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# Effects of nitrogen fertilizer on *Capoeta capoeta*: an immunohistopathological and biochemical investigation

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**Abstract:** Increased use of fertilizers to increase plant production causes important environmental pollution not only in soil but also in water resources. Soil structure, water quality, water systems, and biodiversity are adversely affected by the unconscious use of fertilizers. In this study, *Capoeta capoeta* (Guldenstaedt 1773) were used to investigate the degenerative effects of nitrogen fertilizer. Twenty-four fish were equally allocated into three groups; group 1 (control), group 2 (15 mg/L nitrogen fertilizer in water) and group 3 (15 mg/L nitrogen fertilizer in water). Fish were exposed to the fertilizer for 15 days and then the tissues of gill, liver and intestine were investigated by immunohistopathological and biochemical means to assess the tissue degenerative effects of nitrogen fertilizers in water. Varying degrees of degenerative changes were observed in all of the investigated organs. In histopathological examinations, hydropic degeneration was observed in hepatocytes in fish exposed to fertilizer. Blunting and association in the gill tissue and occasional blunting in the intestinal tissue were detected. Catalase and SOD immunoreactivities increased in liver and gill tissues. There was also an increase in SOD immunoreactivity in the intestine of nitrogen fertilizer exposed fish. Total antioxidant status (TAS) also increased in both of the experimental groups ($p < 0.05$), but total oxidant status (TOS) did not change. The results of the experiment showed that nitrogen fertilizer in water resources may impair health status of fish by adversely affecting tissues in cellular level.

**Key words:** *Capoeta capoeta*, catalase, nitrogen fertilizer, oxidative stress, SOD

## 1. Introduction

Fertilizers are active substances that are commonly and extensively used for restoring plant nutrients in soil, increasing the soil fertility and nutritional quality, and encouraging the development of plants. The need to obtain more products from a unit area brings along the uncontrolled use of chemical fertilizers and pesticides in agricultural areas (Walling and Vaneeckhaute, 2020). Nitrogenous and phosphorus fertilizers take the first place among the fertilizers applied to the soil to increase plant production (Motesharezadeh et al., 2017). Nitrogen fertilizers are applied at increasing levels for agricultural purposes. Thus, alarmingly, nitrate in fertilizers is washed away and mixes with underground water sources that provide drinking water (Karaoğlu, 2021). If the application amount and time of the fertilizer are not chosen appropriately, the nitrogen (N) in nitrogen fertilizers undergoes mineralization. After the excessive use of nitrogen contaminates them. From the contaminated groundwater, nitrate can be transported to surface waters and more importantly negatively affect the fauna in the waters (Kızıloğlu Algan and Bilen, 2005). Fertilizers in the soil are converted to nitrate by microorganisms through nitrification. In this way, the negatively charged nitrate could reach groundwater. Most nitrogen fertilizers cannot be absorbed and therefore contaminate both surface waters and groundwater (Savcı, 2012). Nitrite was shown to induce some genotoxic and histopathological effects on *Capoeta capoeta* (Özcan et al., 2010). Besides nitrogen, many heavy metals such as Cd, Zn, Cu, Ni, and Pb included in the fertilizers may also contribute to the pollution of soil and waters and may cause toxicity in aquatic animals (Wei et al., 2020). The transition and accumulation of heavy metals in aquatic organisms is quite easy. Therefore, the level of environmental pollution can be determined by monitoring the biochemical and physiological markers of aquatic organisms (Kobaza et al., 2021).

Reactive nitrogen produced as a result of human activities causes changes in the global nitrogen cycle and creates alarming environmental effects. The presence of excessive reactive nitrogen in the environment causes nitrate pollution in groundwater, eutrophication in lakes,

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tides in coastal areas and high nitrogen gas emissions into the environment (Shindo et al., 2006). Eutrophication is the response of the ecosystem to the addition of artificial or natural nutrient elements to the aquatic system through detergents, fertilizers or sewage. The increase in substances such as nitrogen and phosphorus in the aquatic ecosystem causes excessive reproduction of algae species. This situation disrupts the oxygen balance in the lake and negatively affects aquatic life (Hasançavuşoğlu and Gündoğdu, 2021). Wild animals and fish in natural resources are primarily and directly affected by the contaminated water (Alkan and Ersin, 2018). Many of the water contaminants are known to induce harmful effects in fish due to induction of excess amount of free radical species (Akbulut et al., 2014).

