



Comparative Toxicity of Alpha and Gamma Iron Oxide Nanoparticles in Rainbow Trout: Histopathology, Hematology, Accumulation, and Oxidative Stress

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Abstract In this study, alpha and gamma iron oxide nanoparticles, characterizations, toxic effects after being exposed to rainbow trout (*Oncorhynchus mykiss*) at 0, 1, 10 and 25 mg/L concentrations for 10 days, and then 10-day recovery period without any nanoparticle exposure were examined for histopathological (kidney, liver and gill), hematological, iron accumulation and potential for oxidative stress (TBARS and GSH). Histopathological damages significant at the exposure of increasing concentrations of both nanoparticles (increase in melanomacrophage aggregations, epithelial tissue deformations, cytoplasmic vacuolizations, fatty changes, necrosis, pyknosis, hyperplasia, hypertrophy, lamellar fusions, capillary dilatations). Gamma nanoparticles were determined to accumulate more than the alpha nanoparticles. The most Fe accumulation was detected in the liver. The findings of oxidative stress parameters showed that both nanoparticles have the potential to generate oxidative stress. It was concluded that the

exposure of alpha and gamma nanoparticles at specified concentrations and durations had a toxic effect on rainbow trout and the toxicity of these nanoparticles was similar.

Keywords Alpha iron oxide · Gamma iron oxide · Nanoparticle · Toxicity · *Oncorhynchus mykiss*

1 Introduction

Nowadays, the science of nanotechnology unfortunately mainly focuses on the formulation and production of new nanomaterials with little or no knowledge of their toxicological properties. Our knowledge of the effects of nanomaterials on the environment and human health is still insufficient, as the potentially harmful effects of nanomaterials on human health and the environment have just come out (Blasco and Corsi 2019; Ottoni et al. 2020). Environmental concerns about nanomaterials are that especially aquatic organisms are more exposed to these nanomaterials.

One of the most important organisms that make up the food chain in biological systems is fish. At the top of the worrying issues is the possibility that people may be exposed to water-borne nanoparticles (NPs) through fish, which constitute an important part of their food supply (Oberdörster 2004). Iron oxide (Fe_2O_3) NPs have become interesting in many industrial activities in favour of their superparamagnetic properties and high catalytic capabilities (Dale 2005; Kadar et al. 2010); radiological applications (MRI), pharmaceutical

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industry, gene therapy, nanosensor applications, cell labeling, cancer treatment and wastewater treatment (Li et al. 2013). It has been reported that the necessity of examining the toxic effects of increased production and use of Fe_2O_3 NPs and their negative impacts on human health in the long term (Ateş et al. 2016). In most of the current studies, the potential toxicity of Fe_2O_3 NPs was investigated, mammalian species (mouse or rat) were used or toxicological examinations were made in different cell cultures (Garcia et al. 2011; Soenen and De Cuyper 2010; Soto et al. 2005; Wang et al. 2009; Zhu et al. 2008). Some studies have investigated the ecotoxicity of Fe_2O_3 NPs on aquatic ecosystems (Ateş et al. 2016; Hedayati et al. 2016; Keerthika et al. 2017; Nations et al. 2011; Özgür et al. 2018; Srinivasan et al. 2016; Villacis et al. 2017).

Fe_2O_3 NPs, hematite (alpha iron oxide, $\alpha\text{-Fe}_2\text{O}_3$ NPs) and maghemite (gamma iron oxide, $\gamma\text{-Fe}_2\text{O}_3$ NPs) are used in different technological implementations (Demir et al. 2015). Similarly, $\alpha\text{-Fe}_2\text{O}_3$ NPs and $\gamma\text{-Fe}_2\text{O}_3$ NPs have the same Fe^{+3} cation. The most important difference between these two NPs is their crystal structure. While $\alpha\text{-Fe}_2\text{O}_3$ NPs is in the rhombohedral crystal structure, $\gamma\text{-Fe}_2\text{O}_3$ NPs is in spherical structure (Can et al. 2012; Randrianantoandro et al. 2001).

In this study, rainbow trout were exposed to different concentrations (0, 1, 10 and 25 mg/L) of $\alpha\text{-Fe}_2\text{O}_3$ NPs and $\gamma\text{-Fe}_2\text{O}_3$ NPs for 10 days in a semi-static regime. At the end of the exposures, the fish were kept in the aquariums for 10-day recovery period without any NP exposures. The study aimed to determine, histopathological changes, hematological alterations, iron accumulation and possible oxidative stress in the fish.

2 Materials and Methods

2.1 Experimental Design

Rainbow trout (*O. mykiss*) ($n = 252$) that are used in this study were provided from Keskin Trout Facility (Ayazma, Çanakkale, Turkey). The fish were adapted to ambient conditions for 15 days in 21 stock aquariums, each with a size of $45 \times 28 \times 80$ cm and 70 L of Çanakkale province tap water in it. The fish were fed with instant trout feed during the adaptation to the laboratory conditions. In the experiments, the average weight of 18 ± 2 g fish was distributed to 21 aquariums with 12 fish each, and experimental semi-static design with three replicates was constituted. The fish were fed on the 7th and 14th days of the experiments to avoid any aggressive behaviour due to nutritional deficiencies and ethical rules. Feeding was performed 1 day before daily water change (70% in the morning, 30% in the evening) and dosing. Fish food and other waste left at the bottom were collected during daily aquarium cleaning. Stock suspensions of $\alpha\text{-Fe}_2\text{O}_3$ NPs and $\gamma\text{-Fe}_2\text{O}_3$ NPs (US Research Nanomaterials, Inc, Houston, TX USA) nanoparticles were dispersed in ultrapure water at 10% (mass/volume). This solution, after vortexing for 20 s, was sonicated in an ultrasonic bath for about 10 min to procure the maximum nanoparticle distribution. The fish were exposed to the $\alpha\text{-Fe}_2\text{O}_3$ NPs and $\gamma\text{-Fe}_2\text{O}_3$ NPs for 10 days in the control group (tap water only) and at concentrations of 1, 10 and 25 mg/L. After the exposures, the fish were kept in aquariums containing water for 10 days, without nanoparticle exposure (recovery). Samplings were

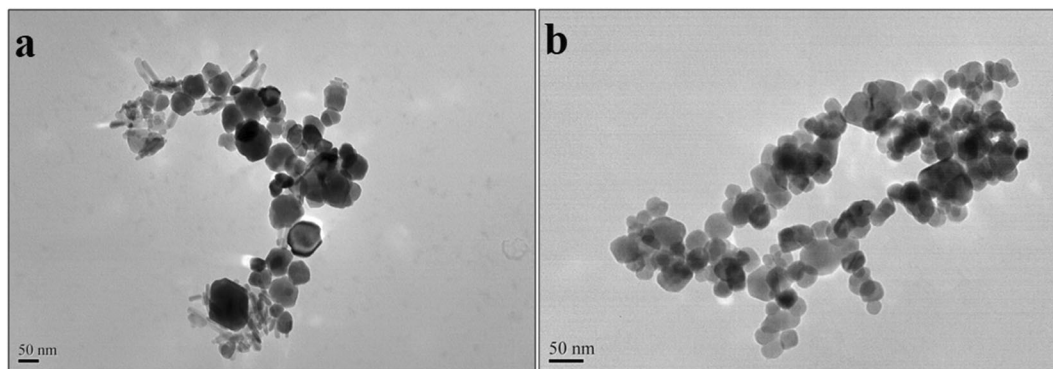


Fig. 1 TEM images of **a** $\alpha\text{-Fe}_2\text{O}_3$ NPs, **b** $\gamma\text{-Fe}_2\text{O}_3$ NPs

Table 1 Size and surface charges of α -Fe₂O₃ NPs and γ -Fe₂O₃ NPs in aqueous medium

Nanoparticles	TEM (nm)	DLS (nm)	Zeta potential (mV)	View (colour)
α -Fe ₂ O ₃	20–110	420 ± 23	− 23.5 ± 1.3	Blurred-yellow/orange
γ -Fe ₂ O ₃	25–130	350 ± 31	− 19.3 ± 1.1	Orange

made four times, day 0, day 4, day 10 and day 20 (recovery). On day 0, day 4, day 10 and day 20, five fish were dissected; blood and tissues of liver, kidney and gill were collected. Temperature and dissolved oxygen were determined with the probe brand YSI MPS 556 (Yellow Spring Instruments, Yellow Springs, OH, USA) as 13.1 ± 0.4 °C and 7.7 ± 0.2 mg/L, and pH was measured with HANNA C 200 (HI 83200, HANNA Instruments, Smithfield, RI, USA) in the daily period as 7.44 ± 0.05 . The hardness of the water every 2 days was measured with a Myron L Poolpro (Carlsbad, USA); total hardness was 121 ± 5 mg CaCO₃/L.

