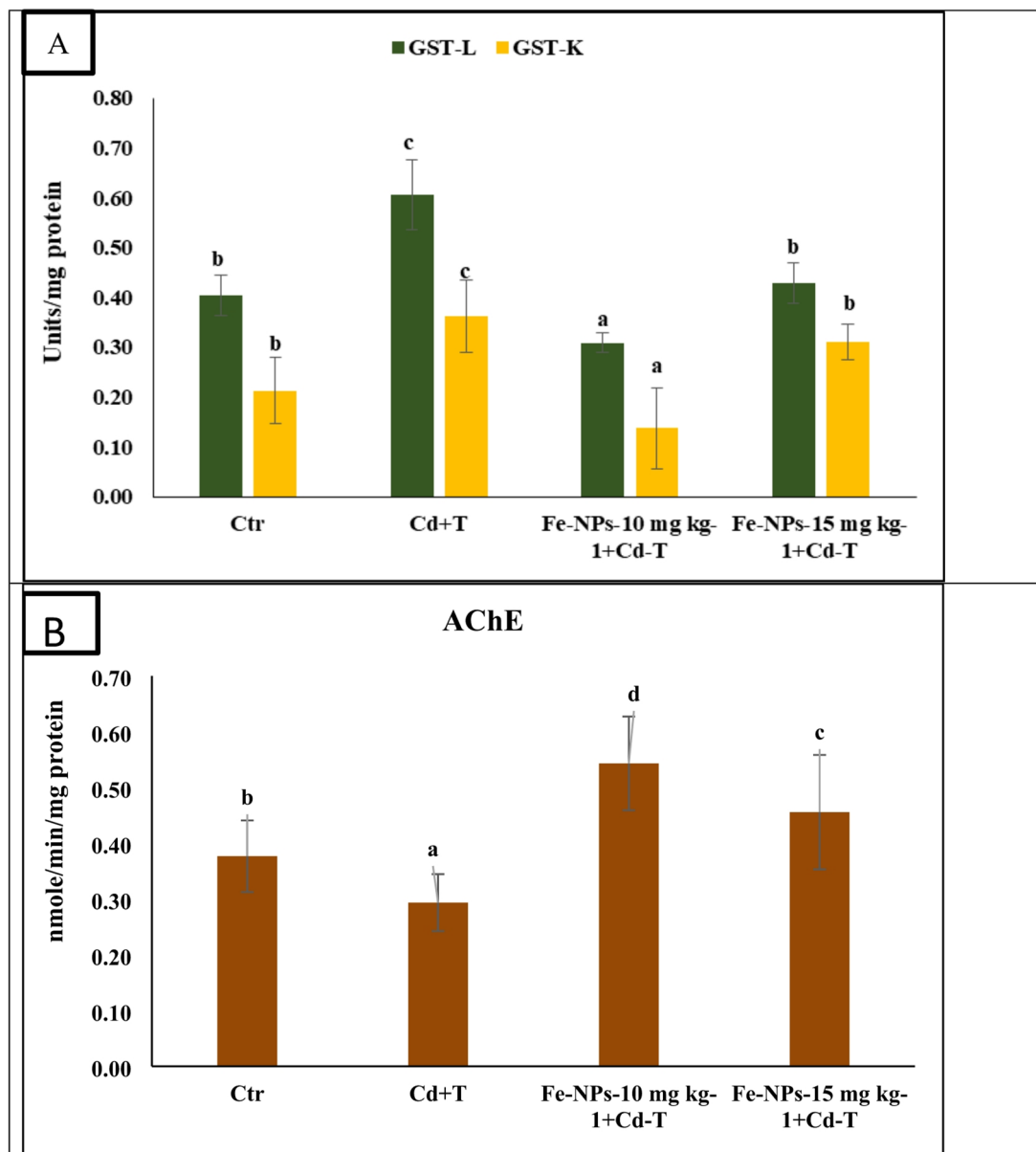
Figure 7A,B and 8 summarize the activities of ALT, AST, LDH, and MDH in the liver and gill tissues of *Pangasianodon hypophthalmus* reared under control conditions and subjected to combined cadmium and high temperature (Cd + T) stress for 40 days. The activity of ALT in the liver was significantly reduced ( $p=0.013$ ) in fish supplemented with Fe-NPs at 10 mg kg<sup>-1</sup> diet. Similarly, ALT activity in the gills was significantly lowered ( $p=0.011$ ) in groups receiving Fe-NPs at both 10 and 15 mg kg<sup>-1</sup> diets under stress conditions, compared to the control and Cd + T groups. AST activity in both liver ( $p=0.017$ ) and gill ( $p=0.001$ ) tissues was markedly elevated in fish exposed to Cd + T stress and fed a control diet. However, supplementation with Fe-NPs at 10 mg kg<sup>-1</sup> significantly reduced AST activity under stress conditions compared to other groups (Fig. 7A–B). In addition, LDH activity in liver ( $p=0.0015$ ) and gill ( $p=0.0011$ ) tissues was significantly decreased in the group fed Fe-NPs at 10 mg kg<sup>-1</sup> diet under stress conditions compared to the control and Cd + T groups. Similarly, MDH activity in the liver was significantly reduced with Fe-NPs supplementation at 10 mg kg<sup>-1</sup> diet. In gill tissue, MDH activity was significantly reduced ( $p=0.014$ ) in the group fed 10 mg kg<sup>-1</sup> Fe-NPs, followed by the 15 mg kg<sup>-1</sup>



