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Original Article

# Toxicity effect of the combined salinity and nitrite on biochemical indices, digestive enzymes, histopathology, and cytochrome P450 gene expression responses in fingerlings of stellate sturgeon, *Acipenser stellatus* Pallas, 1771

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**Abstract:** Reactive nitrogen compounds, such as nitrite ($NaNO_2$), are highly toxic to aquatic animals. In some fish species, low levels of sodium chloride significantly reduce the toxicity of nitrite. *Acipenser stellatus*, one of the sturgeon species in the Caspian Sea, can tolerate the salinity of Caspian Sea water (12 $g.L^{-1}$), and chloride ions are predicted to compete with nitrite. Therefore, we conducted this research to determine whether salinity can decrease the effects of nitrite in stellate sturgeon fingerlings. This research was conducted in two stages: the first step involved determining the median lethal concentration ($LC_{50}$) of sodium nitrite at salinities of 0, 4, 8, and 12 $g.L^{-1}$ over a period of 4 days. The LC50-96h values for sodium nitrite ($NaNO_2$) at salinities of 0 to 12 $g.L^{-1}$were found to be 75.11, 93.54, 241.60, and 353.31 $mg.L^{-1}$, respectively. In the second stage, 240 fish (19.1±1.7 cm and 15.23±2.17 g) were exposed to 50% of the LC50-96h of sodium nitrite (at salinities of 0 to 12 $g.L^{-1}$: 37.56, 46.77, 120.80, and 176.579 $mg.L^{-1}$, respectively) for 4 days, with 4 controls and 4 experimental groups in triplicate. In this stage, as salinity and nitrite concentration increased, the activity of superoxide dismutase, alanine aminotransferase, and aspartate aminotransferase in blood serum increased significantly ($P \leq 0.05$). Conversely, the levels of catalase, alkaline phosphatase, and lactate dehydrogenase in blood serum, as well as amylase, alkaline protease, and lipase in the intestine, decreased significantly ($P \leq 0.05$). High nitrite concentrations associated with salinity had damaged the fish's gills, kidneys, liver, and intestine. The highest expression of the cytochrome P450 gene was observed in the liver of *A. stellatus* exposed to a salinity of 12 $g.L^{-1}$ and a nitrite concentration of 176.579 $mg.L^{-1}$ ($P \leq 0.05$). The results indicated that the toxic effects of nitrite on blood indices, intestinal digestive enzymes, tissues, and gene expression did not decrease as salinity increased.

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## Introduction

*Acipenser stellatus* is a native sturgeon species of the Caspian Sea basin, with its caviar and meat of high nutritional and economic value. This species migrates to the Sefid River, in the southern basin of the Caspian Sea, to spawn. All sturgeon species are collapsed and listed under Acipenseridae I or II CITES due to pollution, habitat destruction, overexploitation, and illegal trade in their habitats. Therefore, the development of sturgeon farming can provide meat and caviar while helping conserve their natural resources. By supplying the meat and caviar needed by society and the global market, the pressure on natural marine reserves will be reduced. Under such conditions, in addition to protecting sturgeon stocks in the Caspian Sea basin, attention to the commercial farming of these fish for meat and caviar production is particularly important (Fazli et al., 2020).

Nitrite ($NO_2^-$) is an important environmental pollutant that harms aquatic organisms and is a significant product of the nitrogen cycle. An increase in nitrite levels affects the physiology of aquatic

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animals and can lead to their mortality (Kroupova et al., 2005; Jensen and Rohde, 2010). Elevated nitrite levels in fish farming systems, resulting from disruptions in the water purification system in the Recirculating Aquaculture System (RAS) or from external nitrite sources, impede bacterial metabolic activity, leading to nitrite accumulation (Kamstra et al., 1996). Nitrite inhibits the absorption of chlorine ($Cl^-$) by gill epithelial cells (Larsen et al., 1992; Jensen et al., 2002). Consequently, elevated nitrite levels in the water facilitate its passage through gill epithelial cells, gradually accumulating in the blood plasma (Jensen and Rohde, 2010). Its accumulation in blood plasma affects immune function, red blood cells, tissues, physiological metabolism, and ion regulation (Aggeraard, 2001; Jensen, 2003; Kroupova et al., 2005; Lavado et al., 2014). Exposure to nitrite at different salinities affects hematological, tissue, and molecular parameters in fish, making them effective tools for monitoring fish health in saline water (Abdel-Tawwab and Monier, 2018). Salinity is another environmental variable to consider (Kir and Sunar, 2018).