Free radical species are naturally formed as byproducts of cellular metabolism. They are mostly composed of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are formed as a result of cellular redox reactions (Pham-Huy et al., 2008). High tissue concentrations of ROS and RNS cause oxidative and nitrosative stress, respectively, which mediate degenerations in cellular structures (Taysı et al., 2021). Cells are evolved to coup with these free radical species through some enzymatic and nonenzymatic mechanisms. Among the many of them, superoxide dismutase (SOD) and catalase are the main enzymes playing role in defense against oxidative damage. SOD converts the superoxide anion ($O_2^{\bullet-}$) to $H_2O_2$ and catalase reduces $H_2O_2$ to water, and detoxifies both $H_2O_2$ and organic hydroperoxides (Ighodaro and Akinloye, 2018).

Fishes are affected by pollution and stress factors in their environment (Parlak et al., 2021). Therefore, they are commonly used as indicator organisms in determining toxicity in aquatic environments. They are also good in giving an idea that the environmental toxicants may have adverse effects on human beings since some cellular metabolic pathways show similarities (Stegeman and Lech, 1991). Thus, fishes provide more advantages than other species in diagnosing diseases and monitoring environmental pollution (Parlak et al., 2021). In this study, *Capoeta capoeta* was used to assess the toxic effects of nitrogen fertilizer. Histopathological changes in liver, gill and intestine tissues were evaluated, changes in catalase and SOD activities were compared, and oxidant-antioxidant status were determined to assess the toxic damage in fish.

## 2. Material and methods

### 2.1. Animals and experimental design

The study protocol was approved (Protocol number: 2015-068) by the Institutional Animal Ethics Committee of Kafkas University. Twenty-four *Capoeta capoeta* (Guldenstaedt, 1773) weighing between 120 and 170 g were allocated into three equal groups after a 10-day acclamation period. Fish were kept in 300-L water tanks in the laboratory environment throughout the experiment. 5 ± 0.3 mg/L dissolved oxygen was given to the tanks and the water temperature was adjusted to 18 ± 1 °C. Fish in the first group were used as control and kept in fresh water containing no nitrogen fertilizer. Nitrogen fertilizer at 15 mg/L and 30 mg/L concentrations was included into the tank water of group 2 and group 3, respectively. Nitrogen fertilizer contained 16.5% nitrate nitrogen and 16.5% ammonium nitrogen summing up total of 33%. Fish were exposed to nitrogen fertilizer for 15 days. At the end of the study period, blood samples were taken from the dorsal aorta of fish and then centrifuged at + 4 °C and 3000 rpm for 10 min for biochemical analysis. Tissue samples of liver, gill and intestine were collected and fixed in 10% phosphate buffered formaldehyde solution for immunohistochemical investigations.

### 2.2. Biochemical analysis

Total oxidant status (TOS) and total antioxidant status (TAS) in serum samples were measured spectrophotometrically by commercial kits as suggested by the manufacturer's protocols (Rel Assay Diagnostics, Gaziantep, Turkey).

### 2.3. Histopathology

Formalin fixed tissue samples of liver, gill and intestine were paraffin embedded, and then 5-µ thick sections were cut and regularly stained with hematoxylin-eosin for microscopic view to assess histopathological changes. Olympus BX53 microscope with Olympus DP74 camera attachment was used.

### 2.4. Immunohistochemistry

Catalase and SOD activities in liver, gill and intestine tissues were assessed by avidin-biotin peroxidase immunohistochemical staining method. For this purpose, 4-µ thick sections taken from the paraffin blocks were placed on poly–L-lysine coated slides and then routinely processed through alcohol and xylene. Tissue sections were treated with 3% $H_2O_2$ to block endogen peroxidase activity and then with nonimmune serum to block nonspecific antibody binding. The sections were incubated with primary antibodies; anticatalase antibody (Catalog Number: GTX110704; GENETEX) diluted 1:500 or anti-SOD 1 antibody (Catalog Number: GTX100659; GENETEX) diluted 1:500 with phosphate buffer solution. Then, biotinylated secondary antibodies and horseradish peroxidases were applied consecutively following phosphate buffer washes. Immunoreactivity of the primary antibodies was assessed by diaminobenzidine/$H_2O_2$ treatment. After the reaction was stopped, the sections were stained with hematoxylin, cover slipped, and observed under a light microscope. The results were evaluated

semiquantitively based on the staining intensity; 0: no immunoreactivity, 1: weak immunoreactivity, 2: moderate immunoreactivity, and 3: strong immunoreactivity. For each animal, 10 microscopic fields from each tissue sections were evaluated.

### 2.5. Statistical analysis

Statistical analyses were performed using the SPSS software program version 20.0 for Windows. Results of biochemical investigations were analyzed by one-way ANOVA followed by the Tukey posthoc test. The results of immunohistochemical investigation were evaluated by Kruskal–Wallis H test followed by Conover posthoc test. The data were shown as means ± standard error of means (SEM) and $p < 0.05$ was accepted statistically significant.