**Fig. 5.** (A,B) Potential role of Fe-NPs diets on CAT, and SOD in liver and kidney tissues of fish reared under control or cadmium and high temperature stress for 40 days. Within endpoints and groups, bars with different superscripts differ significantly (a–d). Data expressed as Mean  $\pm$  SE ( $n=6$ ).

Fe-NPs group, compared to control and Cd + T-exposed groups. Notably, both LDH and MDH activities were significantly elevated in fish concurrently exposed to Cd + T and fed a control diet, highlighting the detrimental effect of the combined stress (Fig. 8).

The activities of protein metabolic enzymes such as ALT and AST in liver and gill tissues were elevated under concurrent exposure to cadmium and high temperature (Cd + T) stress. This elevation may be attributed to disruptions in amino acid metabolism<sup>82</sup>. Increased levels of ALT and AST are recognized indicators of tissue damage, particularly in the liver<sup>83,84</sup>. Additionally, the elevated activities of LDH and MDH observed under Cd + T stress suggest increased physiological stress in fish, often associated with reduced oxygen availability<sup>85</sup>. Under such stress, anaerobic metabolic pathways become more active, leading to elevated lactate concentrations in tissues<sup>85,86</sup>. The increased LDH activity may also reflect metabolic disturbances and reduce nutrient conversion efficiency. Interestingly, dietary supplementation with Fe-NPs significantly reduced ALT and AST activities, suggesting a protective role against Cd + T induced stress. This reduction could be due to the ability of Fe-NPs to mitigate cellular degeneration and necrosis<sup>87</sup>. Iron plays an essential role in maintaining fish health and enhancing resistance to environmental stressors<sup>88</sup>. Moreover, Fe-NPs improve antioxidant capacity, which helps scavenge harmful free radicals and alleviates oxidative damage, thereby protecting liver function and enhancing immune response in fish exposed to Cd + T stress<sup>89</sup>.