The effects of salinity on nitrite have scarcely been investigated. Sodium and Sodium chloride in water tend to decrease the toxic effects of nitrite on freshwater fish. Since the exchange process in the gill of freshwater fish involves chloride absorption (with OH and $HCO_3^-$ disposal), nitrite competes with chloride ($CL/HCO_3^-$ interaction) to be absorbed and can thus be replaced with chloride. Nitrite toxicity decreases with increasing chloride concentration in water (Rmesh, 2012). It has been reported that fish tolerance to nitrite exposure may increase in environments with greater salinity (Barbieri and Doi, 2012; Kir and Sunar, 2018; Kir et al., 2019). The key factor in such results is the attenuating effect of monovalent ions, such as chloride ($Cl^-$), on the toxicity of $NO_2^-$ to organisms (Shinn et al., 2013). There is no report on the effects of nitrite in different salinities on physiological indicators in sturgeon fish. But there are many studies suggesting that nitrite interaction in both saline water and fresh water can have an effect on biochemical indices (Das et al., 2004; Zhang et al., 2020), digestive enzymes (Xu et al., 2013; Long et al., 2015), tissue (Duan et al., 2018), and expression of cytochrome p 450 gene (Lavado et al., 2014; Zhang et al., 2011) of fishes. Thus, any changes in these parameters could represent the presence of nitrite pollutants in water (de Olivera et al., 2002). The intensity and duration of responses may vary with concentration, time, and fish species (Heath, 1995).

Since *A. stellatus* can tolerate the salinity of Caspian Sea water (12 $g.L^{-1}$) and chloride ions predicted to compete with nitrite (Wang et al., 2018; Mahmoudi et al., 2024), we hypothesize that salinity reduces nitrite damage in this species. Therefore, this work was conducted to find out if salinity can decrease the effects of nitrite in stellate sturgeon fingerlings. The results of this research can be used to select the best sites for restocking operations. Hence, the present study aims to investigate the effects of nitrite at different salinities on serum biochemical indicators, identify the best sites for restocking operations, and examine cytochrome P450 gene expression in the livers of stellate sturgeon during both rearing and restocking.

## Materials and Methods

This research was conducted at the Chaboksar sturgeon research station in Guilan, Iran, south of the Caspian Sea. Seven hundred twenty specimens, supplied by the International Sturgeon Research Institute in Rasht, Iran, were designated for the study. The intended salinity levels of the water included 0 $g.L^{-1}$ (well water), 4 $g.L^{-1}$ (a mixture of well water and Caspian Sea water), 8 $g.L^{-1}$ (another mixture of well water and Caspian Sea water), and 12 $g.L^{-1}$ (Caspian Sea water). Before starting the experiment, the fish were acclimatized for 4 weeks at salinities of 0, 4, 8, and 12 $g.L^{-1}$. The experiment was conducted in two stages:

**First stage: Determining the $LC_{50}$-96h of sodium nitrite in salinities 0, 4, 8, and 12 $g.L^{-1}$ for stellate sturgeon fingerlings:** At this stage, 480 *A. stellatus* (14.56±1.84 g and 12.8±1.19 cm) were used in toxicity testing to determine the LC50-96h of nitrite using a semi-static renewal technique (e.g., renewal