2.2 Characterization of Nanoparticles

For the characterization of α -Fe₂O₃ and γ -Fe₂O₃ nanoparticles, transmission electron microscope (TEM), dynamic light scattering (DLS) and zeta analyses were used.

2.3 Histopathological Analysis

The fish were anesthetized with clove oil before dissection. On sampling days, tissues were taken from 4 fish in each experiment group. Tissues were taken from the liver, gill and kidney. Tissues were fixed in Bouin's fixative for 8–12 h according to their size. Tissues were cut with a 5 μ m

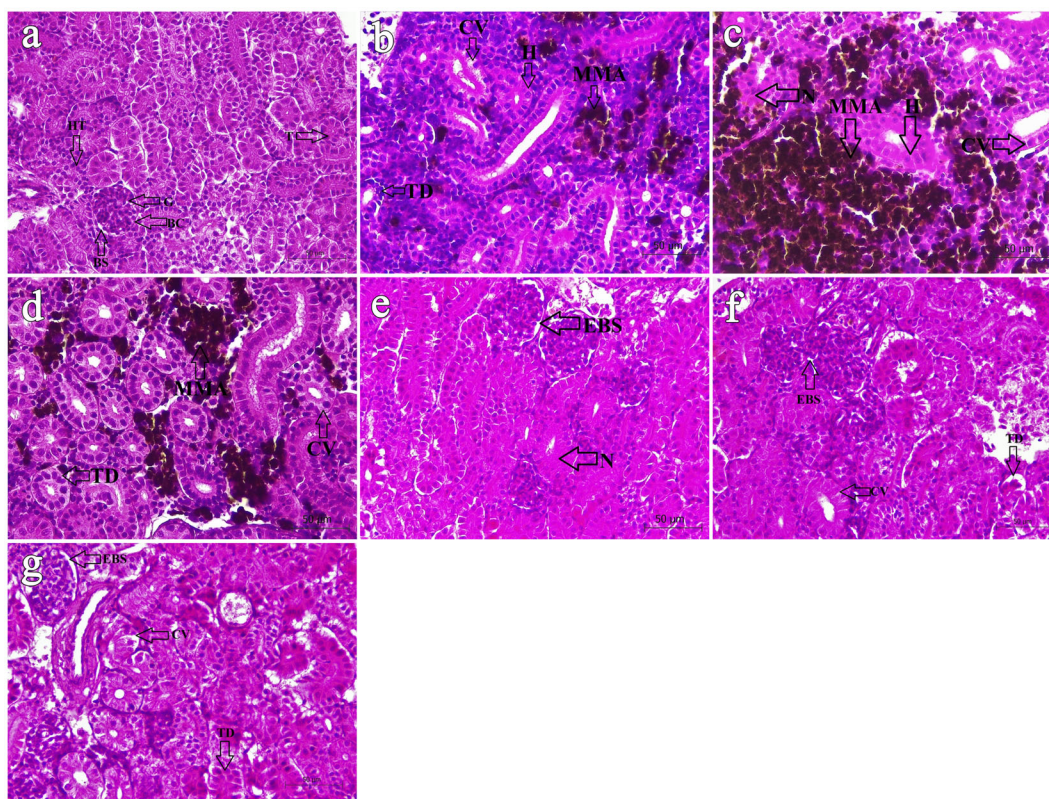


Fig. 2 Histopathological changes in *O. mykiss* kidney, exposed to α -Fe₂O₃ NPs, **a** control, **b** 1 mg/L (10th day), **c** 10 mg/L (10th day), **d** 25 mg/L (10th day), **e** 1 mg/L (20th day), **f** 10 mg/L (20th day), **g** 25 mg/L (20th day). HT: hematopoietic tissue, G:

glomerulus, BS: Bowman's space, BC: Bowman's capsule, T: tubule, CV: cytoplasmic vacuolation, MMA: melanomacrophage aggregations, TD: tubular deformation, H: hypertrophy, N: necrosis, EBS: enlargement of Bowman's space, scale bar 50 μ m, H&E

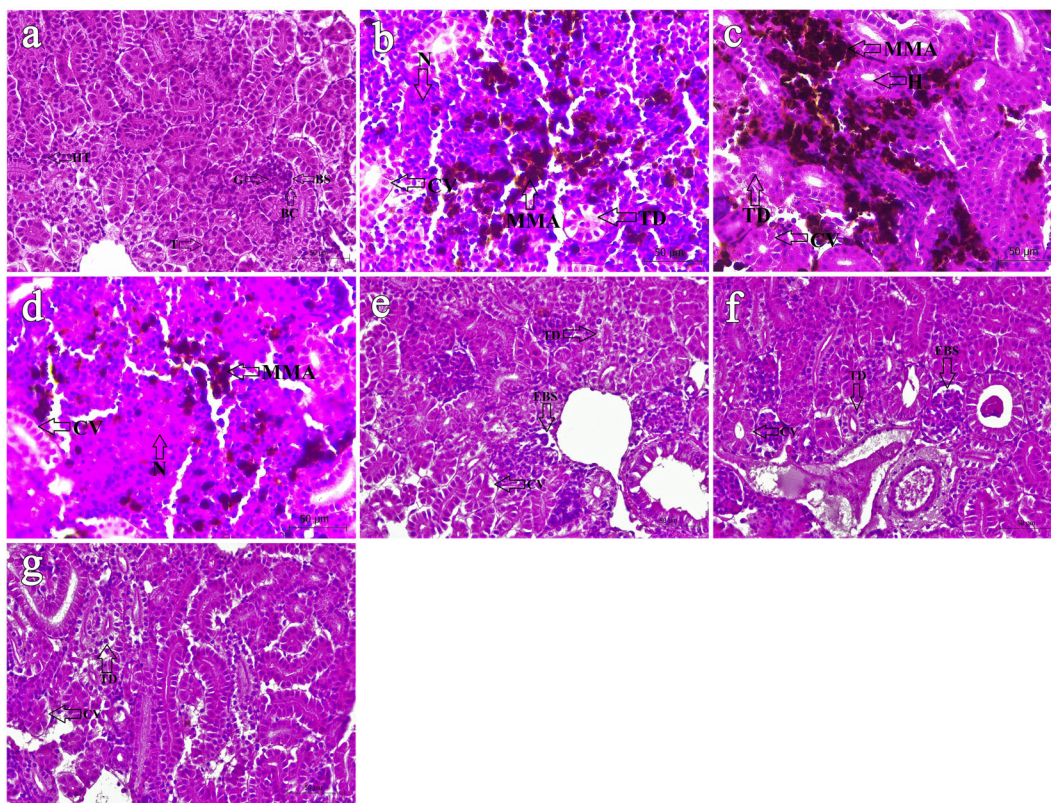


Fig. 3 Histopathological changes in *O. mykiss* kidney, exposed to $\gamma\text{-Fe}_2\text{O}_3$ NPs, **a** control, **b** 1 mg/L (10th day), **c** 10 mg/L (10th day), **d** 25 mg/L (10th day), **e** 1 mg/L (20th day), **f** 10 mg/L (20th day), **g** 25 mg/L (20th day). HT: hematopoietic tissue, G:

glomerulus, BS: Bowman's space, BC: Bowman's capsule, T: tubule, CV: cytoplasmic vacuolation, MMA: melanomacrophage aggregations, TD: tubular deformation, H: hypertrophy, N: necrosis, EBS: enlargement of Bowman's space, scale bar 50 μm , H&E

thickness in a Leica rotary microtome (Leica Biosystems, Leider Lane, USA) and stained with Hematoxylin&Eosin.