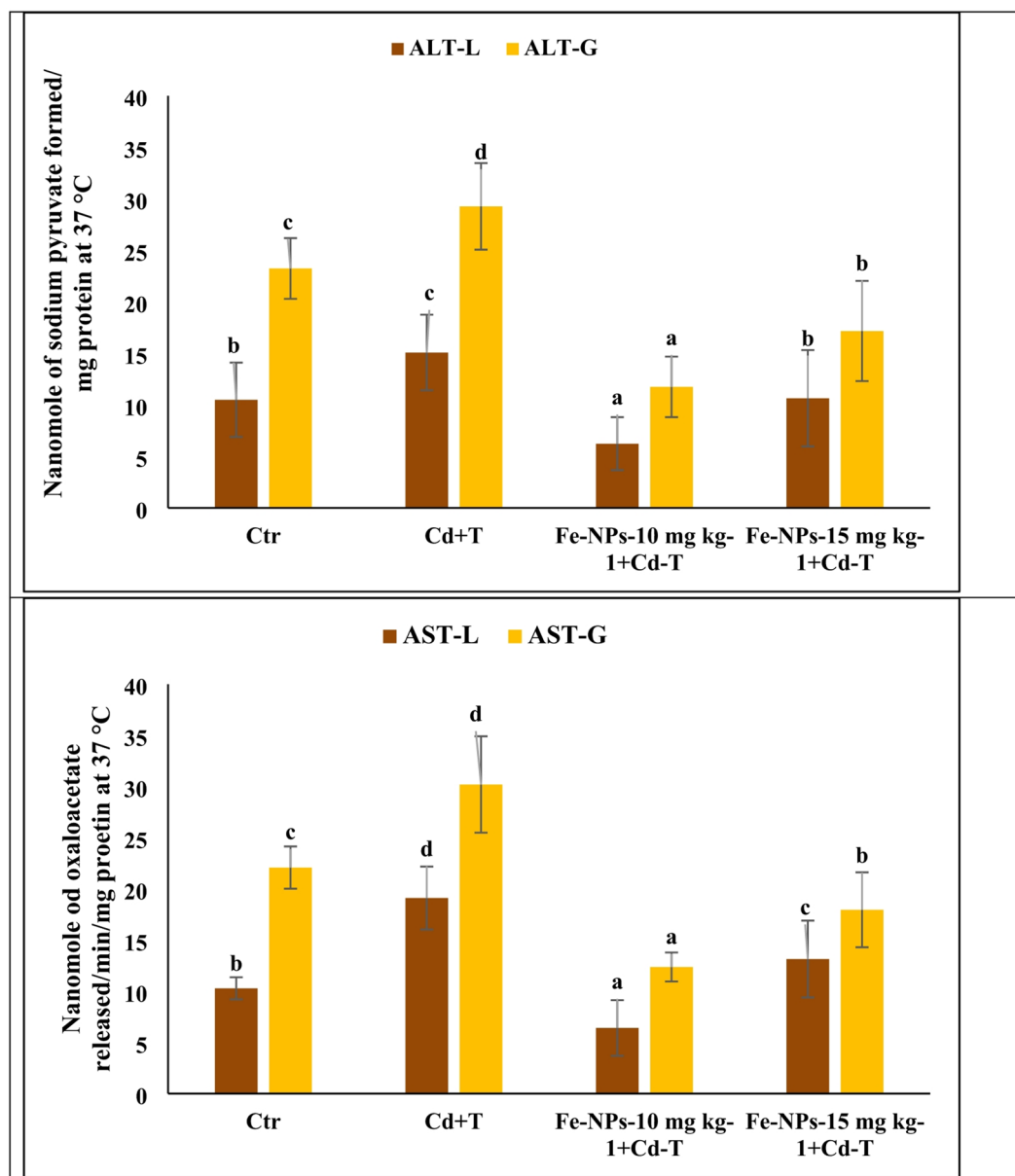


**Fig. 6.** (A,B) Potential role of Fe-NPs diets on GST in liver and kidney tissues and AChE in brain of fish reared under control or cadmium and high temperature stress for 40 days. Within endpoints and groups, bars with different superscripts differ significantly (a–d). Data expressed as Mean  $\pm$  SE ( $n=6$ ).

### Effect of Fe-NPs on bioaccumulation of cd in water and fish tissues

The concentration of cadmium (Cd) in the experimental water samples ranged from  $0.2 \mu\text{g L}^{-1}$  in the control group to  $1480 \mu\text{g L}^{-1}$  in the group concurrently exposed to Cd and high temperature (Cd + T) and fed a control diet. In comparison, the Cd concentrations were reduced to  $236 \mu\text{g L}^{-1}$  and  $210 \mu\text{g L}^{-1}$  in the groups supplemented with Fe-NPs at 10 and 15  $\text{mg kg}^{-1}$  diet, respectively, under Cd + T stress. Similarly, Cd bioaccumulation in muscle, liver, gill, kidney, and brain tissues was assessed at the end of the 40-day experimental period. The highest Cd accumulation was observed in the kidney ( $14.11 \text{ mg kg}^{-1}$ ) and liver ( $12.52 \text{ mg kg}^{-1}$ ), followed by gill ( $6.15 \text{ mg kg}^{-1}$ ), muscle ( $1.35 \text{ mg kg}^{-1}$ ), and brain ( $0.65 \text{ mg kg}^{-1}$ ) tissues. Notably, dietary supplementation with Fe-NPs at both 10 and 15  $\text{mg kg}^{-1}$  significantly reduced Cd bioaccumulation across all examined tissues (Table 5).

