Original article DOI: 10.2478/aiht-2024-75-3826

The effects of iron oxide nanoparticles on antioxidant capacity and response to oxidative stress in Mozambique tilapia (*Oreochromis mossambicus*, Peters 1852)

Puthan Variyam Vidya Balakrishnan[1], Goran Gajski[2], and Kumari Chidambaran Chitra[1]

[1] University of Calicut, Department of Zoology, Endocrinology and Toxicology Laboratory, Malappuram, India
[2] Institute for Medical Research and Occupational Health, Division of Toxicology, Zagreb, Croatia

[Received in February 2024; Similarity Check in February 2024; Accepted in May 2024]

Recent research has raised concern about the biocompatibility of iron oxide nanoparticles (IONPs), as they have been reported to induce oxidative stress and inflammatory responses, whilst prolonged exposure to high IONP concentrations may lead to cyto-/genotoxicity. Besides, there is concern about its environmental impact. The aim of our study was to investigate the effects of IONPs on the antioxidant defence system in freshwater fish Mozambique tilapia (*Oreochromis mossambicus,* Peters 1852). The fish were exposed to IONP concentration of 15 mg/L over 1, 3, 4, 15, 30, and 60 days and the findings compared to a control, unexposed group. In addition, we followed up the fish for 60 days after exposure had stopped to estimate the stability of oxidative stress induced by IONPs. Exposure affected the activity of antioxidant and marker enzymes and increased the levels of hydrogen peroxide and lipid peroxidation in the gill, liver, and brain tissues of the fish. Even after 60 days of depuration, adverse effects remained, indicating long-term nanotoxicity. Moreover, IONPs accumulated in the gill, liver, and brain tissues. Our findings underscore the potential health risks posed to non-target organisms in the environment, and it is imperative to establish appropriate guidelines for safe handling and disposal of IONPs to protect the aquatic environment.

KEY WORDS: antioxidant enzymes; bioaccumulation; environmental health; oxidative stress; toxicity

Iron oxide nanoparticles (IONPs) are usually composed of magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3). They are highly adsorptive due to small size and large surface area-to-volume ratio and have magnetic properties, which make them useful in numerous applications such as biomedicine, environmental remediation, magnetic storage and recording media, catalysis, and magnetic fluids. They can be synthesised using various methods, including co-precipitation, thermal decomposition, sol-gel synthesis, and microemulsion techniques, which can tune IONP properties to specific application by changing their size, shape, surface chemistry, and crystalline structure during synthesis. Although they are characterised by low toxicity and cost, they may still be toxic and entail environmental risks, especially with biomedical and environmental applications (1–9).

Such concerns are well grounded due to their widespread use and potential release into the environment. IONPs can easily reach air, water, and soil thanks to small size and high surface area-to-volume ratio, which enhance their mobility and persistence in the environment, leading to potential long-term exposure and accumulation in various environmental compartments. One of the key environmental hazards associated with IONPs is their toxicity to aquatic organisms, including fish, algae, and other aquatic life, with adverse effects including impaired growth, reproduction, and survival of organisms exposed to high concentrations. Besides, they have the potential to bioaccumulate in aquatic organisms through ingestion or absorption and to biomagnify, which means that IONP concentrations increase as they move up the food chain, posing higher risks to higher trophic levels, including humans (4, 10–15). Moreover, IONPs may interact with other pollutants or environmental stressors, leading to synergistic or antagonistic effects on ecosystems (10, 16–18).

The profound effect of IONPs on haematology, ion regulation, and gill Na/K ATPase activity has been demonstrated in the Indian major carp (*Labeo rohita)* (19), while other studies have reported oxidative stress and genetic damage in Japanese medaka embryos (*Oryzias latipes*) (20), zebrafish (*Danio rerio*) (21), and rainbow trout (*Oncorhynchus mykiss*) (22).

Fish tissues contain a large amount of polyunsaturated fatty acids, which are highly vulnerable to oxidative stress, followed by tissue damage. Oxidative balance in cells or tissues is maintained by a well-equipped prooxidant/antioxidant defence system that scavenges free radicals, yet oxidative stress varies between tissues and may affect them differently.

Despite considerable research of IONP toxicity in fish, there are still knowledge gaps concerning their long-term adverse effects. Therefore, one aim of this study was to investigate the effects of IONPs on antioxidant defence and susceptibility to oxidative stress in the gill, liver, and brain tissues, and whether the nanoparticles get

Corresponding author: Kumari Chidambaran Chitra, University of Calicut, Department of Zoology, Endocrinology and Toxicology Laboratory, Malappuram, India, E-mail: *kcchitra@yahoo.com*, ORCID: 0000-0002-5090-8709

accumulated inside these tissues of the juvenile Mozambique tilapia (*Oreochromis mossambicus*, Peters 1852). Our second aim was to estimate long-term (60 day) presence of oxidative stress induced by IONPs, once the exposure has stopped, and consequent tissue damage using specific marker enzymes.

MATERIALS AND METHODS

Test organism and exposure conditions

For this study we used the juvenile Mozambique tilapia (*Oreochromis mossambicus,* Peters 1852), weighing 6.0±1.5 g and measuring 6.5±1.0 cm in average, obtained from the Safa Aquarium (Kozhikode, India). During the two-week acclimatisation period, a total of 160 fish were housed in a 180 L cement tank under a 12-hour light/dark cycle, where they were provided with a continuous supply of dechlorinated tap water. The fish were fed standard commercial pellets (Taiyo, Uthukkottai, India) on a daily basis, which contained the appropriate amount of nutrients including 41 % crude protein, 6 % crude lipids, 7 % crude fibre, 10 % carbohydrate, 15 % ash, <8.5 % phosphorus, <1.5 % water, and trace amounts of vitamins and minerals. Their health status was monitored throughout the experiment following the guidelines set forth by the Indian Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (23).

Optimal physico-chemical characteristics of the tap water were maintained and monitored in line with the standard methods published by the American Public Health Association (APHA) (24) and the OECD guideline on acidity and alkalinity (25) as follows: temperature 28±2 °C, pH 7.0±0.5, dissolved oxygen 8.6±0.6 mg/L, water hardness 48 mg/L calcium carbonate, total organic carbon 0.04 mg/L, non-ionised ammonia 0.01 mg/L, nitrate 10 mg/L, chlorine 4 mg/L, metallic impurities <1 mg/L, and chemical oxygen demand 3 mg/L. Water analyses were done to ensure that water did not affect the experimental outcomes.

Characterisation of the test chemical

Preparation and characterisation of IONPs used in this study has been described in detail in our previous publications (26, 27). Briefly, IONPs (Fe_3O_4-NPs; Cat. No. 637106) were obtained from Sigma (Darmstadt, Germany). X-ray diffraction (XRD; Rigaku Miniflex, Tokyo, Japan) and transmission electron microscopy (TEM; Jeol/JEM, Tokyo, Japan) were performed to confirm the size and purity of IONPs. IONPs were suspended in double-distilled water and sonicated in an ultrasonic bath (GT Sonic, Guangdong, China) at a frequency of 100 kHz for 30 min to ensure uniformity. All the other chemicals used were of analytical grade and purchased from Himedia (Thane, India).

Selection of test concentration and grouping

The test concentration was selected according to IONP dispersion, as mentioned in our previous publication (27). Following the acclimatisation phase, 160 fish were randomly distributed into eight 40-litre glass tanks (30 cm width, 60 cm length, and 30 cm depth), each holding 20 fish. The control group was not exposed to IONPs. Treatment groups were exposed to IONPs at 15 mg/L for 1, 3, 4, 15, 30, and 60 days (IONP1, 3, 4, 15, 30, and 60, respectively). The depuration group was first exposed to IONPs (15 mg/L) for 60 days, and then followed up in IONP-free water for another 60 days.