## 3. Results

Fish did not show any changes in movement behavior throughout the experimental period in all of the groups. However, slightly increased mucus secretion on the surface of fish exposed to fertilizer was observed.

### 3.1. Serum TOS and TAS

Total oxidant status (TOS) and total antioxidant status (TAS) in serum samples were recorded and the results were shown in Table. TAS in serum increased statistically significant in fertilizer applied groups as compared to control group ($p < 0.05$). TAS increased both in group 2 and group 3 where 15 mg/L and 30 mg/L, respectively, nitrogen fertilizer was added to the tank water. However, no significant difference was observed between these two groups. Serum TOS did not show any significant changes among all three groups ($p > 0.05$).

### 3.2. Histopathological findings

Histopathological findings revealed by nitrogen fertilizer application are shown in Figure 1. Normal histomorphology of liver tissue was observed in fish in control group (Figure 1a). Liver tissue morphology was significantly distorted which was significant by the disruption of hepatic cords in fertilizer exposed fish. Hydropic degeneration, which was characterized by the swollen cytoplasm and occasional necrotic hepatocytes were noted in these fish. Degenerative changes in liver tissue varied from moderate to severe in severity. It was less pronounced in group 2 (Figure 1b) and more pronounced in group 3 (Figure 1c).

Gill tissue of fish in control group had a normal histomorphology (Figure 1d). Varying degrees of degenerative changes in gill tissue were observed in fertilizer exposed fish. Blunting and association in secondary lamellae and vacuolation in some lamellae epithelia were observed in gill tissue of fertilizer given fish. Degenerative changes in both of group 2 (Figure 1e) and group 3 (Figure 1f) were similar and did not show any changes in severity.

Intestinal villi, enterocytes and goblet cells show normal morphology in the control group (Figure 1g). In fertilizer applied groups, occasional blunting and shortening of intestinal villi and increased cellularity in lamina propria were observed. No degenerative changes were noted in columnar epithelial cells. Histomorphological changes in intestine tissue did not show any changes between group 2 (Figure 1h) and group 3 (Figure 1i).

### 3.3. Catalase immunoreactivity

Catalase immunoreactivity resulting from nitrogen fertilizer application is shown in Figure 2. No or very little catalase immunoreactivity was observed in liver tissue of control fish (Figure 2a). There were many catalase immunopositive hepatocytes in liver tissue of fertilizer exposed fish. In locations where degenerative changes are prominent, these immunoreactive cells were seen more frequently and the staining intensity was stronger in cells with pyknotic nuclei. Catalase immunoreactivity was less potent in group 2 (Figure 2b) while it was stronger in group 3 (Figure 2c).

Catalase immunoreactivity was not seen in the gills of control fish (Figure 2d). Varying degrees of catalase immunoreactivity were detected in the secondary lamellar epithelial cells of the gills in fish exposed to the fertilizer. Catalase immunoreactivity was mostly observed in the regions close to the bases where the secondary lamellae connected to the primary lamella. However, no immunostaining was found in the primary lamella sections. Catalase immunoreactivity was less severe in group 2 (Figure 2e) and more severe in group 3 (Figure 2f).

No significant catalase immunoreactivity was found in any of the intestinal sections of the fish in three of the groups (Figures 2g–2i).

**Table.** Total antioxidant status (TAS) and total oxidant status (TOS) in blood serum samples of fish in group1 (control), group 2 (exposed to 15 mg/L nitrogen fertilizer for 15 days) and group 3 (exposed to 15 mg/L nitrogen fertilizer for 30 days). The data are shown as means ± standard error of means and $p < 0.05$ was accepted statistically significant. Different letters in a row indicate statistically significant difference among groups.

| | Group 1 | Group 2 | Group 3 | p value |
|---|---|---|---|---|
| TAS (mmol Trolox Equiv./L) | $1.65 \pm 1.87^{a}$ | $1.83 \pm 0.44^{b}$ | $1.86 \pm 0.09^{b}$ | 0.003 |
| TOS (μmol $H_2O_2$ Equiv./L) | $13.48 \pm 2.73^{a}$ | $15.51 \pm 5.83^{a}$ | $15.47 \pm 5.45^{a}$ | 0.639 |