Cadmium (Cd) bioaccumulation in various fish tissues such as liver, gill, muscle, kidney, and brain reflect the extent of metal bioavailability in the aquatic environment<sup>90</sup>. In the present study, Cd accumulation was notably higher in the liver and kidney, indicating that the rate of Cd uptake exceeded its elimination, consistent with earlier findings<sup>91</sup>. This accumulation pattern also corresponded with reduced digestive enzyme activity in the intestine, suggesting that Cd toxicity impairs gastrointestinal function and results in a “spill-over” effect, thereby exacerbating systemic toxicity<sup>92</sup>. Cd uptake in gill tissue occurs primarily via passive diffusion and subsequent binding to metallothionein-like proteins (MTLPs), which serve a protective role<sup>93</sup>. Furthermore, fish may detoxify Cd through conversion into less biologically active forms via endogenous detoxification

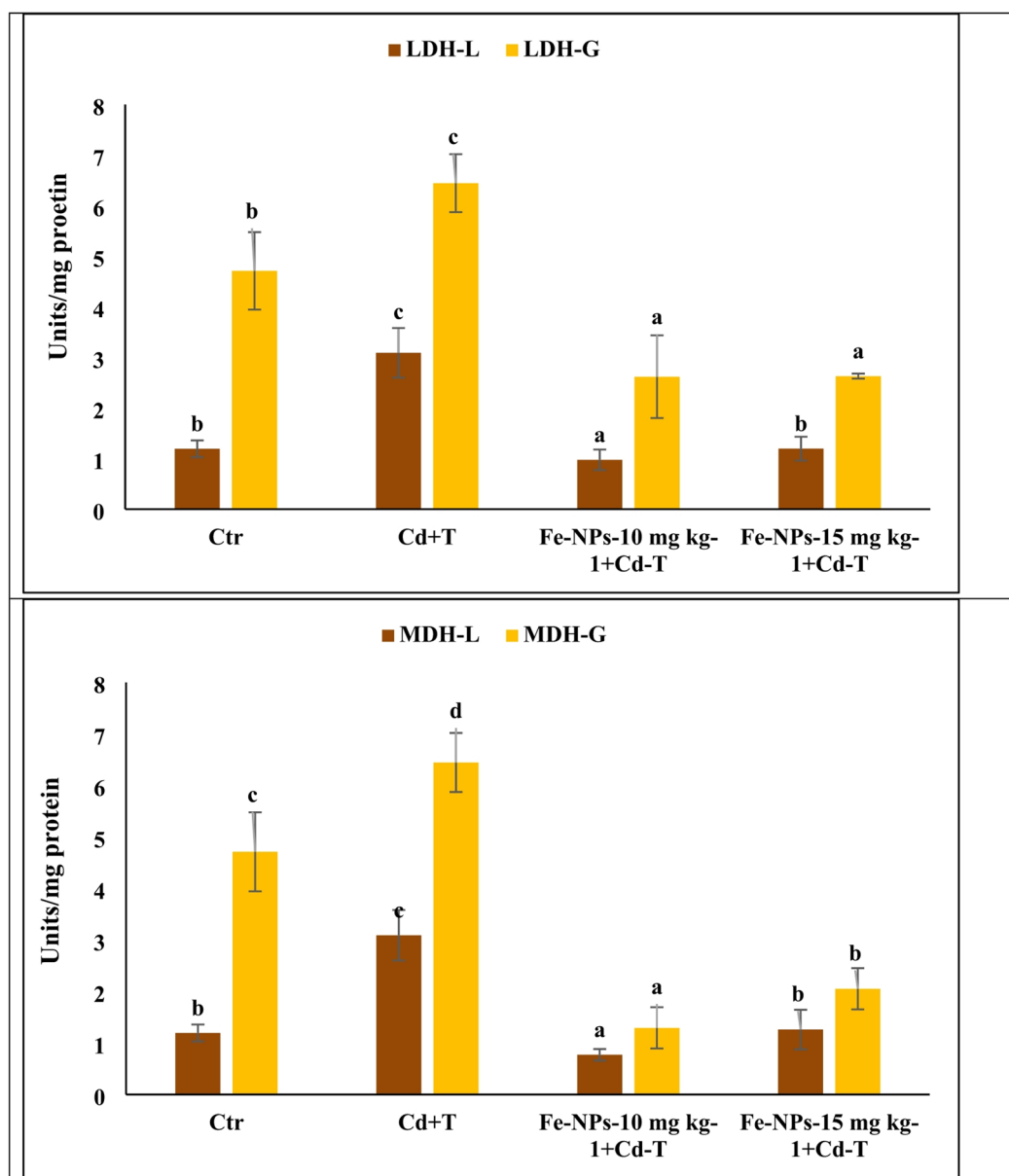


**Fig. 7.** (A,B) Potential role of Fe-NPs diets on ALT and AST in liver and gill tissues of fish reared under control or cadmium and high temperature stress for 40 days. Within endpoints and groups, bars with different superscripts differ significantly (a–d). Data expressed as Mean  $\pm$  SE ( $n = 6$ ).