Determination of the bioaccumulation factor (BAF)

IONP tissue accumulation was analysed in fish groups treated for four days (IONP4), 60 days (IONP60), and in the 60-day depuration group using a method described by Arslan et al. (28) with slight modifications. Fish from each group were captured with a small dip net to avoid additional stress, euthanised, and dissected. Gill, liver, and brain were excised and rinsed with cold saline before weighing. Wet tissue samples were weighed and digested with a mixture of concentrated nitric and hydrochloric acid (3:1) at 200 °C for 1 h, and the digested material was evaporated to remove any contaminants or residues that might interfere with analytical measurements. The remaining tissue was diluted with deionised water to a known volume. Total iron content, consisting of both iron nanoparticles and any other forms of iron within the samples, was quantified with inductively coupled plasma mass spectrometry (ICP-MS; Thermo Fisher, Waltham, MA, USA). The IONP content in total iron concentration was estimated based on the known molar mass of Fe_3O_4 and atomic mass, as follows:

$$Fe_3O_4\ mass = \frac{Fe\ concentration \times Fe_3O_4\ molar\ mass}{Fe\ atomic\ mass} \quad [1]$$

This conversion allowed us to determine the number of nanoparticles corresponding to the measured mass, which was used to calculate the bioaccumulation factor (BAF), expressed as micrograms per gram of wet tissue weight.

$$BAF = \frac{\text{IONP concentration in tissue } (\mu g/g)}{\text{IONP concentration in water } (mg/L)} \quad [2]$$

Preparation of tissue samples for biochemical analysis

At the end of every treatment, fish were captured as described above, weighed, euthanised, and dissected to remove the gill, liver, and brain. The tissues were then rinsed with cold saline and weighed. The hepatosomatic index (HSI) of the fish was calculated using the following equation:

$$HSI = \frac{\text{Liver weight } (mg)}{\text{Total body weight } (mg)} \times 100 \quad [3]$$

Tissue homogenates (1 % w/v) were prepared separately in normal saline on ice using a motor-driven tissue homogeniser (Remi, Mumbai, India) and the homogenates centrifuged at 800 *g* for 15 min at 4 °C to collect supernatants used for biochemical analyses. Total protein was estimated using bovine serum albumin (BSA) for the standard as described elsewhere (29). Activities of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and glutathione peroxidase (GPx) were analysed in the gill, liver, and brain tissues. Activities of marker enzymes alkaline phosphatase (ALP) in the gill and liver and acetylcholinesterase (AChE) in the brain were estimated as described below.

Superoxide dismutase (SOD) assay

SOD activity was determined as described by Marklund and Marklund (30). The assay mixture contained tris-hydrochloric acid buffer (50 mmol/L, pH 7.6) consisting of 1 mmol/L EDTA, 0.2 mmol/L pyrogallol, and 100 µL of enzyme source. The increase in absorbance was measured at 420 nm against an enzyme-free blank at 10-second intervals over 3 min using an ultraviolet (UV)-visible spectrophotometer (Shimadzu, Kyoto, Japan). Enzyme activity is expressed as nmol of oxidised pyrogallol per min per mg of protein.

Catalase (CAT) assay

CAT activity was determined using the method described by Claiborne (31). A total mixture of 3 mL was composed of phosphate buffer (50 mmol/L, pH 7.0), hydrogen peroxide (19 mmol/L), and 50 µL of enzyme source. The decrease in absorbance was measured at 240 nm against an enzyme-free blank at 10-second intervals over 3 min using a UV-visible spectrophotometer (Shimadzu, Kyoto, Japan). Enzyme activity was expressed as mol of hydrogen peroxide consumed per min per mg of protein.

Glutathione reductase (GR) assay

The activity of GR was assayed as described by Carlberg and Mannervik (32). The assay mixture comprised phosphate buffer (100 mmol/L, pH 7.6), NADPH (0.2 µmol/L), oxidised glutathione (2 mmol/L), EDTA (0.01 mol/L), and the enzyme source. The reduction of NADPH was monitored by measuring the decrease in absorbance at 340 nm against an enzyme-free blank at 10-second intervals over 3 min using a UV-visible spectrophotometer (Shimadzu). Enzyme activity is expressed as nmol of oxidised NADPH per min per mg of protein.

Glutathione peroxidase (GPx) assay

The activity of GPx was determined with the method described by Mohandas et al. (33) utilising hydrogen peroxide and NADPH as substrates. The assay mixture comprised phosphate buffer (100 mmol/L, pH 7.6), EDTA (0.01 mol/L), sodium azide, GR, reduced glutathione, and NADPH (0.2 µmol/L). Enzyme assay was added and NADPH reduction monitored by measuring the decrease in its absorbance at 340 nm against an enzyme-free blank at 10-second intervals over 3 min using a UV-visible spectrophotometer (Shimadzu). Enzyme activity is expressed as nmol of oxidised NADPH per min per mg of protein.

Quantification of hydrogen peroxide

The levels of hydrogen peroxide were assessed following the method described by Pick and Keisari (34). The assay is based on the H_2O_2-mediated and horseradish peroxidase-dependent oxidation of phenol red to a product. The incubation mixture comprised phosphate buffer (50 mmol/L, pH 7.6), horseradish peroxidase (8.5 units), phenol red (0.28 nmol/L), dextrose (5.5 nmol/L), and enzyme source (100 µL). The reaction was carried out at room temperature for 30 min, after which it was terminated by adding 10 eq/L sodium hydroxide. The absorbance was measured at 610 nm against the blank. A standard curve was prepared using known concentrations of H_2O_2, and the results are expressed as nmol of generated hydrogen peroxide per mg of protein.

Lipid peroxidation (LPO) assay

LPO was measured using thiobarbituric acid as outlined by Ohkawa et al. (35). A working solution was prepared by combining 15 % w/v trichloroacetic acid, 0.37 % 2-thiobarbituric acid, and 0.25 eq/L hydrochloric acid in a 1:1:1 ratio. The enzyme source was added to the working solution at a ratio of 1:2 and incubated in a boiling water bath for 10 min. Followed the measurement of absorbance at 535 nm against the blank. Malondialdehyde (MDA) solution served as the standard, and the results are expressed as nmol of MDA per mg of protein.

Alkaline phosphatase (ALP) assay

The activity of ALP in gill and liver tissues was determined following the method described by Bessey et al. (36). Pre-incubation involved *p*-nitrophenyl phosphate and glycine buffer at pH 10.5, maintained at 37 °C for 5 min. Subsequently, the sample and 0.02 eq/L NaOH were added to the mixture and incubated for 30 min. The resultant colour was measured at 420 nm using a UV-visible spectrophotometer (Shimadzu) against the blank. Upon the addition of 0.1 mL of concentrated hydrochloric acid, the mixture was thoroughly mixed and the difference in absorbance recorded as the measure of enzyme activity. The activity of ALP was determined from the calibration curve generated using the *p*-nitrophenol standard. Enzyme activity is expressed as µmol of liberated *p*-nitrophenol per min per mg of protein.

Acetylcholinesterase (AChE) assay

The activity of AChE in the brain tissue was determined as described by Ellman et al. (37) by monitoring the increase in yellow colour resulting from the reaction between thiocholine and dithiobisnitrobenzoate (DTNB; 0.01 mol/L). Brain tissue was

homogenised and dissolved in phosphate buffer (0.1 mol/L, pH 8.0), 15 mg of sodium bicarbonate, and DTNB. Enzyme activity is expressed as nmol of hydrolysed acetylthiocholine per min per mg of protein.

Statistical analyses

Statistical analyses were run on the IBM SPSS Statistics version 21.0 for Windows (SPSS Inc., Chicago, MI, USA). Differences between the means were determined with one-way analysis of variance (ANOVA), followed by Duncan's multiple range *post-hoc* test. Normality of distribution was tested with the Kolmogorov-Smirnov test and the homogeneity of variance between subsets analysed with Levene's test. The significance was set at $P<0.05$ vs control. Data are presented as means ± standard deviations (SD) for 20 animals per group.