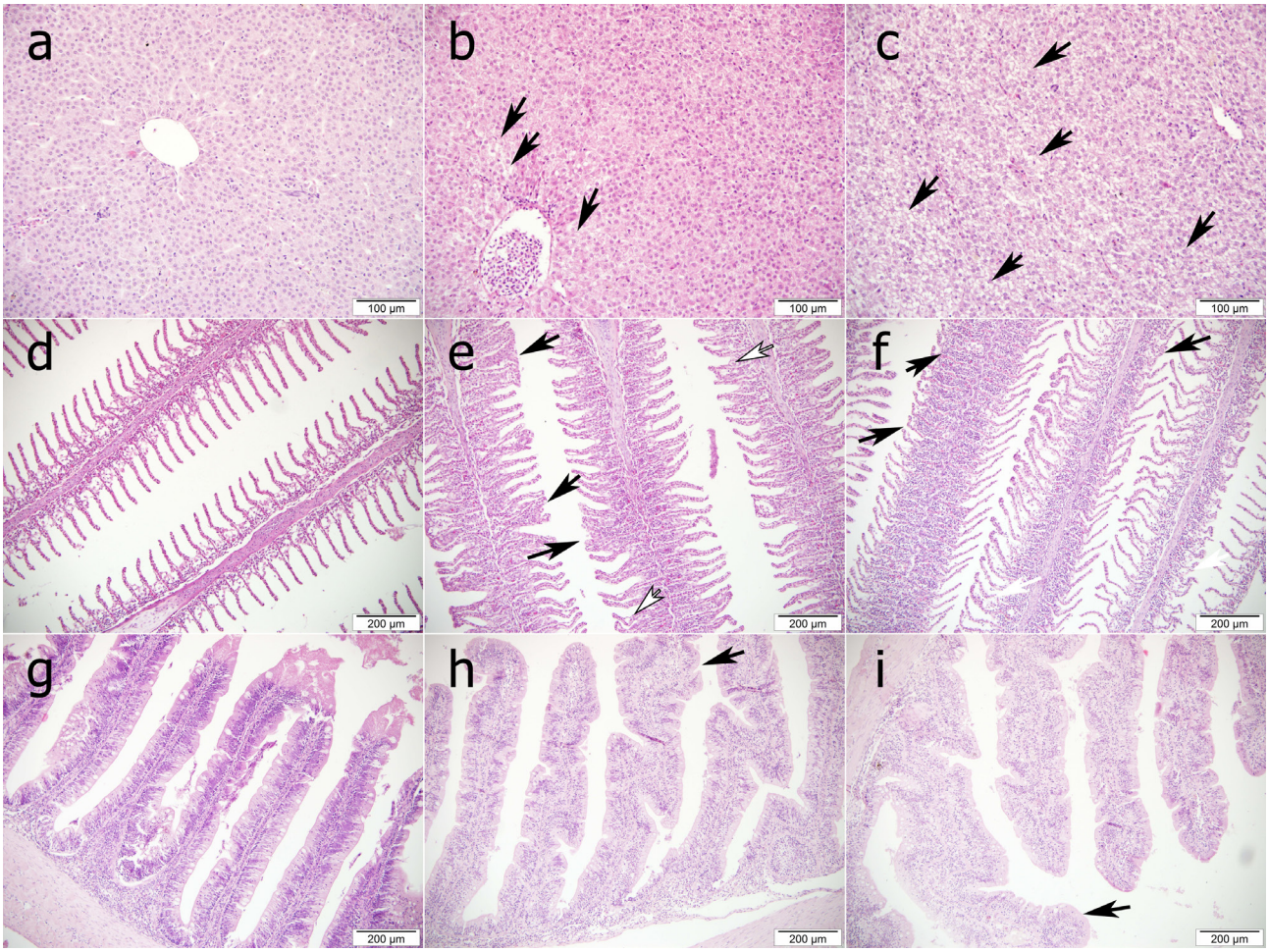


**Figure 1.** Histopathology of liver tissue in group 1 (a), group 2 (b), and group 3 (c). Hydropic degeneration in hepatocytes (black arrows) in nitrogen fertilizer exposed fish. In gill tissue, normal histomorphology in group 1 (d) while blunting and association in some secondary lamellae (black arrows) and some vacuolated epithelia (white arrows) in group 2 (e) and group 3 (f). Normal intestinal morphology in group 1 (g) while occasional blunting in intestinal villi (black arrows) in group 2 (h) and group 3 (i). Group 1: control, group 2: fish exposed to 15 mg/L nitrogen fertilizer for 15 days, and group 3: fish exposed to 30 mg/L nitrogen fertilizer for 15 days. Hematoxylin-eosin.

### 3.4. SOD immunoreactivity

SOD immunoreactivity resulting from nitrogen fertilizer application is shown in Figure 3. SOD immunoreactivity was not observed in liver of fish in control group (Figure 3a). Weak to moderate SOD immunoreactivity was seen in group 2 (Figure 3b) while it was moderate to severe in group 3 (Figure 3c).

In gill tissue, SOD immunoreactivity was mostly not observed, except in few cells in control group (Figure 3d). In group 2 (Figure 3e) and group 3 (Figure 3f), some secondary lamellae cells showed SOD immunoreactivity. They were weakly to moderately stained in both groups and there was no significant difference between the fertilizer exposed groups.