2.4 Hematological Analysis

The back of the anal fin was thoroughly cleaned with alcohol and $\sim 400 \mu\text{L}$ blood was taken from the caudal vein with a 2.5-mL plastic syringe without damaging the fish. Hematological analyses were performed using an automatic blood count device (Mindray BC-3000Plus, Shenzhen, China).

2.5 Nanoparticle Accumulation

At the end of the experiments, tissues are taken from different organs (liver, kidney and gill). Tissues were washed with cold water to remove surface pollution. For ICP-MS analysis, approximately 0.1 g of wet tissue was weighed from cleaned samples and it was dispersed in 10 mL HNO_3 in Teflon containers under a fume hood at

200 $^\circ\text{C}$ on a heating table. The content was visually checked to ensure that the nanoparticles were completely dispersed. And after the cooling process, it was diluted with pure water to 15 mL. For the final stage, the acid ratio was diluted with pure water for a maximum of 5% for ICP-MS analysis.

2.6 Oxidative Stress (TBARS and GSH)

Determination of thiobarbituric acid-reactive substances (TBARS) was performed in a 96 cell microplate using 40 μL tissue homogenate. The microplate was read by a microplate reader (Thermo Fisher Scientific, Waltham, USA) at a wavelength of 530 nm. Total glutathione content was measured by the enzymatic cycle procedure in the presence of DTNB, NADPH and GR. The absorbance changes were read by a microplate reader (Thermo Fisher Scientific, Waltham, USA) at a wavelength of 412 nm every 15 s that were taken and recorded for 10 min.

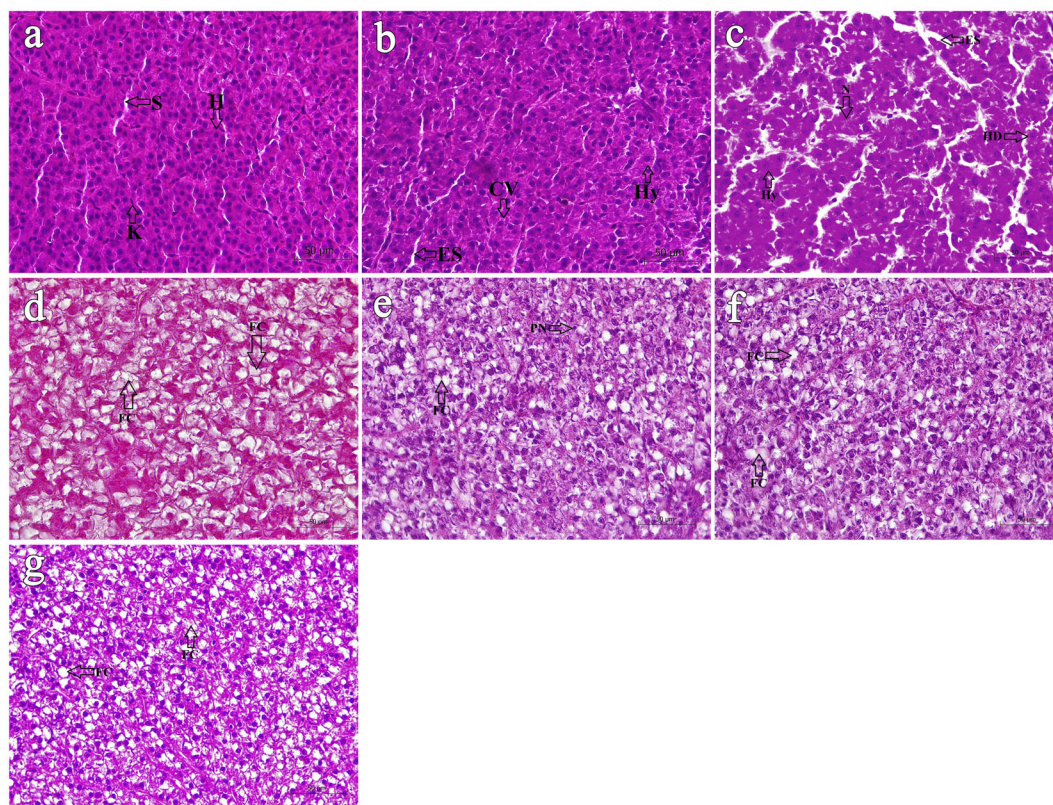


Fig. 4 Histopathological changes in *O. mykiss* liver, exposed to α -Fe₂O₃ NPs, **a** control, **b** 1 mg/L (10th day), **c** 10 mg/L (10th day), **d** 25 mg/L (10th day), **e** 1 mg/L (20th day), **f** 10 mg/L (20th day), **g** 25 mg/L (20th day). H: hepatocyte, K: Kupffer cell, S:

sinusoid, Hy: hypertrophy, CV: cytoplasmic vacuolation, ES: enlargement of sinusoidal cavity, N: necrosis, FC: fatty change, PN: pyknotic nucleus, scale bar 50 μ m, H&E

2.7 Statistical Analysis

The statistical analysis was using the SPSS 21.0 package program. ANOVA with Tukey post-test (one-way ANOVA for comparison between the exposure groups and the control group) was used. The significance level was considered to be 0.05.

3 Results

3.1 Nanoparticle Characterization

In TEM images that are obtained for both nanoparticles, it was determined that nanoparticles have homogeneous morphology and generally round structure but there were also cubes, cylinders and stick-like shapes that are not exactly round edged. Although it was determined that rod-like structures were more in α -Fe₂O₃ NPs, spherical structures were determined to be more

dominant and similarly sized in γ -Fe₂O₃ NPs (Fig. 1). Also, in both NPs, it was determined that they make clusters even a little. These results are similar to the dynamic light scattering (DLS) findings.

The size distribution and surface charge of both nanoparticles in a liquid environment are presented in Table 1.

3.2 Histopathological Findings

Histopathological changes in kidney, liver and gills on the 10th and 20th (recovery) days of the experiments are presented in Figs 2, 3, 4, 5, 6 and 7. The kidneys from control fish showed normal histology, presenting the typical structure epithelial tissue, glomerulus, Bowman's capsule and hematopoietic tissue (Figs. 2a and 3a). On the 10th day of α -Fe₂O₃ NP-treated groups, histopathological changes such as epithelial deformations, hypertrophy, cytoplasmic vacuolation, melanomacrophage aggregations and focal necrosis