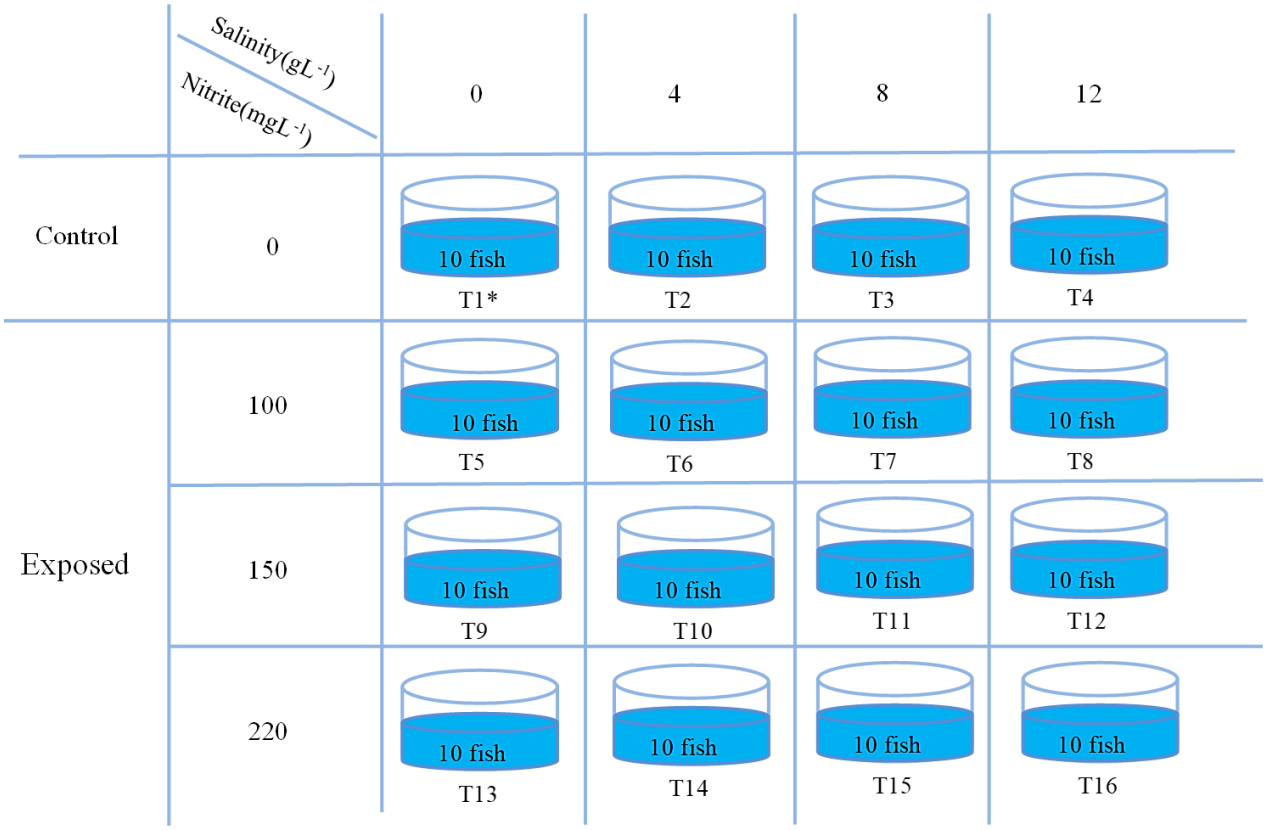


Figure 1. Treatments for determining the LC50-96h (median lethal concentration) of $NaNO_2$ in different salinities in fingerling of stellate sturgeon, *Acipenser stellatus* (*T1-T16: Treatment 1- 16).

every 24 hrs) based on OECD guidance document (Long et al., 2015). We used sodium nitrite ($NaNO_2$; Merck, Darmstadt, Germany; purity ≥ 98%) to prepare nitrite at different concentrations. Fish were kept in 40 circular plastic tanks (76 cm diameter, 0.3 cm height, and a volume of 60 liters, filled with 40 liters of water, with 10 fish per tank) and exposed to different nitrite concentrations (100, 150, and 220 $mg.L^{-1}$) (Golipour et al., 2017) in water with salinities of 0, 4, 8, and 12 $g.L^{-1}$ for 96 hrs (Fig. 1). For each treatment, three replicates were set up. The fish remained unfed during this time to avoid the unwanted effects of feed and/or fish feces. After 96 hours, the death rate and fish mortality were calculated. Using a Probit analysis, the LC50 (96 h) for nitrite was determined in water at salinities of 0, 4, 8, and 12 $g.L^{-1}$ (Ghafarifarsani et al., 2021). There was a daily 75% water renewal using a $NaNO_2$ solution. Temperature (21.37±0.39°C), pH (8.04±0.13), and DO (dissolved oxygen) (7.67±0.17 $mg.L^{-1}$) were measured daily during the experiment using a Hach Multi-parameter liquid analyzer (HQ 40d, Hach-Lange Company, USA). Salinity was measured using a refractometer (S/Mill-E Ophthalmometer- Japan).