In intestine tissue, no SOD immunoreactivity was observed in control group (Figure 3g). In group 2 (Figure 3h) and group 3 (Figure 3i), however, moderate to strong SOD immunoreactivity was observed in some enterocyte columnar cells with no difference between the two groups.

## 4. Discussion

Nitrogen compounds in aquatic environments are composed of nitrogen, organic nitrogen, nonionized ammonia, ammonium, total ammonia, nitrite and nitrate (Benli, 2006). Among them ammonia and nitrite are the most toxic nitrogen compounds in aquatic environments (Lawson, 1995). Ammonia is mostly produced as a waste product of fish and this affects the nitrogen concentration in aquatic environment. High concentrations of molecular ammonia are known to pass from water through the gill epithelium, and then to the blood and tissues causing toxication (Svobodova et al., 1993). Nitrogen in organic

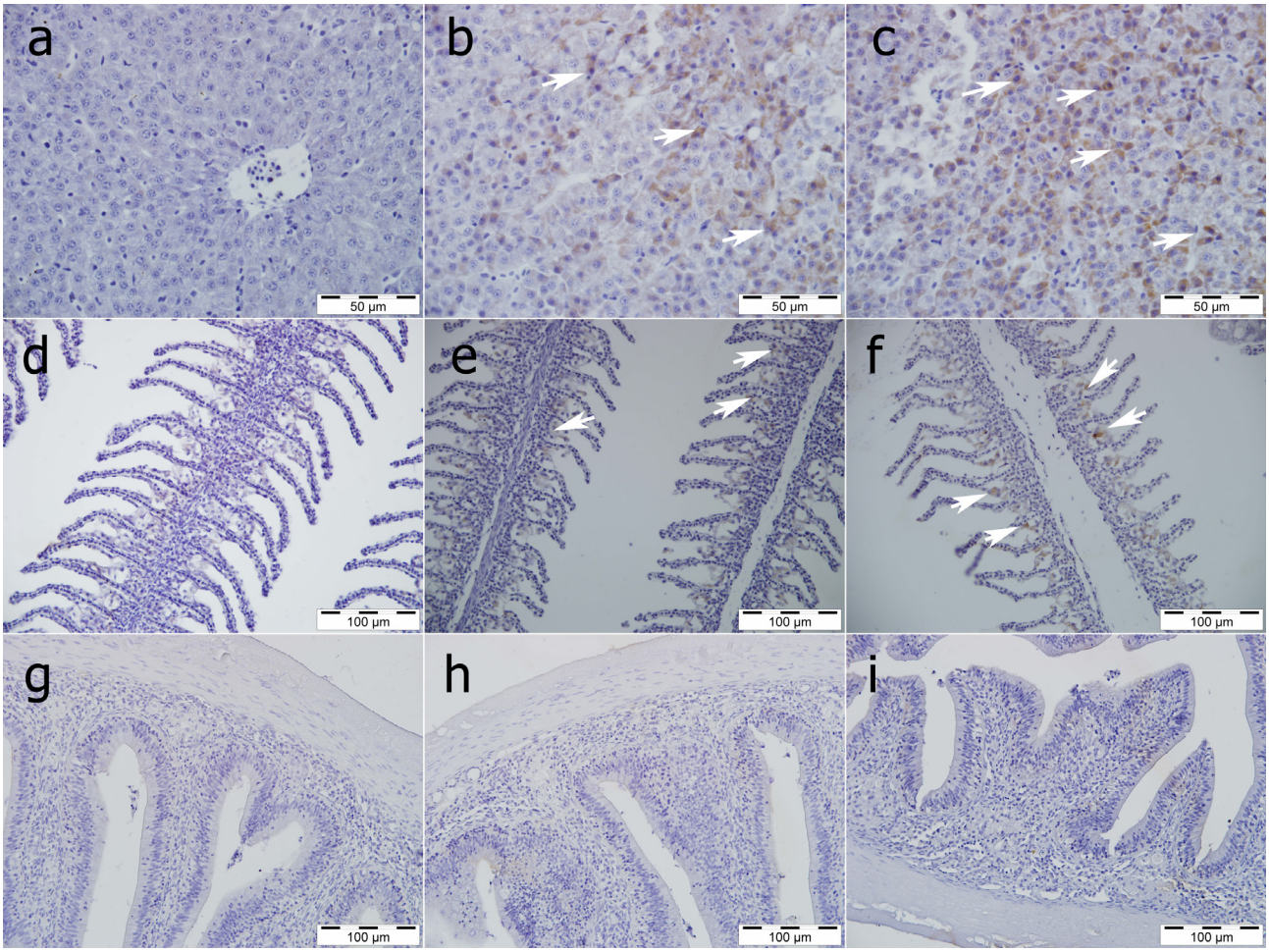


**Figure 2.** Immunohistochemistry for catalase. In liver tissue, no catalase immunoreactivity in group 1 (a) while there are many hepatocytes showing strong immunoreactivity (arrows) in group 2 (b) and group 3 (c). No catalase immunoreactivity is present in gill tissue in group 1 (d). Light to moderate catalase immunoreactive cells were seen in lamellar epithelia (arrows) in group 2 (e) and group 3 (f). No catalase immunoreactivity in intestine tissue in group 1 (g), group 2 (h), and group 3 (i). Group 1: control, group 2: fish exposed to 15 mg/L nitrogen fertilizer for 15 days, and group 3: fish exposed to 30 mg/L nitrogen fertilizer for 15 days.

and inorganic fertilizers are of great interest in aquatic pollution since excess use of nitrogen fertilizers is increasingly in progress where high yield plant production is aimed. Nitrogen concentration in aquatic environments can be affected by various factors and the use of nitrogen fertilizers and their wash off can greatly influence the level, causing toxicity to aquatic animals (Hargreaves, 1998). Different fish species show different level of toxicity and the affected organs and its outcomes vary (Twitchen and Eddy, 1994). Hence, in this study toxic effect of nitrogen fertilizer on *Capoeta capoeta,* which is an important inhabitant of Kars Creek (Turkey), was investigated, giving emphasis on changes in cellular histomorphology and some oxidant and antioxidant markers.

High levels of ammonia in waters can cause various adverse effects in aquatic animals, including degenerative changes in liver, gill, and kidney tissues, decrease in reproduction and growth rate, changes in cerebral energy metabolism, hematological parameters and plasma components, and ultimately death (Twitchen and Eddy, 1994; Randall and Tsui, 2002; Shin et al, 2016). Oxidative stress and impairment in oxygen delivery in ammonia toxicity is an important underlying mechanism in tissue degeneration and death (Wilkie, 1997). Nitrogenous compounds cause varying degrees of degenerative changes in various organs depending on the fish species and the toxic dose and composition of the toxic agent. In the present study, degenerative changes characterized by hydropic degeneration in liver, blunting and association in secondary lamellae and vacuolation in gill epithelia, and blunting and shortening of intestinal villi and increased cellularity in intestine tissues were observed.

Histopathological changes due to various nitrogenous compounds in many fish species and in various organs