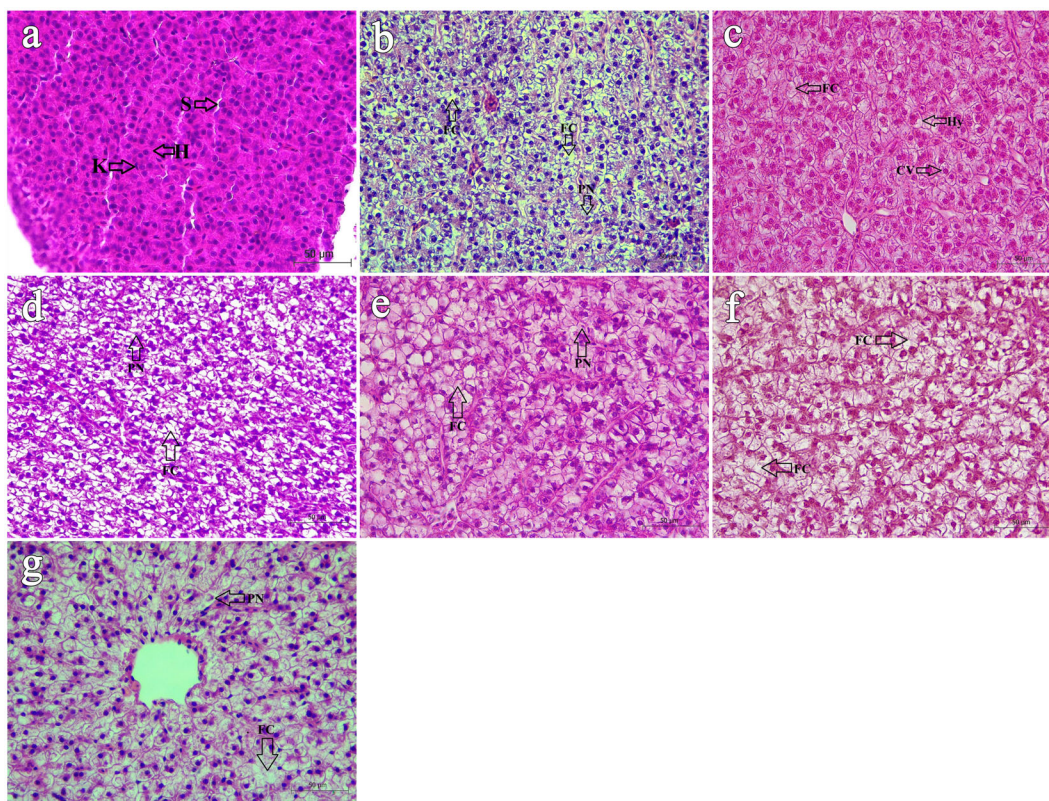


Fig. 5 Histopathological changes in *O. mykiss* liver, exposed to γ -Fe₂O₃ NPs, **a** control, **b** 1 mg/L (10th day), **c** 10 mg/L (10th day), **d** 25 mg/L (10th day), **e** 1 mg/L (20th day), **f** 10 mg/L (20th day), **g** 25 mg/L. H: hepatocyte, K: Kupffer cell, S: sinusoid, Hy:

hypertrophy, CV: cytoplasmic vacuolation, ES: enlargement of sinusoidal cavity, N: necrosis, FC: fatty change, PN: pyknotic nucleus, scale bar 50 μ m, H&E

were observed (Fig. 2 b–d). In all the α -Fe₂O₃ NP recovery groups, enlargement of Bowman's space, cytoplasmic vacuolation and focal necrosis were observed (Fig. 2e–g). In these groups, melanomacrophages were not observed. Figures 3 b, c and d show cytoplasmic vacuolation, hypertrophy, tubular deformation, enlargement of Bowman's space, melanomacrophage aggregations and focal necrosis on the 10th day of γ -Fe₂O₃ NP exposures (1, 10 and 25 mg/L). In all the γ -Fe₂O₃ NP recovery groups, melanomacrophages were not observed, but enlargement of Bowman's space, cytoplasmic vacuolation and focal necrosis were identified (Fig. 3e–g).

Hepatocyte, Kupffer cell and sinusoid were observed in normal structure in the liver sections of individuals from control group (Figs. 4a and 5a). On the other hand, severe histological damages were found in liver sections of 1, 10 and 25 mg/L α -Fe₂O₃ NP exposure groups (Fig. 4b–d). At the 10th day of experiment, enlargement of sinusoidal cavity, necrosis and fatty changes were

observed in liver of fish exposed to 10 and 25 mg/L α -Fe₂O₃ NPs. Pyknotic nucleus and fatty changes were observed in all the liver sections of α -Fe₂O₃ NP recovery groups (Fig. 4e–g). Cytoplasmic vacuolation, pyknotic nucleus, hypertrophy and fatty changes were observed in the liver on the 10th day of γ -Fe₂O₃ NP exposures (Fig. 5b–d). Pyknotic nucleus and fatty changes were observed in all the liver sections of γ -Fe₂O₃ NP recovery groups (Fig. 5e–g).

In the gill sections, the control fish showed normal histological structure (Figs. 6a and 7a). In groups exposed to 1, 10 and 25 mg/L α -Fe₂O₃ NPs, the observed damages were a lamellar fusion, hyperplasia of the basal cell, capillary dilatation and clubbing at the tips of the secondary lamellae (Fig. 6b–d). Especially in 10 and 25 mg/L α -Fe₂O₃ NP exposure groups, lamellar fusions observed frequently. Lamellar deformation, hyperplasia of the basal cell capillary dilatation and clubbing at the tips of the secondary lamellae were observed in all the gill sections of α -Fe₂O₃ NP recovery groups (Fig. 6e–

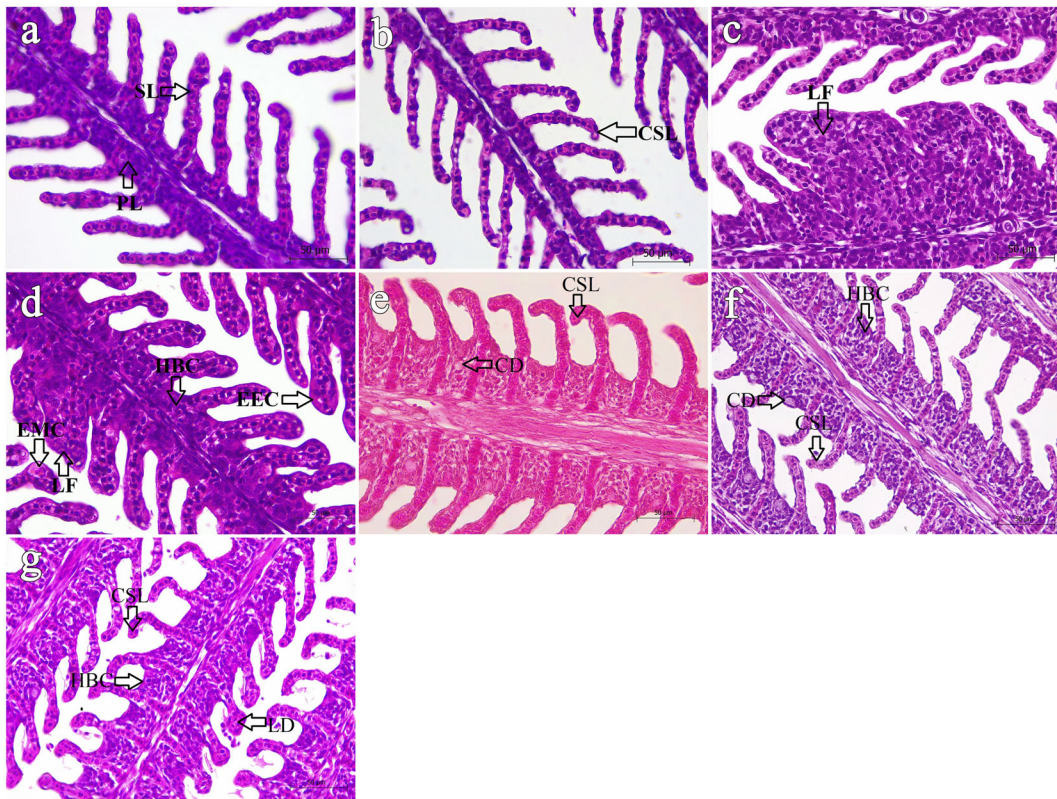


Fig. 6 Histopathological changes in *O. mykiss* gill, exposed to α -Fe₂O₃ NPs, **a** control, **b** 1 mg/L (10th day), **c** 10 mg/L (10th day), **d** 25 mg/L (10th day), **e** 1 mg/L (20th day), **f** 10 mg/L (20th day), **g** 25 mg/L (20th day). PL: primary lamellae, SL: secondary lamellae, LF: lamellar fusion, LD: lamellar deformation, EMC: enlargement of the mucous cell, HBC: hyperplasia of the basal cell, EEC: enlargement of the epithelial cell, CD: capillary dilatation, scale bar 50 μ m, H&E

g). Figures 7 b, c and d show lamellar fusion, hyperplasia of the basal cell, capillary dilatation and clubbing at the tips of the secondary lamellae on the 10th day of γ -Fe₂O₃ NP exposures (1, 10 and 25 mg/L). Similar histopathological findings were observed in gill sections of γ -Fe₂O₃ NP recovery groups (Fig. 7e–g).

3.3 Hematological Findings

There was no statistically significant change in the hematological parameters until the 20th day of the experiment. Mean values of some hematological parameters (WBC, RBC, HGB, HCT, MCV, MCH and MCHC) are presented in Table 2 ($p < 0.05$).