RESULTS

Characterisation of iron oxide nanoparticles

The XRD results confirmed that IONPs used in our study were free from impurities. TEM analysis revealed a crystalline structure, with irregular and roughly symmetrical morphology, ranging in size from 1 to 100 nm. The average crystallite size calculated with the Scherrer equation was 15.65 nm. These results corroborate the manufacturer's specifications (Figure 1).

Body and tissue weights

Fish exposed to IONPs did not exhibit significant changes in body weight. However, gill weight increased while the hepatosomatic index decreased significantly ($P<0.05$) after 30 and 60 days of exposure (Table 1). Brain weight remained unchanged throughout the experiment. The hepatosomatic index returned to control levels in the depuration group, while body and organ weights remained unchanged (Table 1).

Bioaccumulation of nanoparticles in tissues

The ICP-MS analysis confirms the presence of IONPs in the gill, liver, and brain tissues of the fish after 4 and 60 days of exposure (Table 2). The gill tissue exhibited the highest bioaccumulation factor, followed by the liver and brain. The 60-day depuration failed to restore IONP levels to control.

Biochemical analysis

The activities of SOD (Figure 2), CAT (Figure 3), GR (Figure 4), and GPx (Figure 5) showed a significant ($P<0.05$) decrease in the gill, liver, and brain in a time-dependent manner compared to respective controls. Conversely, the levels of hydrogen peroxide (Figure 6) and LPO (Figure 7) increased significantly ($P<0.05$) in

Table 1 The Effect of iron oxide nanoparticles (IONPs; 15 mg/L) on mean (±SD) body and tissue weights of the fish Mozambique tilapia (*Oreochromis mossambicus,* Peters 1852) by groups (N=20 each)

Parameters	IONPs (15 mg/L)							Depuration group (60 days)
	Control	1 day	3 days	4 days	15 days	30 days	60 days	
Body weight (g)	6.71±0.09	6.25±0.40	6.31±0.35	6.51±0.26	6.59±0.38	6.47±0.38	6.63±0.40	6.99±0.13
Gill weight (mg)	143±11.9	146±3.25	152±3.88	152±1.19	159±3.20	**161±2.98***	**172±1.41***	**186±1.50***
Hepatosomatic index (%)	13.53±1.86	13.78±1.75	13.22±1.06	12.06±1.42	11.98±1.53	**11.90±1.75***	**9.84±1.26***	13.22±0.48
Brain weight (mg)	16.5±1.81	16.6±2.23	16.3±3.56	15.2±1.61	15.1±0.90	15.3±2.83	15.0±1.66	19.5±1.08

*P<0.05 compared to the control group

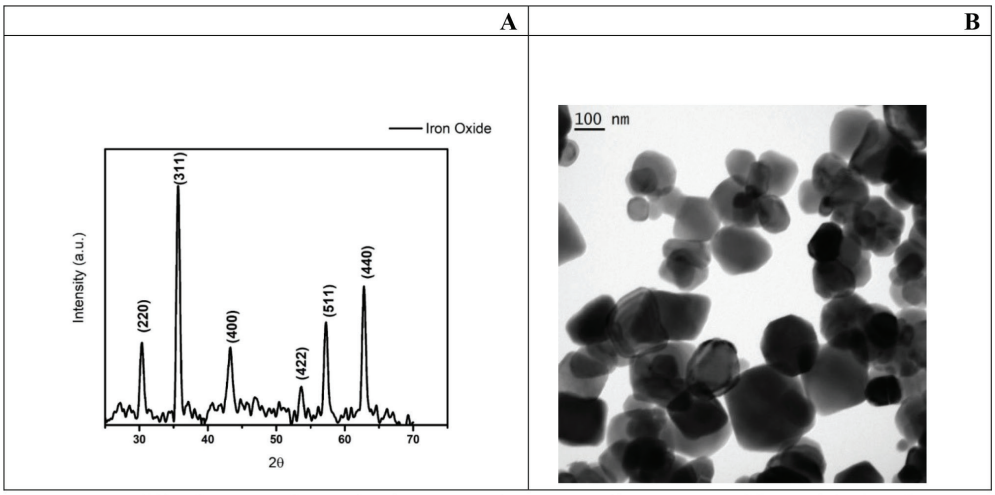

Figure 1 X-ray diffraction (XRD) image showing XRD peaks corresponding to IONPs with a particle size of 15.65 nm (A) and the transmission electron microscopy (TEM) image showing the morphology of IONP aggregates dispersed in double distilled water. Scale bar = 100 nm

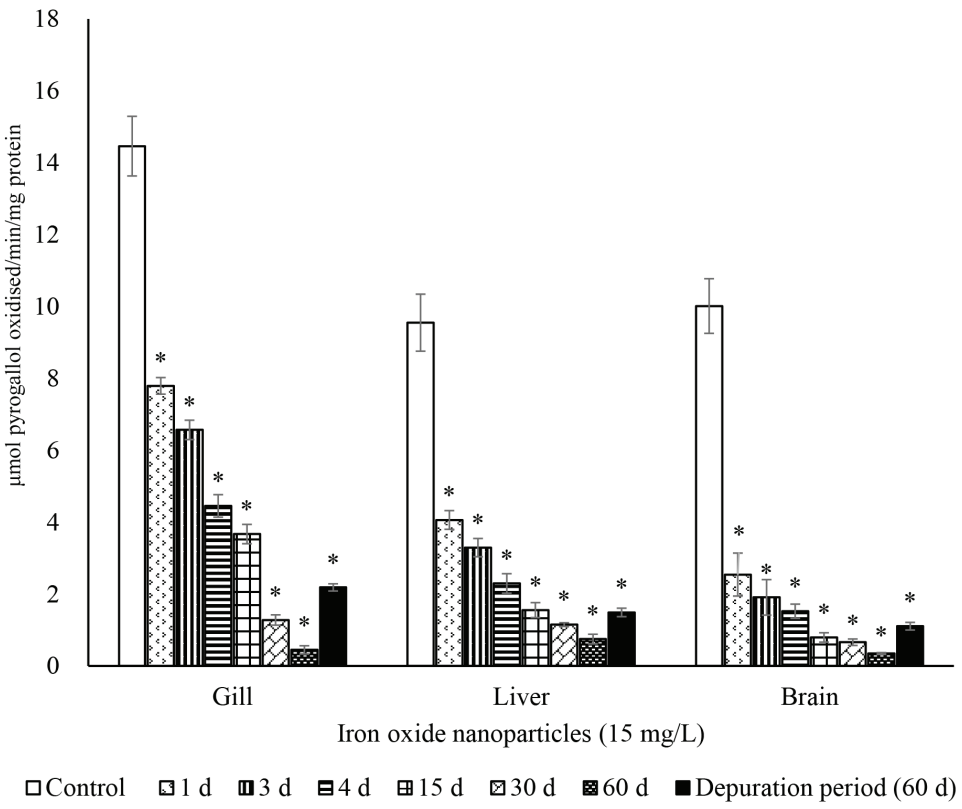

Figure 2 The effect of iron oxide nanoparticles (IONPs; 15 mg/L) on mean (±SD) activity of superoxide dismutase (SOD) in the fish Mozambique tilapia (*Oreochromis mossambicus*, Peters 1852) by groups (N=20 each). *P<0.05 vs control

Table 2 Mean (±SD) Bioaccumulation of iron oxide nanoparticles (IONPs; 15 mg/L) in the gill, liver, and brain tissues of the fish Mozambique tilapia (*Oreochromis mossambicus,* Peters 1852) exposed for 4 and 60 days, and fish followed up for 60 days after 60-day exposure had stopped (depuration period) (N=20 per group)

Concentration of IONPs in water (mg/L)	Exposure duration	Tissues	IONP tissue concentrations (µg/mg)	BAF
0 (Control)	60 days	Gill, liver, and brain	Below detection limit	Below detection limit
15	4 days	Gill	0.53±.017	0.04
		Liver	0.19±0.01	0.01
		Brain	0.15±0.01	0.01
	60 days	Gill	7.87±0.26	0.53
		Liver	6.25±0.13	0.42
		Brain	1.86±0.10	0.13
	Depuration (60 days)	Gill	2.20±0.08	0.15
		Liver	1.90±0.08	0.13
		Brain	0.92±0.13	0.06

BAF – bioaccumulation factor

all tissues in a time-dependent manner. Upon 60-day depuration, these levels did not return to control (Figures 2–7).