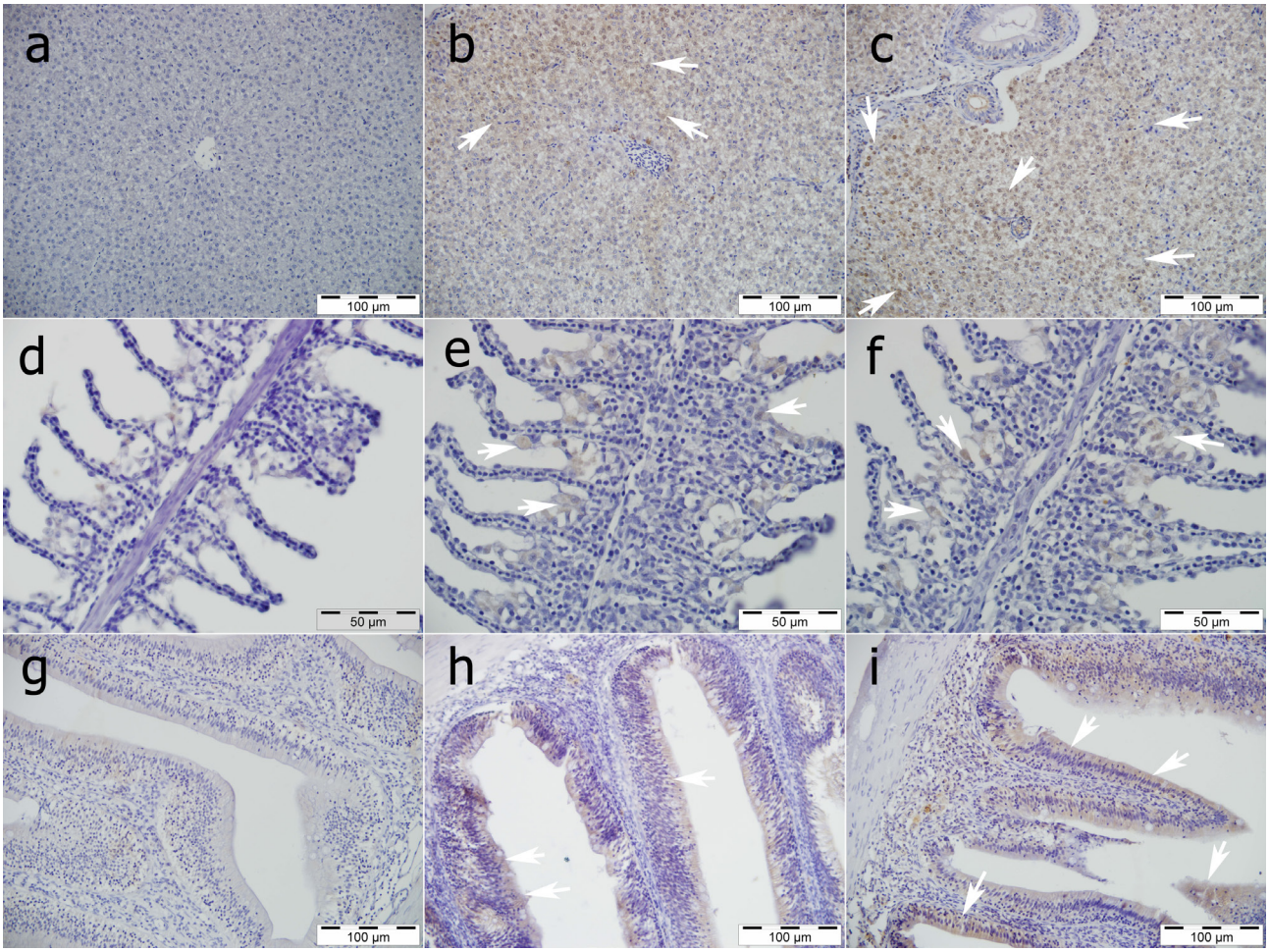


**Figure 3.** Immunohistochemistry for SOD. No SOD immunoreactivity in liver tissue in group 1 (a). Moderate and strong SOD immunoreactivity (arrows) in hepatocytes in group 2 (b) and group 3 (c), respectively. In gill tissue, no SOD immunoreactivity in group 1 (d) while few lamellar epithelial cells show light to moderate SOD immunoreactivity (arrows) in group 2 (e) and group 3 (f). In intestine tissue, no SOD immunoreactivity in group 1 (g) while moderate to strong reactivity (arrows) in intestinal villi epithelia in group 2 (h) and group 3 (i). Group 1: control, group 2: fish exposed to 15 mg/L nitrogen fertilizer for 15 days, and group 3: fish exposed to 30 mg/L nitrogen fertilizer for 15 days.

were recorded and described in the literature. However, descriptive terminology in evaluation of each lesion for every single case seems to change greatly, and this makes their comparison to other studies quite difficult and confusing. In a study investigating the effects of sublethal composite nitrogen fertilizers compounds on rainbow trout (*Oncorhynchus mykiss*), it was indicated that the fertilizers induce degenerative changes in skin, liver, gill and kidney tissues (Capkin et al., 2009). Degenerative changes in gill tissue showed similarities to that of our findings where liver changes in the current investigation showed some differences where hyalin formations were not observed in our study. In a chronic toxicity study where juvenile sea bream (*Sparus aurata*) was exposed to 13 mg/L total ammonia-N for 20 days, hepatocyte atrophy and heterogeneous staining pattern were described; however, no changes were reported in gill and kidney