3.4 Fe (Iron) Accumulation

Fe accumulation (μ g/g) levels in kidney, liver and gill are presented in Fig. 8. According to the analysis of the

10th day of the experiments in kidney tissue, it was assessed that the group exposed to 25 mg/L γ -Fe₂O₃ NPs had the highest concentration of Fe accumulation compared with all other groups ($p < 0.05$). At the end of the 20th day, it was determined that the Fe in the kidney was partially excreted and there was no statistical difference between the control and all treatment groups ($p > 0.05$) (Fig. 8a).

In the liver, on the 4th day of the experiments in terms of Fe accumulation, it was determined that there was an increase in all groups compared with the control group except 1 mg/L α -Fe₂O₃ NP exposure ($p < 0.05$). At the end of the 10th day, in terms of Fe accumulation, the difference between all groups and the control group was statistically significant ($p < 0.05$). At the end of the 20th day, the Fe in the liver could not be removed from the tissue yet; it was determined that there was a difference between all treatment groups and the control group ($p < 0.05$). The highest concentrations of element Fe

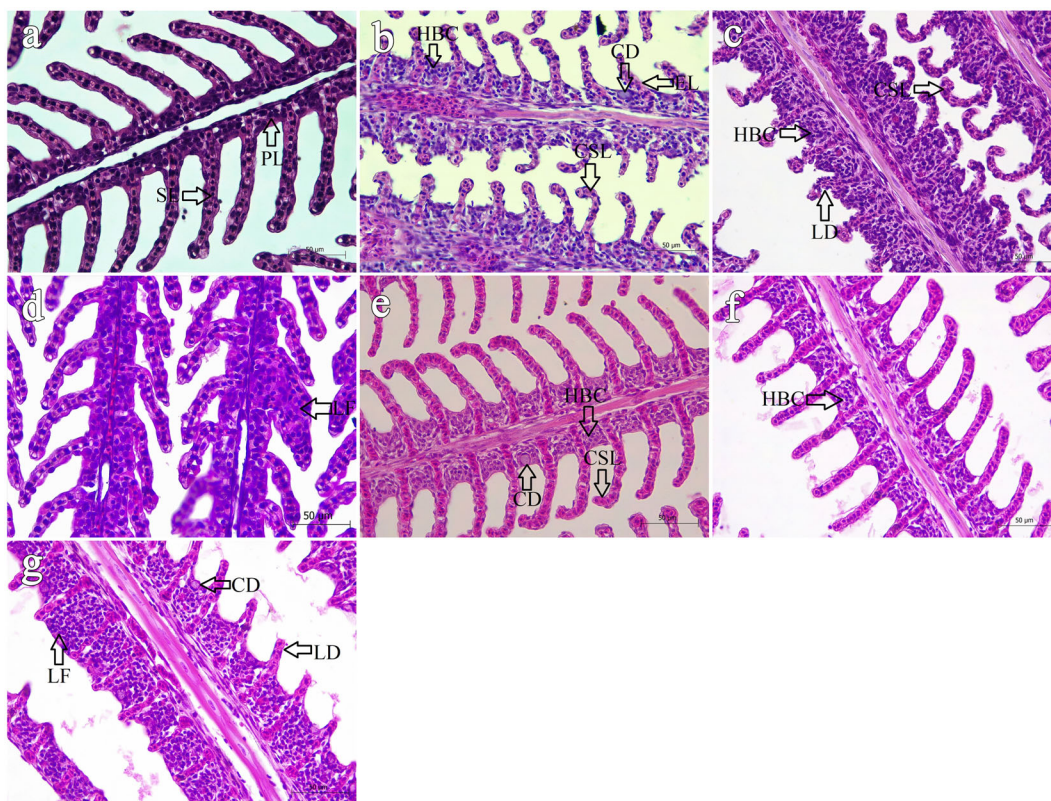


Fig. 7 Histopathological changes in *O. mykiss* gill, exposed to γ - Fe_2O_3 NPs, **a** control, **b** 1 mg/L (10th day), **c** 10 mg/L (10th day), **d** 25 mg/L (10th day), **e** 1 mg/L (20th day), **f** 10 mg/L (20th day), **g** 25 mg/L (20th day). PL: primary lamellae, SL: secondary

lamellae, CSL: clubbing at the tips of the secondary lamellae, LF: lamellar fusion, LD: lamellar deformation, HBC: hyperplasia of the basal cell, CD: capillary dilatation, EL: epithelial lifting, scale bar 50 μm , H&E

were found in groups exposed to 25 mg/L α - Fe_2O_3 and γ - Fe_2O_3 NPs (Fig. 8b).

Compared with other organs in the study (kidney and liver), Fe accumulation was found to be least in the gills. At the end of the 10th day, Fe accumulation in all treatment groups increased compared with the control group, and Fe accumulation increased significantly in the group exposed to 25 mg/L γ - Fe_2O_3 NPs ($p < 0.05$). At the end of the 20th day, the Fe in the gill was partially removed including all treatment groups, and there was no difference between the treatment groups and the control group ($p > 0.05$) (Fig. 8c).

3.5 Oxidative Stress (TBARS and GSH Analysis)

TBARS activity in the kidney, liver and gill is presented in Fig. 9. On the 4th day of the experiments, TBARS levels in the kidney, which exposed to 25 mg/L α - Fe_2O_3 and 1, 10 and 25 mg/L γ - Fe_2O_3 NPs, increased compared with the control group ($p < 0.05$). At the end of the

10th day, it was found that TBARS levels increased in all treatment groups compared with the control group ($p < 0.05$). On the 20th day of the experiments, TBARS levels were similarly increased in all treatment groups compared with the control group ($p < 0.05$) (Fig. 9a).

TBARS activity levels in the liver were found to increase in all treatment groups at the end of the 10th day compared with the control group ($p < 0.05$). It was determined that the highest TBARS levels were in the groups exposed to 25 mg/L α - Fe_2O_3 and 25 mg/L γ - Fe_2O_3 NPs. On the 20th day of the experiments, it was found that TBARS levels increased at the highest concentrations in the groups exposed to 25 mg/L α - Fe_2O_3 and 10 and 25 mg/L γ - Fe_2O_3 NPs ($p < 0.05$) (Fig. 9b).

There was no difference between the control group and all other treatment groups in terms of TBARS activity levels in the gill, on the 4th and 10th days of the experiments ($p > 0.05$). The highest TBARS level was detected in the group exposed to 25 mg/L γ - Fe_2O_3 NPs at the end of the 20th day (Fig. 9c).

Table 2 Effects of α -Fe₂O₃ and γ -Fe₂O₃ nanoparticles (1, 10 and 25 mg/L) on hematological variables in *O. mykiss*, on the days 0, 4, 10 and 20