In both gill and liver, ALP activity dropped significantly ($P<0.05$) in a time-dependent manner. A similar significant and time-dependent drop was observed for brain tissue AChE activity. Again, 60-day depuration did not reverse the activities of these enzymes (Figure 8).

DISCUSSION

The accumulation of the IONPs in the gill, liver, and brain tissues of *Oreochromis mossambicus* determined in our study underscores their ability to easily enter the organism, accumulate in vital organs, and potentially alter their functions (38, 39). As reported by Ates et al. (40), who studied chronic exposure of tilapia (*Oreochromis niloticus*) to IONPs, the uptake, assimilation, and immunotoxic effect much depend on the morphological properties of IONPs. Small nanoparticle size facilitates penetration into organisms and subsequent accumulation in tissues, a phenomenon that has less been explored in aquatic bioaccumulation studies. It is crucial to understand whether nanoparticles directly incorporate into tissues, cells, and biomolecules, leading to toxicological responses, or undergo chemical changes and transformations that indirectly contribute to toxicity. In a previous study, we also reported histopathological changes induced by IONPs in these tissues of *Oreochromis mossambicus* (26). Our findings align with those of Murali et al. (41) on the accumulation of three different concentrations of aluminium oxide nanoparticles in the liver tissue resulting in severe histopathological changes.

Like all aerobic organisms, fish have well-defined and functional prooxidant/antioxidant systems to counter oxidative stress and the formation of reactive oxygen species (ROS) (42). In our study, exposure to IONPs resulted in depletion of antioxidant enzymes such as SOD, CAT, GR, and GPx in all tissues. This depletion resulted in the accumulation of free radicals, as evidenced by increased levels of hydrogen peroxide. Similar results have been observed in the gill and liver tissues of freshwater orange chromide (*Pseudetroplus maculatus*) upon exposure to fullerene C_{60} for 96 h (43, 44). Furthermore, the 60-day recovery from nanoparticles exposure (depuration period) did not significantly improve antioxidant enzyme activity or restore it to control levels, which suggests that these effects of nanotoxicity may be long-term. They may be owed to changes in vital protein conformations (45), irreversible cytoskeleton damage (46), or induced adsorption of nanoparticles by lipid bilayers as a result of lipophilicity of cationic ligands (47).

Nanoparticle toxicity is owed to oxygen depletion and accumulation of free radicals (48, 49). Lipids, polyunsaturated fatty acids (PUFA) in particular, possess several double bonds and are highly susceptible to free radical attacks. Exposure to IONPs for 60 days resulted in a significant rise in LPO levels in all three tissues of our test organism, which suggests that gill, liver, and brain are equally targeted by oxidative stress and corroborates our earlier findings of elevated LPO levels in the liver of the same species (50).

Considering that ALP is involved in the hydrolysis of exogenous materials, transphosphorylation, and membrane transport (51), its decreased activity in the gill and liver tissues points to impaired membrane transport and cellular toxicity (50).

The activity of the brain tissue marker, AChE, the key enzyme involved in the breakdown of the neurotransmitter acetylcholine to terminate synaptic neurotransmission (51), dropped in a time-dependent manner, indicating the neurotoxic effects of IONPs. The activities of these marker enzymes remained depleted throughout the depuration period, which corroborates long-term nanotoxic effects.

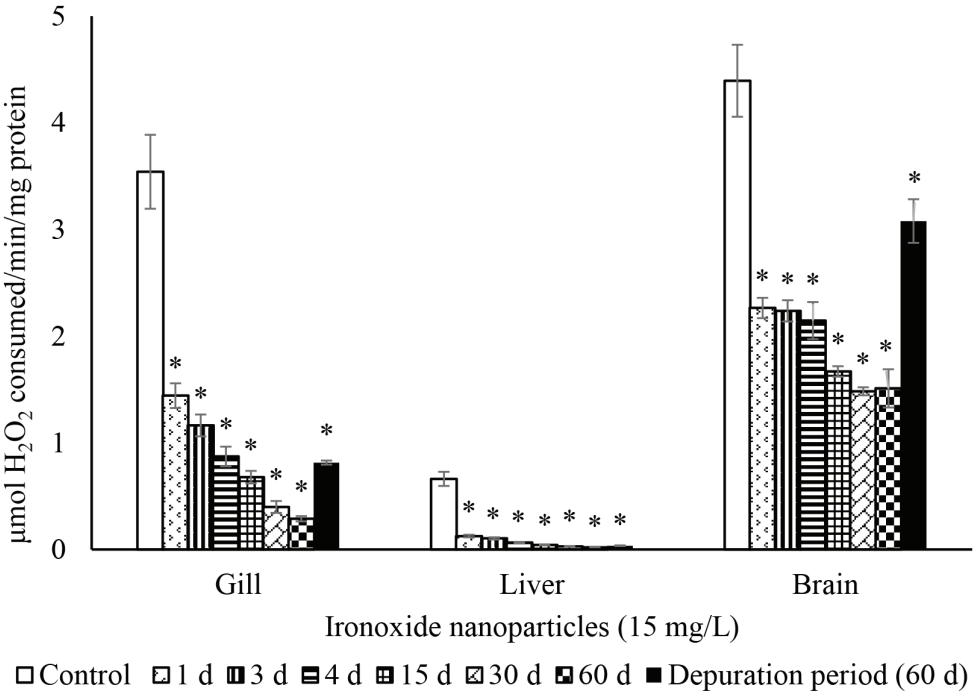

Figure 3 The effect of iron oxide nanoparticles (IONPs; 15 mg/L) on mean (±SD) activity of catalase (CAT) in the fish Mozambique tilapia (*Oreochromis mossambicus,* Peters 1852) by groups (N=20 each). *P<0.05 vs control

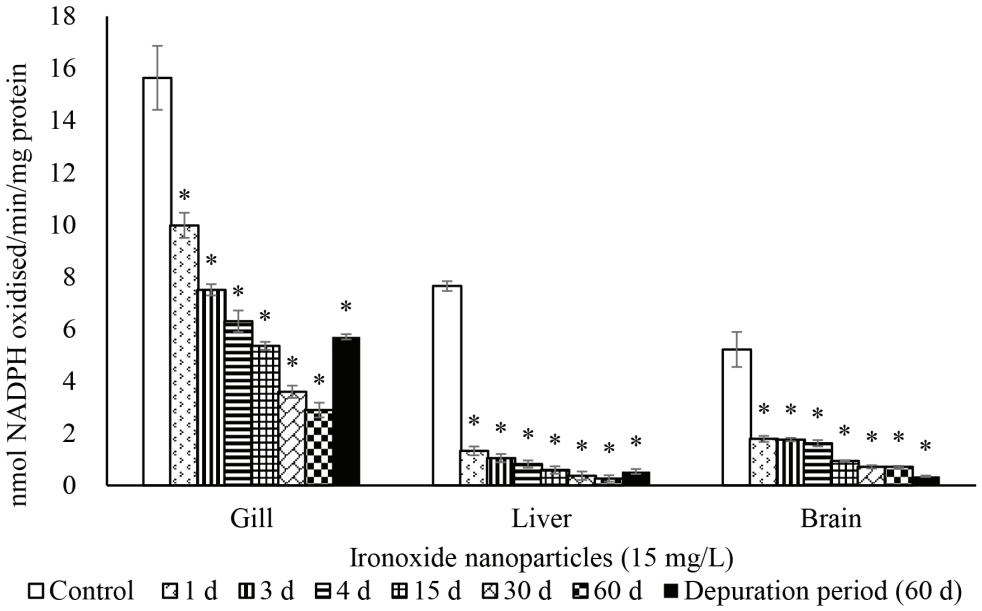

Figure 4 The effect of iron oxide nanoparticles (IONPs; 15 mg/L) on mean (±SD) activity of glutathione reductase (GR) in the fish Mozambique tilapia (*Oreochromis mossambicus,* Peters 1852) by groups (N=20 each). *P<0.05 vs control