tissues (Wajsbrot et al., 1993). In another chronic study, hypertrophy followed by pyknosis and necrosis in hepatocytes was also reported in *Channa punctatus* exposed to fertilizer ammonium sulfate for 6 months (Ram and Stahyanesan, 1987). In scaly carp (*Cyprinus carpio*) hypertrophy and necrosis were also described in liver, gill and kidneys (Mallet and Sims, 1994).

In an acute toxicity study of ammonia on tilapia (*Oreochromis niloticus L.*) fingerlings, gill hyperplasia and lamella fusion were reported (Karasu Benli and Köksal, 2005). In *Cyprinus carpio var. communis*, ammonia toxicity was shown to induce lamellar fusion, edema, hyperplasia and proliferation of chloride cells in gill lamellae (Chezhian et al., 2012). In *Cyprinus carpio*, ammonia was indicated to cause hyperemia and edema, swelling, aneurysm,

telangiectasia, hypertrophy and hyperplasia in gill tissue (Malik et al., 1986; Peyghan and Takamy, 2002). Curling towards the upper side in the secondary lamellae resulting in the joining with each other in gill tissue was described in *Mystus M. vittatus* exposed to nitrogen-phosphorus-potassium composite fertilizer (Pande and Pande, 1988). In digestive system, swollen intestinal villi were reported in *Ctenopharyngodon idella* exposed to 50 mg/L total ammonia nitrogen (Cao et al., 2021). Therefore, overall, the histopathological findings show some similarities and differences with the literature. However, intestinal lesions described in our study seem to be less common finding. Changes in these findings might be due to different species, concentrations and composition of toxic agent, duration of exposure and many other minor factors in experimental design.