Day	Groups	WBC ($\times 10^3 \text{ mm}^{-3}$)	RBC ($\times 10^6 \text{ mm}^{-3}$)	HGB (g/dL)	HCT (%)	MCV (μm^3)	MCH (pg/cell)	MCHC (%)
0	Control	40.6 $\pm$ 1.3	1.5 $\pm$ 0.1	9.1 $\pm$ 1.3	25.1 $\pm$ 2.1	166.4 $\pm$ 5.9	60.4 $\pm$ 2.1	36.2 $\pm$ 1.7
4	Control	43.7 $\pm$ 2.2	1.4 $\pm$ 0.1	9.3 $\pm$ 0.4	24.4 $\pm$ 0.9	167.4 $\pm$ 5.5	63.7 $\pm$ 1.9	38.1 $\pm$ 1.2
	α -1 mg/L	41.7 $\pm$ 2.0	1.3 $\pm$ 0.1	8.6 $\pm$ 0.4	24.5 $\pm$ 1.3	179.4 $\pm$ 3.3	63.4 $\pm$ 1.4	35.3 $\pm$ 0.2
	α -10 mg/L	38.0 $\pm$ 2.9	1.2 $\pm$ 0.1	8.0 $\pm$ 0.7	21.6 $\pm$ 2.0	175.5 $\pm$ 2.1	64.9 $\pm$ 1.5	36.9 $\pm$ 0.4
	α -25 mg/L	45.5 $\pm$ 1.6	1.4 $\pm$ 0.0	9.4 $\pm$ 0.4	25.7 $\pm$ 1.3	179.9 $\pm$ 2.7	65.8 $\pm$ 1.6	36.5 $\pm$ 0.5
	γ -1 mg/L	42.7 $\pm$ 3.0	1.3 $\pm$ 0.1	8.8 $\pm$ 0.6	23.7 $\pm$ 1.9	176.2 $\pm$ 3.3	65.5 $\pm$ 1.1	37.2 $\pm$ 0.4
	γ -10 mg/L	43.0 $\pm$ 1.6	1.4 $\pm$ 0.0	8.9 $\pm$ 0.4	25.0 $\pm$ 1.1	180.2 $\pm$ 3.3	64.5 $\pm$ 1.6	35.7 $\pm$ 0.4
	γ -25 mg/L	38.0 $\pm$ 5.6	1.2 $\pm$ 0.1	8.9 $\pm$ 0.6	21.9 $\pm$ 3.0	179.5 $\pm$ 2.0	81.1 $\pm$ 7.4	45.0 $\pm$ 9.4
10	Control	43.6 $\pm$ 1.8	1.4 $\pm$ 0.0	9.4 $\pm$ 0.5	25.3 $\pm$ 1.8	172.0 $\pm$ 2.0	64.3 $\pm$ 0.8	37.4 $\pm$ 0.6
	α -1 mg/L	42.6 $\pm$ 0.9	1.4 $\pm$ 0.0	9.2 $\pm$ 0.1	25.8 $\pm$ 0.6	177.6 $\pm$ 4.5	63.6 $\pm$ 2.0	35.8 $\pm$ 0.6
	α -10 mg/L	40.6 $\pm$ 1.9	1.3 $\pm$ 0.0	8.8 $\pm$ 0.3	24.5 $\pm$ 1.4	183.7 $\pm$ 1.3	66.7 $\pm$ 1.4	36.3 $\pm$ 0.8
	α -25 mg/L	39.7 $\pm$ 1.9	1.2 $\pm$ 0.0	8.3 $\pm$ 0.5	22.4 $\pm$ 1.4	174.2 $\pm$ 3.8	64.8 $\pm$ 1.2	37.2 $\pm$ 0.4
	γ -1 mg/L	37.1 $\pm$ 2.7	1.2 $\pm$ 0.1	8.0 $\pm$ 0.4	21.9 $\pm$ 1.2	179.1 $\pm$ 4.5	65.7 $\pm$ 2.6	36.6 $\pm$ 0.5
	γ -10 mg/L	41.5 $\pm$ 1.6	1.3 $\pm$ 0.0	9.0 $\pm$ 0.4	24.1 $\pm$ 1.1	179.0 $\pm$ 3.5	66.5 $\pm$ 0.3	37.2 $\pm$ 0.8
	γ -25 mg/L	39.2 $\pm$ 5.0	1.3 $\pm$ 0.1	8.6 $\pm$ 1.1	23.5 $\pm$ 3.3	176.5 $\pm$ 4.2	65.7 $\pm$ 1.6	37.2 $\pm$ 0.7
20	Control	33.5 $\pm$ 2.1	1.5 $\pm$ 0.1	8.9 $\pm$ 0.6	28.5 $\pm$ 2.7	184.2 $\pm$ 4.1	57.4 $\pm$ 1.2	31.2 $\pm$ 1.1
(recovery)	α -1 mg/L	45.6 $\pm$ 1.5	1.3 $\pm$ 0.1	8.8 $\pm$ 0.5	27.6 $\pm$ 2.4	198.7 $\pm$ 5.1	63.7 $\pm$ 1.6	32.0 $\pm$ 0.8
	α -10 mg/L	41.9 $\pm$ 2.1	1.4 $\pm$ 0.0	9.1 $\pm$ 0.4	21.9 $\pm$ 0.8	153.7 $\pm$ 3.8	63.9 $\pm$ 1.0	41.6 $\pm$ 0.9
	α -25 mg/L	41.5 $\pm$ 0.8	1.2 $\pm$ 0.0	8.5 $\pm$ 0.1	25.8 $\pm$ 1.5	200.7 $\pm$ 2.6	66.2 $\pm$ 0.9	33.0 $\pm$ 1.5
	γ -1 mg/L	39.5 $\pm$ 1.1	1.3 $\pm$ 0.1	9.1 $\pm$ 0.8	24.6 $\pm$ 1.7	188.3 $\pm$ 4.5	69.4 $\pm$ 1.8	36.8 $\pm$ 1.6
	γ -10 mg/L	40.1 $\pm$ 2.1	1.5 $\pm$ 0.1	8.5 $\pm$ 0.9	25.9 $\pm$ 1.9	166.5 $\pm$ 2.6	54.8 $\pm$ 1.4	32.9 $\pm$ 1.6
	γ -25 mg/L	39.1 $\pm$ 2.9	1.4 $\pm$ 0.0	8.2 $\pm$ 1.1	23.8 $\pm$ 2.4	166.5 $\pm$ 2.1	57.6 $\pm$ 1.8	34.5 $\pm$ 1.5

WBC white blood cell, RBC red blood cell, HGB hemoglobin, HCT hematocrit, MCV mean cell volume, MCH mean corpuscular hemoglobin, MCHC mean cell hemoglobin concentration; mean $\pm$ SD

GSH levels in kidney, liver and gill are presented in Fig. 10. GSH levels in the kidney increased in the groups exposed to 10 and 25 mg/L α -Fe₂O₃ and 1, 10 and 25 mg/L γ -Fe₂O₃ NPs compared with the control group on the 4th day of the experiments ($p < 0.05$). At the end of the 10th day and 20th day, GSH levels increased in all treatment groups compared with the control group ($p < 0.05$). GSH level was found to be at the highest concentration in the group exposed to 25 mg/L γ -Fe₂O₃ NPs (Fig. 10a).

The GSH activity level in the liver increased significantly in only the group exposed to 25 mg/L γ -Fe₂O₃ NPs on day 4 of the experiments ($p < 0.05$). At the end of the 10th day, GSH levels increased in all treatment groups compared with the control group ($p < 0.05$). It was determined that there was no difference between the control and all treatment groups in terms of GSH levels on the 20th day ($p > 0.05$) (Fig. 10b).

It was determined that there was a difference in GSH activity levels in the gill between the control and all treatment groups on the 4th and 10th days of the trials ($p < 0.05$). It was determined that there was a difference in GSH levels on the 20th day between all treatment groups and the control group, except the group that was exposed to 10 mg/L α -Fe₂O₃ NPs ($p < 0.05$). The highest GSH level increase was detected in the group exposed to 25 mg/L γ -Fe₂O₃ NPs (Fig. 10c).

4 Discussion

4.1 Histopathology

In kidney sections, it was determined that the most distinct pathological findings of 1, 10 and 25 mg/L α -Fe₂O₃ and γ -Fe₂O₃ NP exposure are the increase in the