**Second stage: Effects the 50% of LC50-96h [median lethal concentration] of sodium nitrite [$NaNO_2$] in salinities 0, 4, 8, and 12 $g.L^{-1}$ for fingerling of stellate sturgeon:** After specifying the LC50-96h of nitrite in the first stage, *A. stellatus* were subjected to the 50% of $LC_{50}$-96h of nitrite for 4 days (Fig. 2). A total number of 240 fish with an average weight of 14.56±1.84 g were used in this experiment. The fish were randomly distributed into 24 plastic tanks (76 cm in diameter, 0.3 cm in height, and a volume of 60 liters), which were filled with 40 liters of water, and stocked with 10 fish per tank. The tank's water was constantly aerated and replaced. For each treatment, three replicates were devised. This experiment included the use of the semi-static renewal technique (75% water renewal per 24 hrs. through the addition of sodium nitrite ($NaNO_2$). Temperature (16.1±0.52°C), dissolved oxygen (7.81±0.11 $mg.L^{-1}$), and pH (8.04±0.12) were measured daily during the experiment using a Hach Multi-parameter liquid

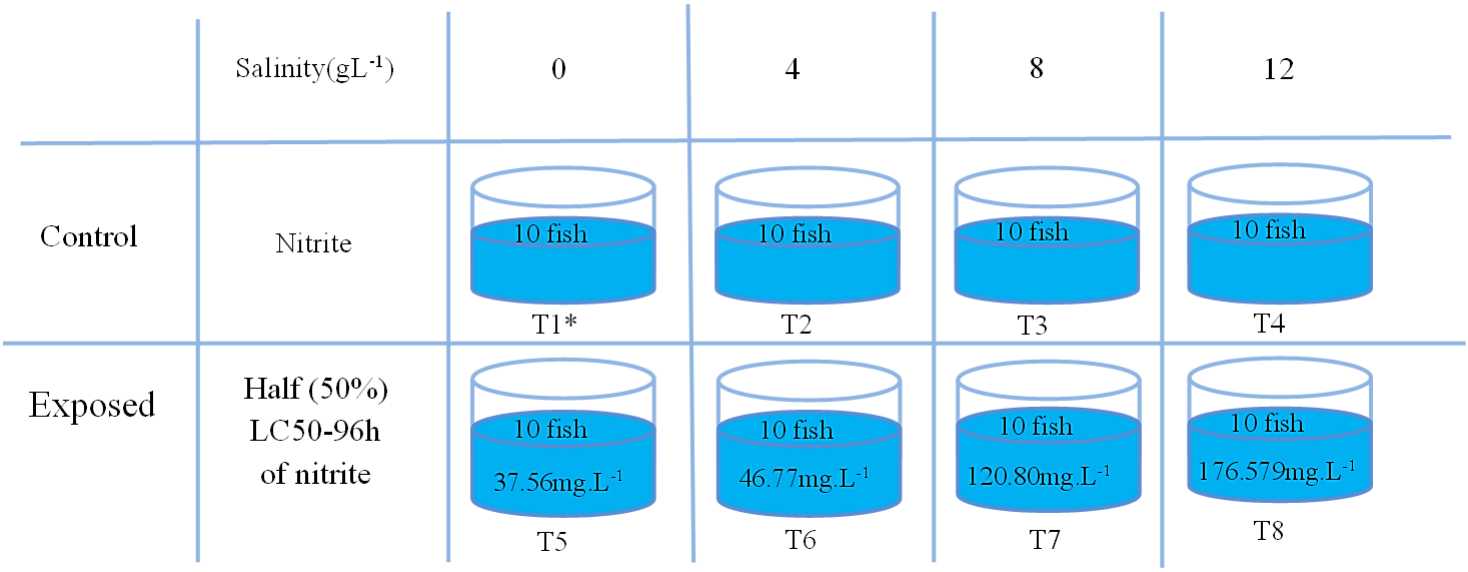


Figure 2. Treatments for study of the effects of the 50% of LC50-96h (median lethal concentration) of $NaNO_2$ in different salinities in fingerling of stellate sturgeon, Acipenser stellatus (*T1-T8: Treatment 1-8).

analyzer (HQ 40d, Hach-Lange Company, USA). A refractometer (Atago, S/Mill-E, Handheld Salinity, Japan) was used to measure water salinity. Sampling was performed on day 4 for the experimental groups. For each treatment, three replicates were devised. The fish remained unfed during this time so as to avoid the unwanted effects of feed and/or fish feces. After 4 days, 17 fish from each replicate of each group were selected randomly for the biochemical, digestive enzyme activity, histopathological, and gene expression p450 assays. First, the fish were anesthetized with a fresh clove powder solution (0.5 $g.L^{-1}$; clove powder dissolved in one liter of water) (Rahbar et al., 2019). Then, blood samples were collected from the caudal vein using a heparinized syringe. Serum was obtained by centrifuging blood samples at 3000 rpm for 10 min at 4 °C and stored at -80°C for further biochemical analyses (Adel et al., 2015).