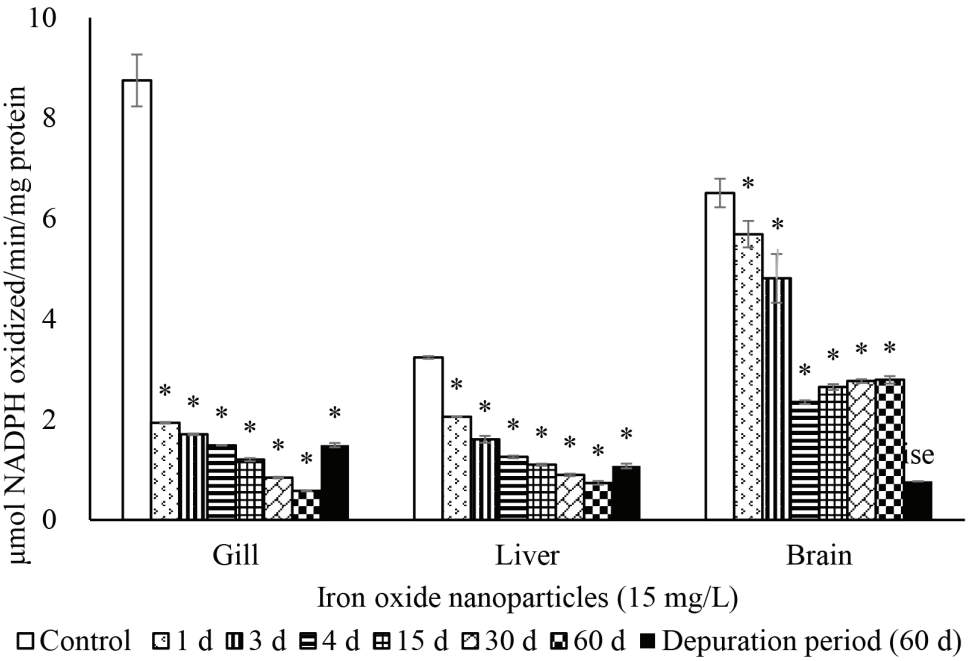

Figure 5 The effect of iron oxide nanoparticles (IONPs; 15 mg/L) on mean (±SD) activity of glutathione peroxidase (GPO) in the fish Mozambique tilapia (*Oreochromis mossambicus,* Peters 1852) by groups (N=20 each). *P<0.05 vs control

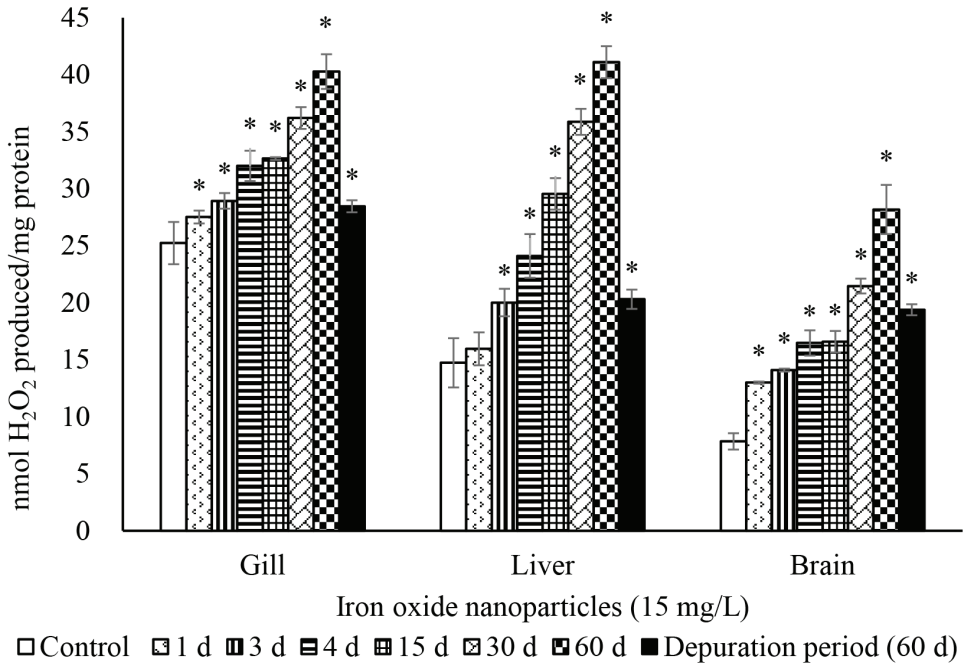

Figure 6 The effect of iron oxide nanoparticles (IONPs; 15 mg/L) on mean (±SD) mean (±SD) hydrogen peroxide levels in the fish Mozambique tilapia (*Oreochromis mossambicus,* Peters 1852) by groups (N=20 each). *P<0.05 vs control

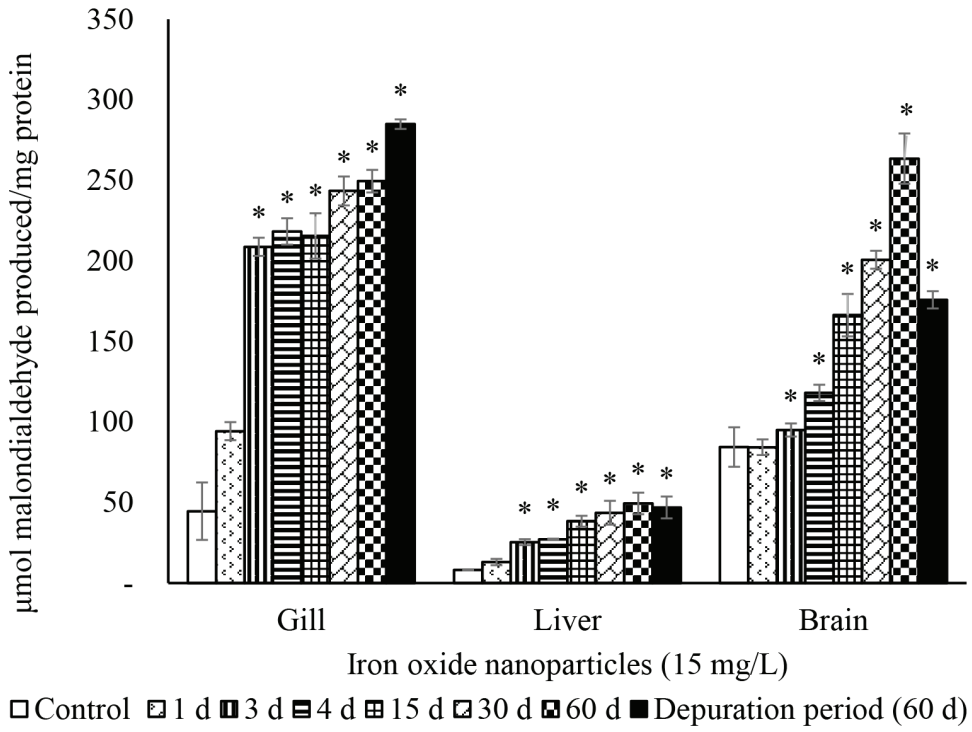

Figure 7 The effect of iron oxide nanoparticles (IONPs; 15 mg/L) on mean (±SD) lipid peroxidation (LPO) in the fish Mozambique tilapia (*Oreochromis mossambicus,* Peters 1852) by groups (N=20 each). *P<0.05 vs control