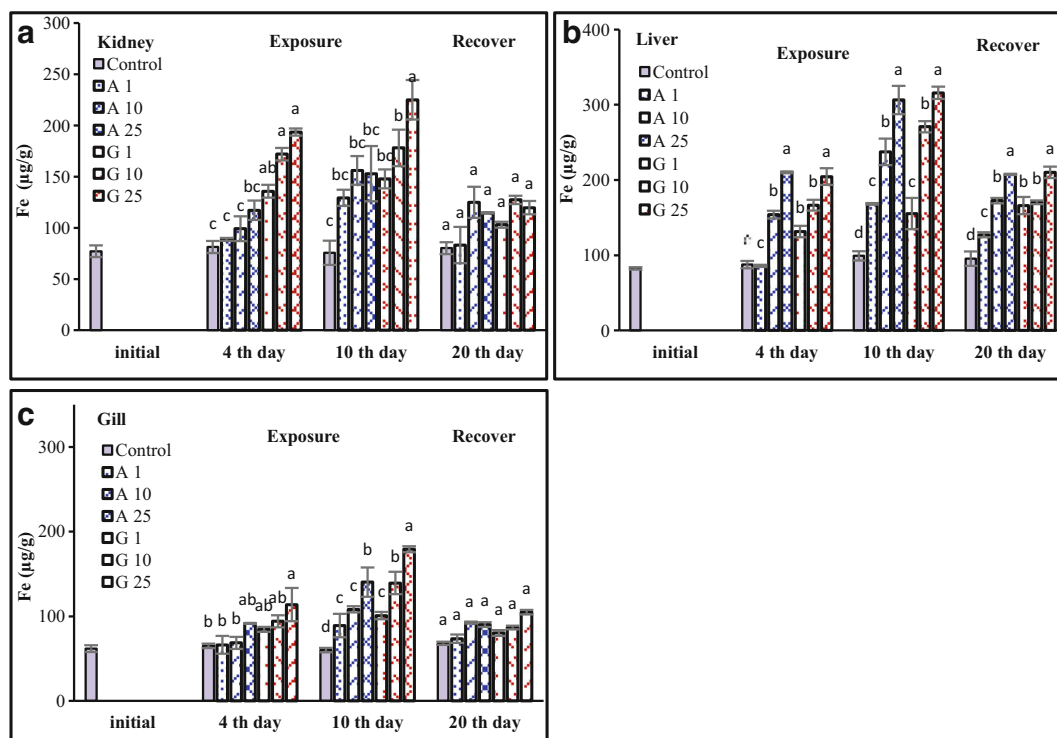


Fig. 8 Fe accumulation ($\mu\text{g/g}$), **a** Kidney, **b** Liver, **c** Gill. 0, 4, 10 and 20 days. For given times, concentrations with different lower-case letters are significantly different ($p < 0.05$); one-way

ANOVA followed by Tukey post hoc test (A: alpha iron oxide, G: gamma iron oxide)

intensity of melanomacrophage aggregations. It was determined that increased concentrations of both NPs are significant deformations, cytoplasmic vacuolizations and hypertrophies in kidney tubular epithelium. At the end of the exposures, necrosis was observed in the kidney of the fish which especially 10 and 25 mg/L $\alpha\text{-Fe}_2\text{O}_3$ and $\gamma\text{-Fe}_2\text{O}_3$ NPs were exposed. In the kidney sections of the recovery group fish, melanomacrophage aggregations were not observed. However, it was determined that the pathological findings that were observed in the application groups are still present, and in addition to these, enlargements in the bowman space are observed. It was determined that the NPs of $\alpha\text{-Fe}_2\text{O}_3$ and $\gamma\text{-Fe}_2\text{O}_3$ caused similar histopathological damages to the kidney. In the current literature, no study identified the pathological damage caused by iron oxide nanoparticles in fish kidneys. After all, in studies that have different NPs (copper and zinc) used, similar damage to the findings has been reported in our study (Al-Bairuty et al. 2013; Kaya et al. 2015). In the liver sections, it was determined that the exposure of 1 and 10 mg/L $\alpha\text{-Fe}_2\text{O}_3$ and $\gamma\text{-Fe}_2\text{O}_3$ NPs caused hypertrophy and cytoplasmic vacuolization, sinusoidal expansion

and an increase in Kupffer cell numbers in hepatocytes. Pyknosis and fatty changes were frequently observed in livers of fish that were applied 25 mg/L $\alpha\text{-Fe}_2\text{O}_3$ and $\gamma\text{-Fe}_2\text{O}_3$ NPs. It was determined that the NPs of $\alpha\text{-Fe}_2\text{O}_3$ and $\gamma\text{-Fe}_2\text{O}_3$ caused similar histopathological damages to the liver. Pathological findings, increasing concentrations and time were more clearly observed. In the liver sections of the recovery group fish, pyknosis and fatty changes in the entire tissue were observed. In trout liver, exposure of single-walled carbon nanotubes for 10 days has been reported to cause apoptosis, morphological changes in hepatic nuclei, fragmentation in nuclear material and cellular necrosis (Smith et al. 2007). Similar findings were detected after exposure of copper and TiO_2 NPs to rainbow trout (Al-Bairuty et al. 2013). Besides, Kaya et al. (2015) reported that different sizes (40–60 nm vs 80–100 nm) of 1 and 10 mg/L zinc NP exposure to tilapia, for 14 days, caused sinusoidal enlargement, cell infiltration, fatty change and cytoplasmic vacuolization. In our study, histopathological findings that are detected in liver sections are compatible with toxicological studies with other nanoparticles in the current literature. It was determined that all exposures

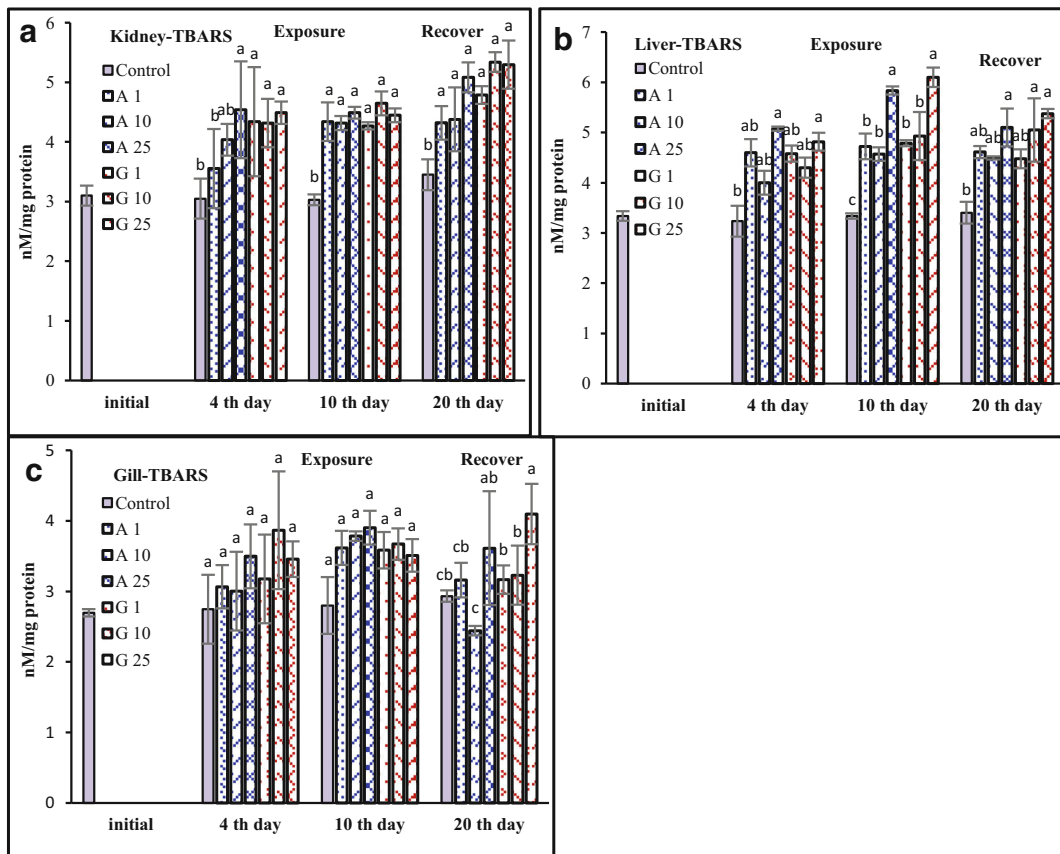


Fig. 9 Effects of exposure to α -Fe₂O₃ and γ -Fe₂O₃ NPs (1, 10 and 25 mg/L) on the level of TBARS (nM/mg protein) at the experiment 0, 4, 10 and 20 days. **a** Kidney, **b** liver, **c** gill. For given

times, concentrations with different lowercase letters are significantly different ($p < 0.05$); one-way ANOVA followed by Tukey post hoc test (A: alpha iron oxide, G: gamma iron oxide)

of α -Fe₂O₃ and γ -Fe₂O₃ NPs caused undifferentiated basal cell hyperplasia, hypertrophy in mucous cells, separation and deformations in the secondary lamellar epithelium in the gill sections. Especially on the 10th day of exposures of α -Fe₂O₃ and γ -Fe₂O₃ NPs at concentrations of 10 and 25 mg/L, lamellar fusion, tips at the ends of secondary lamellae and capillary dilatations were frequently observed. It was determined that hyperplastic activity is continued, epithelial damage in secondary lamellae could not be compensated, partial lamellar fusions are continued and clubbings at the tips of secondary lamellae are still existing in all healing groups. In the current literature, no study on histopathological damage that is caused by iron oxide NPs in fish gills has been found. The damage that is caused by various nanoparticle exposures to the gills was found to be similar to the findings in our study. After 48 h of exposure of 1.5 mg/L copper NPs to *D. rerio*, proliferation in gill epithelial cells and edema primary and

secondary lamellae have been reported (Hao et al. 2009). In a different study, Federici et al. (2007) reported that 14 days of TiO₂ NP exposure caused hyperplasia in the gills of *O. mykiss*. Besides, 24 and 48 h exposures of copper NPs caused an increase in filament width in *D. rerio* (Griffitt et al. 2007).