**Determination of biochemical indexes:** The levels of aspartate aminotransferase (AST), lactate dehydrogenase (LDH), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) in serum were determined using commercial kits (ZellBio GmbH Company, Germany) by a biochemical auto-analyzer (Prestige 24i, Boeki, Japan) (Mansourghanaei et al., 2022). Activities of glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) in serum were determined using commercial kits (ZellBio GmbH Company, Germany (ELIZA Reader, BI OTEK, RS232, USA), based on the method described by Bolann and Ulvik (1991), Paglia and Valentine (1967), and Goth (1991), respectively.

**Determination of intestinal tissue digestive enzymes:** At the end of the fourth day, 3 fish were selected from each treatment, and their intestines were removed to measure the activities of amylase, lipase, and alkaline protease. The intestines were cut into pieces and homogenized in 0.9% NaCl solution (de Paiva-Maia et al., 2013). The homogenized solutions were centrifuged at 5,000g for 5 min. The supernatants were used for the enzymatic evaluations. The protein ratio in tissues was determined using the Bradford method (1976), expressed as U/mg (Lakwani et al., 2022). All digestive enzyme analyses were performed with a Photometer (EMRA, E-600, Japan). Lipase, Amylase, and alkaline protease activity were assayed according to Iijima et al. (1998), Bernfeld (1951), and Anson (1938), respectively.

**Histopathological changes in gill, liver, kidney, and Intestine:** The gills, kidney, liver, and intestine tissues were removed and fixed in aqueous Bouin's solution. After fixation for 48 h, tissues were dehydrated through a graded series of ethanol, cleared in xylene, and infiltrated in paraffin. Sections of 5 m were prepared from paraffin blocks by using a rotary

Table 1. The characteristics of the primers used in the study of cytochrome P450 gene expression in fingerlings of stellate sturgeon, *Acipenser stellatus* (Safari et al., 2016).

| Primer name | Primer sequence | Tm | Product length | Primer performance |
|---|---|---|---|---|
| Ap p450q-PCRF | GTCATCTGTGCCATGTGCTT | 56 | 237 | 99% |
| Ap p450q-PCRR | TCTTGTCGAAGGAGCGGTAG | 56 | | |
| β- actin q-PCRF | TTGCCATCCAGGCTGTGCT | 56 | 215 | 99% |
| β- actin q-PCRR | TCTCGGCTGTGGTGAA | 56 | | |

Table 2. The LC50-96h (median lethal concentration) of sodium nitrite in fingerlings of stellate sturgeon, *Acipenser stellatus*, at different salinities.

| Nitrite concentration (with 95% confidence ) (mg.L$^{-1}$) | | | | | 50%, half of LC50-96h (median lethal concentration) of sodium nitrite (mg.L$^{-1}$] |
|---|---|---|---|---|---|
| Time / Salinity (g.L$^{-1}$) | 24 h | 48 h | 72 h | 96 h | |
| 0 | 180.61 | 105.56 | 90.44 | 75.11 | 37.56 |
| 4 | 347.15 | 215.48 | 114.41 | 93.54 | 46.77 |
| 8 | 899.09 | 304.32 | 277.54 | 241.60 | 120.80 |
| 12 | 1582.31 | 857.11 | 616.14 | 353.31 | 176.579 |

microtome. These sections were then stained with hematoxylin–eosin (H&E). At the end of the experiment, all prepared sections were examined under an optical microscope equipped with a camera (BEL photonic BIO2T, Monza, Italy) (Anson, 1938).