pathways<sup>94</sup>. Remarkably, dietary supplementation with Fe-NPs effectively reduced Cd accumulation across all tissues, highlighting their potential role in enhancing metal detoxification. To the best of our knowledge, this is the first report demonstrating a significant reduction in Cd bioaccumulation in multiple tissues of fish through the dietary inclusion of Fe-NPs.

## Conclusion

The present investigation addresses pressing environmental concerns such as cadmium pollution and high temperature stress. Dietary supplementation with iron-nanoparticles (Fe-NPs) showed promising results for mitigation of cadmium and high temperature stress in fish. Fe-NPs containing diets also modulate stress-responsive gene expression, thereby enhancing the resilience of fish to these environmental challenges. Specifically, Fe-NPs supplementation at 10 mg kg<sup>-1</sup> was effective in promoting cadmium detoxification across various fish tissues. Moreover, Fe-NPs at this level significantly improved the expression of genes associated with growth performance, antioxidant defense, immune response, and apoptosis regulation. Collectively, these findings highlight the potential of Fe-NPs supplementation as a strategic intervention to bolster fish health and performance under the compounded stressors of pollution and climate change.



**Fig. 8.** (A,B) Potential role of Fe-NPs diets on LDH and MDH in liver and gill tissues of fish reared under control or cadmium and high temperature stress for 40 days. Within endpoints and groups, bars with different superscripts differ significantly (a–d). Data expressed as Mean  $\pm$  SE ( $n = 6$ ).

Treatments	Control	Cd + T	Fe-NPs-10 mg kg <sup>-1</sup> -Cd + T	Fe-NPs-15 mg kg <sup>-1</sup> -Cd + T
Water ( $\mu\text{g L}^{-1}$ )	0.2 $\pm$ 0.03	1480.40 $\pm$ 4.21	236.25 $\pm$ 1.08	210.24 $\pm$ 1.05
Muscle ( $\text{mg L}^{-1}$ )	ND	1.35 $\pm$ 0.28	0.14 $\pm$ 0.01	0.11 $\pm$ 0.02
Liver ( $\text{mg L}^{-1}$ )	0.11 $\pm$ 0.02	12.52 $\pm$ 0.31	4.28 $\pm$ 0.14	3.45 $\pm$ 0.04
Kidney ( $\text{mg L}^{-1}$ )	0.25 $\pm$ 0.01	14.11 $\pm$ 0.14	5.22 $\pm$ 0.18	5.26 $\pm$ 0.19
Gill ( $\text{mg L}^{-1}$ )	0.12 $\pm$ 0.03	6.15 $\pm$ 0.16	1.01 $\pm$ 0.01	1.02 $\pm$ 0.13
Brain ( $\text{mg L}^{-1}$ )	ND	0.65 $\pm$ 0.01	0.02 $\pm$ 0.001	0.01 $\pm$ 0.003

**Table 5.** Effect of dietary Fe-NPs on cadmium bioaccumulation in water and different fish tissues of *Pangasianodon hypophthalmus* reared under control or cadmium and high temperature for 40 days. Data expressed as Mean  $\pm$  SE ( $n = 5$ ).

## Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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## Author contributions

Authors Name	Contribution	Raveena Rajpurohit	Conduct the experiment	Neeraj Kumar	Conceived and designed the experiments; performed the experiments; analyzed the data; contributed reagents/materials/analysis tools; wrote the paper
		Kalpna Chandra	more Resources	Prem Kumar	Data Validation
		Ko-tha Sammi Reddy	Supervision.		

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## Declarations

## Competing interests

The authors declare no competing interests.

## Ethics approval

The fish experimental facilities are registered with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CCSEA), under registration number 2190/GO/RRBi/SL/2022. All experimental procedures were approved by the Institute Research Committee (IRC) and were conducted in strict accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

## Additional information

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