4.2 Hematological Changes

In the studies of toxicants on fish hematology, decreases in RBC, Hb and Hct levels were associated with developing the anemic condition due to inhibition of events such as erythropoiesis and hemogenesis, dysfunction in osmoregulation and increased erythrocyte destruction in hematopoietic organs (Kumar et al. 2011; Sepici Dinçel et al. 2007). In this study, we did not find any significant changes in the WBC, RBC, HCT, HGB, MCV, MCH and MCHC levels of rainbow trout. The same results have been reported in tilapia that are exposed to 0.1, 0.5

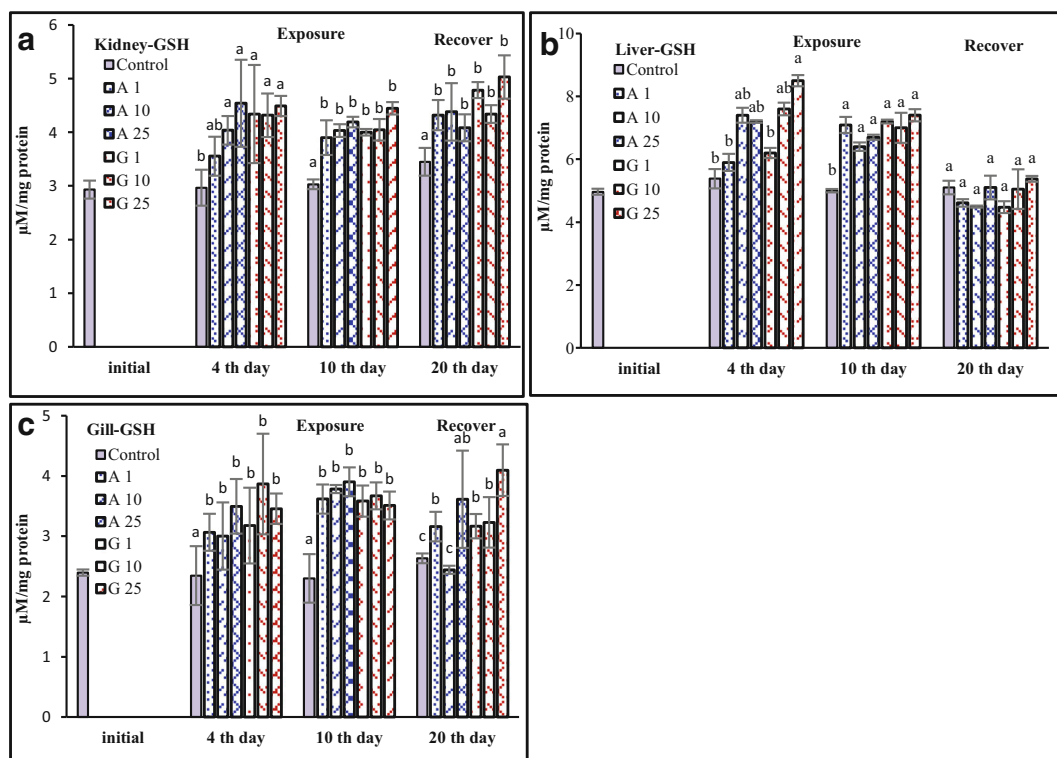


Fig. 10 Effects of exposure to α -Fe₂O₃ and γ -Fe₂O₃ NPs (1, 10 and 25 mg/L) on the level of GSH (μ M/mg protein) at the experiment 0, 4, 10 and 20 days. **a** Kidney, **b** liver, **c** gill. For

given times, concentrations with different lowercase letters are significantly different ($p < 0.05$); one-way ANOVA followed by Tukey post hoc test (A: alpha iron oxide, G: gamma iron oxide)

and 10 mg/L α -Fe₂O₃ and γ -Fe₂O₃ nanoparticles for 60 days (Ateş et al. 2016).

4.3 Iron Accumulation

In our study, an increase was observed in the amount of iron accumulation depending on time and concentration in all organs. The most Fe accumulation was detected in the liver and the lowest Fe accumulation was detected in the gill. On the 20th day of the experiment, it was determined that the level of Fe in the tissues (kidney and gill) of the fish except the liver was similar to the control group. Similar to the findings in our study, according to Zhang et al. (2015), the accumulation in the kidneys of zebra fish that are exposed to Fe₂O₃ and Fe₃O₄ NPs reduced to normal levels after 24 days of the last exposure. When all tissues were evaluated together, it has been detected that γ -Fe₂O₃ NPs accumulate more than α -Fe₂O₃ NPs. It is thought that the difference proceeds from the hydrodynamic and morphological qualification of nanoparticles.

4.4 TBARS and GSH Analysis

TBARS, which is an indicator of lipid peroxidation, is one of the important biomarkers that show oxidative stress (Kehrer 1993). The increase of free radicals and TBARS levels directly causes inhibition of Na⁺, K⁺-ATPase activity (Rauchova et al. 1999). Similarly, changes in GSH levels are valuable biomarkers of the adaptation mechanism in moderate oxidative stress conditions (Zhang et al. 2004). In our study, TBARS activities at the end of the exposures (10th day) increased in tissues other than gill. On the 20th day of the experiment, it was determined that the increase in activity in the kidney and liver continued significantly in growing concentrations of both NPs. On the same day, it has been determined that TBARS activity levels in the gills fluctuated interestingly and an increase was observed especially in fish that are exposed to 25 mg/L γ -Fe₂O₃ NPs compared with the control group. In terms of GSH activities on the 10th day, an increase was

determined compared with the control group in all organs and concentrations that are evaluated in the study. On the 20th day, different reactions were determined in terms of the same activities. It was determined that the GSH levels in the kidneys of the fish in the recovery period were increased compared with the control group; there is similarity shown with the control group in their livers and in the gill they fluctuated.

5 Conclusion

The harmless nanotechnological applications and materials should be produced and used on living organisms. Only in this way can nanotechnology be sustained safely. The findings that are obtained in toxicological studies to clarify the harmful effects of NPs will be the most important reference for the safe production and use of the produced nanomaterial. In this study, α -Fe₂O₃ and γ -Fe₂O₃ NPs whose toxic effects were investigated, after they were exposed to rainbow trout at different concentrations for 10 days and then for 10-day recovery period, all findings suggest that these NPs have similar toxic effects.

Authors' Contributions Mert Gürkan: study conception, histopathological analysis, manuscript preparation, drawing of figures. Selin Ertürk Gürkan: study conception, data analysis, oxidative stress parameters, and accumulation analysis. Sevdan Yılmaz: hematological analysis. Mehmet Ateş: nanoparticle characterization analysis.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethics Approval An official application was made to the Çanakkale Onsekiz Mart University, Animal Experiments Local Ethics Committee for the "Ethics Committee Permit Certificate" before starting the study. Experiments were started after the permit was obtained. All experiments were carried out by experts in accordance with ethical rules.

Code Availability No code availability.

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