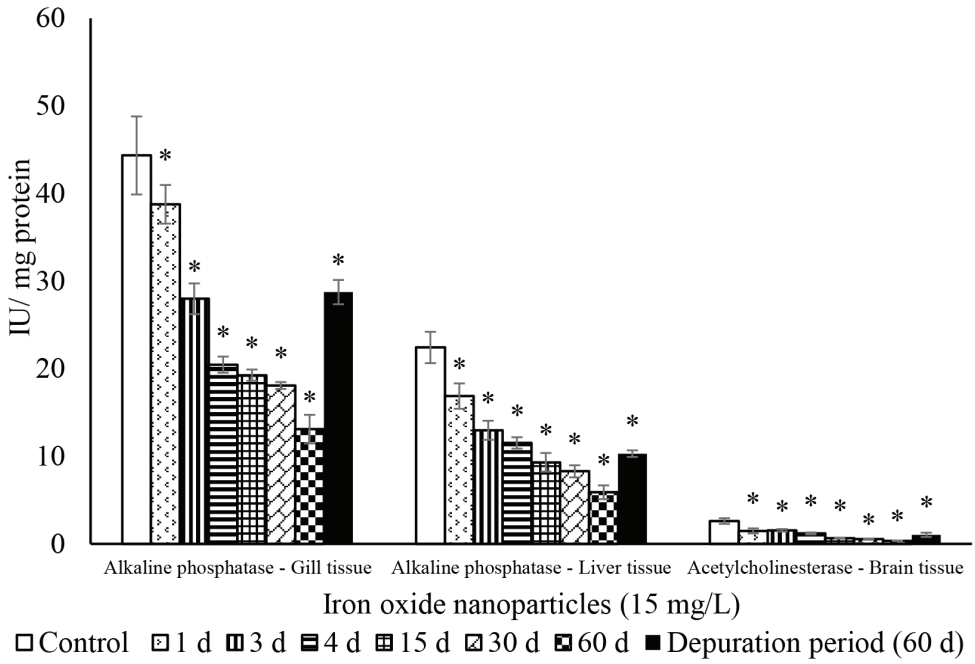

Figure 8 The effect of iron oxide nanoparticles (IONPs; 15 mg/L) on mean (±SD) activity of alkaline phosphatase (ALP) in the fish Mozambique tilapia (*Oreochromis mossambicus,* Peters 1852) by groups (N=20 each). *P<0.05 vs control

CONCLUSION

Clearly, oxidative stress plays a major role in nanoparticle-induced toxicity, and nanoparticles execute it through different mechanisms like generating ROS, disrupting cellular antioxidant defence system, interacting and damaging biomolecules like proteins, lipids, and DNA, as well as by disrupting membrane integrity (52–55). Our findings well illustrate the cascade of events and the relation between different mechanisms that could lead to oxidative stress. IONP accumulation in the gill, liver, and even brain tissue confirms their internalisation by fish and also the ability to cross the blood-brain barrier and induce neurotoxicity. Accumulation of IONPs inside vital organs disrupted the antioxidant defence system by depleting antioxidant enzymes, resulting in redox imbalance, which in turn induced LPO and likely impaired cell membrane integrity.

Our study also highlights the persistence of IONPs and their toxic effects in the fish even after the depuration period. We therefore need to consider long-term exposure to IONPs and their exacerbating toxicity over time, as it has various biological implications for humans. Even though human health risks resulting from occupational and environmental exposure to IONPs are negligible, *in vitro* studies on human cells such as blood (56), endothelial (57, 58), neuroblastoma and glioblastoma (59), alveolar epithelial cells (60), neutrophils (61), and astrocytes (62) point to the potential risks of IONP exposure and the need for caution before applying IONPs in clinical settings. However, further studies are warranted to shed more light on the nature IONP toxicity and its implications for humans.

Furthermore, our findings highlight the need for further investigations into the potential risks associated with nanoparticle exposure in aquatic environments.

Acknowledgments

We gratefully acknowledge the financial support provided by the University Grants Commission – Special Assistance Programme (UGC-SAP), Government of India.

Conflict of interests

None to declare.

REFERENCES

1. Noqta OA, Aziz AA, Usman IA, Bououdina M. Recent advances in iron oxide nanoparticles (IONPs): synthesis and surface modification for biomedical applications. J Supercond Nov Magn 2019;32:779–95. doi: 10.1007/s10948-018-4939-6
2. Jjagwe J, Olupot PW, Carrara S. Iron oxide nanoparticles/nanocomposites derived from steel and iron wastes for water treatment: A review. J Environ Manage 2023;343:118236. doi: 10.1016/J.JENVMAN.2023.118236
3. Baabu PRS, Kumar HK, Gumpu MB, Babu KJ, Kulandaisamy AJ, Rayappan JBB. Iron oxide nanoparticles: A Review on the province of its compounds, properties and biological applications. Materials (Basel) 2022;16(1):59. doi: 10.3390/ma16010059
4. Patiño-Ruiz DA, Meramo-Hurtado SI, González-Delgado ÁD, Herrera A. Environmental sustainability evaluation of iron oxide nanoparticles synthesized via green synthesis and the coprecipitation method: A comparative life cycle assessment study. ACS Omega 2021;6:12410–23. doi: 10.1021/acsomega.0c05246
5. Tai VC, Che HX, Kong XY, Ho KC, Ng WM. Decoding iron oxide nanoparticles from design and development to real world application in water remediation. J Ind Eng Chem 2023;127:82–100. doi: 10.1016/J.JIEC.2023.07.038
6. Oberdörster G, Oberdörster E, Oberdörster J. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ Health Perspect 2005;113:823–39. doi: 10.1289/ehp.7339
7. Piccinno F, Gottschalk F, Seeger S, Nowack B. Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world. J Nanoparticle Res 2012;14:1109. doi: 10.1007/s11051-012-1109-9
8. Zhang C, Liu T, Gao J, Su Y, Shi C. Recent development and application of magnetic nanoparticles for cell labeling and imaging. Mini Rev Med Chem 2010;10:194–203. doi: 10.2174/138955710791185073
9. Xuan L, Ju Z, Skonieczna M, Zhou P, Huang R. Nanoparticles-induced potential toxicity on human health: Applications, toxicity mechanisms, and evaluation models. MedComm 2023;4(4):e327. doi: 10.1002/mco2.327
10. Tao Z, Zhou Q, Zheng T, Mo F, Ouyang S. Iron oxide nanoparticles in the soil environment: Adsorption, transformation, and environmental risk. J Hazard Mater 2023;459:132107. doi: 10.1016/J.JHAZMAT.2023.132107
11. Caixeta MB, Araújo PS, Rodrigues CC, Gonçalves BB, Araújo OA, Bevilaqua GB, Malafaia G, Silva LD, Rocha TL. Risk assessment of iron oxide nanoparticles in an aquatic ecosystem: A case study on *Biomphalaria glabrata*. J Hazard Mater 2021;401:123398. doi: 10.1016/J.JHAZMAT.2020.123398
12. Cotin G, Piant S, Mertz D, Felder-Flesch D, Begin-Colin S. Iron oxide nanoparticles for biomedical applications: synthesis, functionalization, and application. In: Mahmoudi M, Laurent S, editors. Iron oxide nanoparticles biomedical application. Chapter 2. Amsterdam: Elsevier; 2018. p. 43–88. doi: 10.1016/B978-0-08-101925-2.00002-4
13. Mahmoudi M, Laurent S. Iron Oxide Nanoparticles for Biomedical Applications : Synthesis, Functionalization and Application. 1st ed. Imprint: Elsevier; 2017.
14. Cedervall T, Hansson L-A, Lard M, Frohm B, Linse S. Food chain transport of nanoparticles affects behaviour and fat metabolism in fish. PLoS One 2012;7:e32254. doi: 10.1371/journal.pone.0032254
15. Maharramov AM, Hasanova UA, Suleymanova IA, Osmanova GE, Hajiyeva NE. The engineered nanoparticles in food chain: potential toxicity and effects. SN Appl Sci 2019;1:1362. doi: 10.1007/s42452-019-1412-5
16. Forest V. Combined effects of nanoparticles and other environmental contaminants on human health - an issue often overlooked. NanoImpact 2021;23:100344. doi: 10.1016/J.IMPACT.2021.100344
17. Li M, Liu W, Slaveykova VI. Effects of mixtures of engineered nanoparticles and metallic pollutants on aquatic organisms. Environments 2020;7(4):27. doi: 10.3390/environments7040027