Underlying mechanism behind cellular degeneration in most cases is mediated by molecules called free radicals. Reactive oxygen species such as superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide ($H_2O_2$) are the most intensely studied free radical species (Taufenberger and Magistretti, 2021). They can act with cellular proteins, lipids and DNA, and cause degenerative changes in their structure and function, and ultimately death. These reactive species are perpetually produced as a result of cellular redox reactions (Alkadi, 2020). Hence, organisms are evolved with some enzymatic and nonenzymatic antioxidant systems to eliminate or reduce the degenerative effects of these radicals. Superoxide dismutase (SOD), catalase and glutathione peroxidase are present as the first defense line against reactive oxygen species (Ighodaro and Akinloye, 2018). Therefore, cellular expression levels of these antioxidant enzymes are commonly studied in cellular degenerative changes regardless of the toxic or degenerative agents.

SOD and catalase activities were shown immunohistochemically and seen to increase in liver and gill tissues in the nitrogen fertilizer exposed fish in the current study. Increased SOD activity was also noted in intestine tissue of the fertilizer exposed fish. Increased SOD and catalase activities in liver tissue were reported in *Carassius auratus* acutely exposed to ammonia (Yang et al., 2010). Similarly, increased SOD and catalase activities as well as reactive oxygen species in digestive system were shown in *Ctenopharyngodon idella* exposed to different concentrations of total ammonia nitrogen in an acute study (Cao et al., 2021). Adverse effects of pesticides and herbicides have also been commonly studied in fish and antioxidant enzymes were investigated as the signs of toxicity. In *Oreochromis niloticus* exposed to oxyfluorfen, liver catalase activity was shown to fluctuate with time, initially increase and then decrease in pattern, while SOD activity was reported to decrease (Peixoto et al., 2006). Increase in SOD activity in gill tissues of *Oreochromis niloticus* and *Cyprinus carpio* species exposed to 2,4-dichlorophenoxyacetic acid and azinphosmethyl were reported; however, no changes in SOD activity were recorded in kidney and brain tissues, catalase activity was also shown to increase only in kidney tissue of *Cyprinus carpio* (Oruc et al., 2004). Controversial results about SOD and catalase activities may be due to difference in the toxic agents, toxicity level, toxicity duration, tissue type, and the method used to measure them. Fluctuating expression pattern, toxicity levels and the time when the measurements are done may also greatly influence the result making some wrong interpretation, and vast variety of aquatic organisms even makes it more difficult for proper interpretation and comparison. Against all the difficulties, however, it has been stated that these enzymes can be used as appropriate and safe indicators in pollution studies and ecotoxicological risk assessments (Alak et al., 2011). This is also shown to be true for the current study.

Oxidative stress can be shown by measuring oxidant/antioxidant status in serum or tissues (Fazio et al., 2015). In the current study, TAS and TOS were measured in serum samples of *Capoeta capoeta* exposed to two different concentrations of nitrogen fertilizer. Serum TAS was detected to increase significantly in fertilizer exposed groups compared to the control while there was no statistical difference in TOS among the three groups. Increased antioxidant enzyme levels might reduce the oxidative damage resulting no significant change in oxidative status in the present study. However, histopathological changes observed in liver and gill tissues partially contradict with this, and more degenerative changes in many organs might be needed to show an effect on the serum. On the other hand, increased serum TAS, and tissue catalase and SOD activities might be interpreted as signs of tissue reaction against the toxic substance and partially showed preventive effects on tissues. Different levels of serum or tissue TAS and TOS were shown to occur as a result of different insults in fish (Fazio et al., 2015; Taysı et al., 2021).

In conclusion, *Capoeta capoeta* exposed to 15 and 30 mg/L nitrogen fertilizer for 15 days were shown to be adversely affected, which was prominent with histopathological changes, catalase and SOD levels in liver, gill and intestine and serum TAS and TOS levels. Excess use of these nitrogen fertilizer might, therefore, causes significant toxic effects in aquatic organisms if they wash off from soil to reach freshwater resources. The results indicate that predictive and protective measures in use of nitrogen fertilizers should be monitored continuously.

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and antioxidant levels in the tissues of *Capoeta capoeta* (Guldestaedt 1773) by immunohistochemical and biochemical methods", University of Kafkas.

**Conflict of interest**

The authors have no competing interests to declare that are relevant to the content of this article.

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