18. Zhang F, Wang Z, Peijnenburg WJGM, Vijver MG. Review and prospects on the ecotoxicity of mixtures of nanoparticles and hybrid nanomaterials. Environ Sci Technol 2022;56:15238–50. doi: 10.1021/acs.est.2c03333
19. Remya AS, Ramesh M, Saravanan M, Poopal RK, Bharathi S, Nataraj D. Iron oxide nanoparticles to an Indian major carp, *Labeo rohita*: Impacts on hematology, iono regulation and gill Na^+/K^+ ATPase activity. J King Saud Univ - Sci 2015;27:151–60. doi: 10.1016/J.JKSUS.2014.11.002
20. Li H, Zhou Q, Wu Y, Fu J, Wang T, Jiang G. Effects of waterborne nano-iron on medaka (*Oryzias latipes*): Antioxidant enzymatic activity, lipid peroxidation and histopathology. Ecotoxicol Environ Saf 2009;72:684–92. doi: 10.1016/J.ECOENV.2008.09.027
21. Villacis RAR, Filho JS, Piña B, Azevedo RB, Pic-Taylor A, Mazzeu JF, Grisolia CK. Integrated assessment of toxic effects of maghemite (γ-Fe_2O_3) nanoparticles in zebrafish. Aquat Toxicol 2017;191:219–25. doi: 10.1016/J.AQUATOX.2017.08.004
22. Ucar A, Parlak V, Ozgeris FB, Yeltekin AC, Arslan ME, Alak G, Turkez H, Kocaman EM, Atamanalp M. Magnetic nanoparticles-induced neurotoxicity and oxidative stress in brain of rainbow trout: Mitigation by ulexite through modulation of antioxidant, anti-inflammatory, and antiapoptotic activities. Sci Total Environ 2022;838:155718. doi: 10.1016/J.SCITOTENV.2022.155718
23. Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Guidelines of CPCSEA for Experimentation on Fishes [displayed 20 May 2024]. Available at https://ccsea.gov.in/WriteReadData/userfiles/file/Guidelines%20of%20CPCSEA%20for%20Experimentation%20on%20Fishes-2021.pdf
24. Rice EW, Baird RB, Eaton AD, editors. Standard Methods for the Examination of Water and Wastewater. 23rd ed. Washington (DC): American Public Health Association; 2017.
25. Organisation for Economic Co-operation and Development (OECD). Test No. 122: Determination of pH, Acidity and Alkalinity. Paris: OECD; 2013. doi: 10.1787/9789264203686-en
26. Vidya PV, Chitra KC. Irreversible histopathological modifications induced by iron oxide nanoparticles in the fish, *Oreochromis mossambicus* (Peters, 1852). Biol Forum-An Int J 2019;11:1–6.
27. Vidya PV, Chitra KC. Evaluation of genetic damage in *Oreochromis mossambicus* exposed to selected nanoparticles by using micronucleus and comet bioassays. Croat J Fish 2018;76:115–24. doi: 10.2478/cjf-2018-0015
28. Arslan Z, Ates M, McDuffy W, Agachan MS, Farah IO, Yu WW, Bednar AJ. Probing metabolic stability of CdSe nanoparticles: Alkaline extraction of free cadmium from liver and kidney samples of rats exposed to CdSe nanoparticles. J Hazard Mater 2011;192:192–9. doi: 10.1016/J.JHAZMAT.2011.05.003
29. Lowry O, Rosebrough N, Farr AL, Randall R. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75. doi: 10.1016/S0021-9258(19)52451-6
30. Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 1974;47:469–74. doi: 10.1111/j.1432-1033.1974.tb03714.x
31. Claiborne A. Catalase activity. In: Greenwald RA, editors. CRC Handbook of methods for oxygen radical research. Boca Raton (FL): CRC Press; 1985. p. 283–4.
32. Carlberg I, Mannervik B. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. J Biol Chem 1975;250:5475–80. doi: 10.1016/S0021-9258(19)41206-4
33. Mohandas J, Marshall JJ, Duggin GG, Horvath JS, Tiller DJ. Low activities of glutathione-related enzymes as factors in the genesis of urinary bladder cancer1. Cancer Res 1984;44:5086–91. PMID: 6149017
34. Pick E, Keisari Y. Superoxide anion and hydrogen peroxide production by chemically elicited peritoneal macrophages–induction by multiple nonphagocytic stimuli. Cell Immunol 1981;59:301–18. doi: 10.1016/0008-8749(81)90411-1
35. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351–8. doi: 10.1016/0003-2697(79)90738-3
36. Bessey OA, Lowry OH, Brock MJ. A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. J Biol Chem 1946;164:321–9. doi: 10.1016/S0021-9258(18)43072-4
37. Ellman GL, Courtney KD, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88–95. doi: 10.1016/0006-2952(61)90145-9
38. Ates M, Demir V, Adiguzel R, Arslan Z. Bioaccumulation, subacute toxicity, and tissue distribution of engineered titanium dioxide (TiO_2) nanoparticles in goldfish (*Carassius auratus*). J Nanomater 2013;2013:460518. doi: 10.1155/2013/460518
39. Kalman J, Connolly M, Abdolahpur-Monikh F, Fernández-Saavedra R, Cardona-García AI, Conde-Vilda E, Martínez-Morcillo S, Peijnenburg WJGM, Rucandio I, Fernández-Cruz ML. Bioaccumulation of CuO nanomaterials in rainbow trout: Influence of exposure route and particle shape. Chemosphere 2023;310:136894. doi: 10.1016/J.CHEMOSPHERE.2022.136894
40. Ates M, Demir V, Arslan Z, Kaya H, Yılmaz S, Camas M. Chronic exposure of tilapia (*Oreochromis niloticus*) to iron oxide nanoparticles: Effects of particle morphology on accumulation, elimination, hematology and immune responses. Aquat Toxicol 2016;177:22–32. doi: 10.1016/J.AQUATOX.2016.05.005
41. Murali M, Suganthi P, Athif P, Sadiq Bukhari A, Syed Mohamed HE, Basu H, Singhal RK. Histological alterations in the hepatic tissues of Al_2O_3 nanoparticles exposed freshwater fish *Oreochromis mossambicus*. J Trace Elem Med Biol 2017;44:125–31. doi: 10.1016/J.JTEMB.2017.07.001
42. Martínez-Álvarez RM, Morales AE, Sanz A. Antioxidant defenses in fish: biotic and abiotic factors. Rev Fish Biol Fish 2005;15:75–88. doi: 10.1007/s11160-005-7846-4
43. Sumi N, Chitra K. Effects of fullerene (C_{60}) on antioxidant enzyme activities and lipid peroxidation in gill of the cichlid fish, *Pseudetroplus maculatus* (Bloch, 1795). J Zool Stud 2016;3:31–7.
44. Sumi N, Chitra K. Acute exposure to fullerene (C_{60}) altered antioxidant defence system in hepatocytes of the cichlid fish, *Pseudetroplus maculatus*. Int J Res 2017;4:953–62.
45. Mahmoudi M, Shokrgozar MA, Sardari S, Moghadam MK, Vali H, Laurent S, Stroeve P. Irreversible changes in protein conformation due to interaction with superparamagnetic iron oxide nanoparticles. Nanoscale 2011;3:1127–38. doi: 10.1039/c0nr00733a
46. Déciga-Alcaraz A, Delgado-Buenrostro NL, Ispanixtlahuatl-Meráz O, Freyre-Fonseca V, Flores-Flores JO, Ganem-Rondero A, Vaca-Paniagua F, Pilar Ramos-Godinez M del, Morales-Barcenas R, Sánchez-Pérez Y, García-Cuéllar CM, Chirino YI. Irreversible disruption of

the cytoskeleton as induced by non-cytotoxic exposure to titanium dioxide nanoparticles in lung epithelial cells. Chem Biol Interact 2020;323:109063. doi: 10.1016/J.CBI.2020.109063
47. Lochbaum CA, Chew AK, Zhang X, Rotello V, Van Lehn RC, Pedersen JA. Lipophilicity of cationic ligands promotes irreversible adsorption of nanoparticles to lipid bilayers. ACS Nano 2021;15:6562–72. doi: 10.1021/acsnano.0c09732
48. Federici G, Shaw BJ, Handy RD. Toxicity of titanium dioxide nanoparticles to rainbow trout (*Oncorhynchus mykiss*): gill injury, oxidative stress, and other physiological effects. Aquat Toxicol 2007;84:415–30. doi: 10.1016/J.AQUATOX.2007.07.009
49. Afifi M, Saddick S, Abu Zinada OA. Toxicity of silver nanoparticles on the brain of *Oreochromis niloticus* and *Tilapia zillii*. Saudi J Biol Sci 2016;23:754–60. doi: 10.1016/J.SJBS.2016.06.008
50. Vidya PV, Chitra KC. Elevation of reactive oxygen species in hepatocytes of tilapian fish when exposed to silicon dioxide: a potential environmental impact of nanomaterial. Int J Recent Sci Res 2015;6:2990–5.
51. Green MR, Sambrook J. Alkaline phosphatase. Cold Spring Harb Protoc 2020;2020:pdb.top100768. doi: 10.1101/pdb.top100768
52. Temiz Ö, Kargın F. Toxicological impacts on antioxidant responses, stress protein, and genotoxicity parameters of aluminum oxide nanoparticles in the liver of *Oreochromis niloticus*. Biol Trace Elem Res 2022;200:1339–46. doi: 10.1007/s12011-021-02723-0
53. Khan GB, Akhtar N, Khan MF, Ullah Z, Tabassum S, Tedesse Z. Toxicological impact of Zinc Nano Particles on tilapia fish (*Oreochromis mossambicus*). Saudi J Biol Sci 2022;29:1221–6. doi: 10.1016/J.SJBS.2021.09.044
54. Wei X, Jiang W, Yu J, Ding L, Hu J, Jiang G. Effects of SiO_2 nanoparticles on phospholipid membrane integrity and fluidity. J Hazard Mater 2015;287:217–24. doi: 10.1016/J.JHAZMAT.2015.01.063
55. Zhao X, Wang S, Wu Y, You H, Lv L. Acute ZnO nanoparticles exposure induces developmental toxicity, oxidative stress and DNA damage in embryo-larval zebrafish. Aquat Toxicol 2013;136–137:49–59. doi: 10.1016/J.AQUATOX.2013.03.019
56. Wolf-Grosse S, Mollnes TE, Ali S, Stenvik J, Nilsen AM. Iron oxide nanoparticles enhance Toll-like receptor-induced cytokines in a particle size- and actin-dependent manner in human blood. Nanomedicine 2018;13:1773–85. doi: 10.2217/nnm-2017-0362
57. Wu X, Zhang Y, Mao H, Zhang M. Toxic effects of iron oxide nanoparticles on human umbilical vein endothelial cells. Int J Nanomedicine 2010;5:385–99. doi: 10.2147/IJN.S10458
58. Palacios-Hernandez T, Diaz-Diestra DM, Nguyen AK, Skoog SA, Vijaya Chikkaveeraiah B, Tang X, Wu Y, Petrochenko PE, Sussman EM, Goering PL. Cytotoxicity, cellular uptake and apoptotic responses in human coronary artery endothelial cells exposed to ultrasmall superparamagnetic iron oxide nanoparticles. J Appl Toxicol 2020;40:918–30. doi: 10.1002/jat.3953
59. Costa C, Brandão F, Bessa MJ, Costa S, Valdiglesias V, Kiliç G, Fernández-Bertólez N, Quaresma P, Pereira E, Pásaro E, Laffon B, Teixeira JP. *In vitro* cytotoxicity of superparamagnetic iron oxide nanoparticles on neuronal and glial cells. Evaluation of nanoparticle interference with viability tests. J Appl Toxicol 2016;36:361–72. doi: 10.1002/jat.3213
60. Dwivedi S, Siddiqui MA, Farshori NN, Ahamed M, Musarrat J, Al-Khedhairy AA. Synthesis, characterization and toxicological evaluation of iron oxide nanoparticles in human lung alveolar epithelial cells. Colloids Surfaces B Biointerfaces 2014;122:209–15. doi: 10.1016/J.COLSURFB.2014.06.064
61. Couto D, Freitas M, Vilas-Boas V, Dias I, Porto G, Lopez-Quintela MA, Rivas J, Freitas P, Carvalho F, Fernandes E. Interaction of polyacrylic acid coated and non-coated iron oxide nanoparticles with human neutrophils. Toxicol Lett 2014;225:57–65. doi: 10.1016/J.TOXLET.2013.11.020
62. Fernández-Bertólez N, Costa C, Brandão F, Kiliç G, Duarte JA, Teixeira JP, Pásaro E, Valdiglesias V, Laffon B. Toxicological assessment of silica-coated iron oxide nanoparticles in human astrocytes. Food Chem Toxicol 2018;118:13–23. doi: 10.1016/J.FCT.2018.04.058

Učinci nanočestica željezova oksida na antioksidacijski kapacitet i odgovor na oksidacijski stres u mozambičke tilapije (*Oreochromis mossambicus*, Peters 1852)

Nedavna istraživanja izazvala su zabrinutost oko biokompatibilnosti nanočestica željezova oksida (engl. *iron oxide nanoparticles* – IONP), nakon što je utvrđeno da izazivaju oksidacijski stres i upalni odgovor, a produljena izloženost visokim koncentracijama IONP-a može dovesti do cito-/genotoksičnosti. Osim toga, postoji i zabrinutost u pogledu njihova utjecaja na okoliš. Cilj ovog istraživanja bio je proučiti djelovanje IONP-a na antioksidacijski obrambeni sustav slatkovodne ribe mozambičke tilapije (*Oreochromis mossambicus*, Peters 1852). Ribe su bile izložene koncentraciji IONP-a od 15 mg/L tijekom 1, 3, 4, 15, 30 i 60 dana, a usporedno su praćene i jedinke kontrolne, neizložene skupine. Nadalje, praćenje je nastavljeno tijekom 60 dana nakon prestanka izloženosti kako bismo procijenili stabilnost oksidacijskoga stresa izazvanoga IONP-om. Izloženost je utjecala na aktivnost antioksidacijskih i markerskih enzima te povećala razine vodikova peroksida i lipidne peroksidacije u tkivu ribljih škrga, jetre i mozga. Čak i nakon 60 dana „čišćenja" zaostali su štetni učinci, koji upozoravaju na nepovratnu nanotoksičnost. Štoviše, IONP se akumulirao u tkivu škrga, jetre i mozga. Naša otkrića naglašavaju potencijalne zdravstvene rizike za neciljane organizme u okolišu, te je nužno uspostaviti odgovarajuće smjernice za sigurno rukovanje i odlaganje IONP-a kako bi se zaštitio vodeni okoliš.

KLJUČNE RIJEČI: antioksidacijski enzimi; bioakumulacija; oksidacijski stres; toksičnost; zdravlje okoliša