**Samples collection and gene analysis:** A total of 9 fish from each treatment (3 fish of each repetition) were randomly selected. Their livers were removed, and samples were placed into RNAase-free microtubes, immediately frozen in liquid nitrogen, and stored at -80ºC until RNA extraction and gene expression analysis. BIOZOL RNA extraction kit (Bioflux-Bioer, China) was used for total RNA extraction. Agarose gel electrophoresis and spectrophotometric analysis were used to determine RNA quality and quantity. The complementary DNA (cDNA) was synthesized using SuperScript III reverse transcriptase (Invitrogen) with an oligo dT18 primer. Real-time PCR was performed on an iCycler (BioRad, USA) using SYBR Green qPCR master mix (Fermentase, France) as follows: 3 min at 95°C, then 45 cycles of 20 s at 95°C, 20 s at 60°C, and 20 s at 72°C. The reaction was carried out in triplicate for each sample. Table 1 represents the primer used in this study. Beta-actin was used as a reference gene to normalize the expression of the target genes. After verification of primer efficiencies, gene expression was determined using the 2^-ΔΔCt method (Livak and Schmittgen, 2001). Based on the results of our previous studies, the stability of β-actin as a reference gene has been validated (Safari et al., 2016; Safari et al., 2022).

**Statistical analysis:** LC50 values at 24, 48, 72, and 96 h were estimated using Probit Analysis (Finney, 1971). The mortality percentage was calculated and converted to a probit value using the probit table. The data obtained to determine the relative expression of the P450 gene compared to beta-actin by the 2^-ΔΔCt method using REST were analyzed using software (Pfaffl et al., 2002). The Shapiro-Wilk test was applied to assess the normality and homogeneity of data. The experiment was designed for analysis using repeated-measures analysis of variance (two-way ANOVA) to determine the effects of nitrite and salinity exposure on the treatments. The two-way ANOVA first assessed the interaction between nitrite and salinity on responses. As interactions occurred, one-way ANOVA, followed by Tukey's test at a significance level of $P<0.05$, was used to determine treatment responses. All data were reported as mean±SD. All Data were analyzed in SPSS (SPSS 19.0, IBM Software Inc., Chicago, IL).

## Results

**LC50 of sodium nitrite:** After 24, 48, 72, and 96 hours, the LC50-96h of nitrite was determined at salinities ranging from 0 to 12 g.L$^{-1}$ (Table 2). According to the results, as salinity increased from 0

Table 3. Two-way ANOVA results for biochemical indices in the blood serum of fingerling stellate sturgeon, *Acipenser stellatus*, exposed to salinity and the 50% of the LC50-96h (median lethal concentration) of sodium nitrite after 4 days.

| Item | | *P*-value | | |
|---|---|---|---|---|
| | | Salinity | Nitrite | Salinity + Nitrite |
| Antioxidant activities | CAT | 0.000 | 0.690 | 0.021 |
| | SOD | 0.000 | 0.025 | 0.004 |
| | GPX | 0.123 | 0.710 | 0.138 |
| Liver enzymes | ALT | 0.046 | 0.001 | 0.000 |
| | AST | 0.000 | 0.000 | 0.000 |
| | ALP | 0.000 | 0.000 | 0.000 |
| | LDH | 0.000 | 0.000 | 0.000 |

Numbers less than $P<0.05$ indicate the significance of the desired variable.

Table 4. Antioxidant activities change in the blood serum of fingerlings of stellate sturgeon exposed to salinity and the 50% of the LC50-96h of sodium nitrite after 4 days.

| Treatments | CAT(u/mL) | SOD (u/mL) | GPX (u/mL) |
|---|---|---|---|
| Control (Salinity + Nitrite) | | | |
| T1 (0 $g.L^{-1}$+ 0 $mg.L^{-1}$)* | 42.67±2.52 $^{bc}$ | 54.67±1.53 $^{a}$ | 121± 5.57 $^{a}$ |
| T2 (4 $g.L^{-1}$+ 0 $mg.L^{-1}$) | 42.67±3.21 $^{bc}$ | 57.67±2.52 $^{a}$ | 126.33±7.02 $^{a}$ |
| T3 (8 $g.L^{-1}$+ 0 $mg.L^{-1}$) | 36.33±2.08 $^{ab}$ | 59.33±3.21 $^{ab}$ | 120.33±3.78 $^{a}$ |
| T4 (12 $g.L^{-1}$+ 0 $mg.L^{-1}$) | 41.67±2.08 $^{bc}$ | 61.67±3.52 $^{ab}$ | 124.33±4.73 $^{a}$ |
| Exposed (Salinity + half LC50-96h of nitrite) | | | |
| T5 (0 $g.L^{-1}$+ 37.56 $mg.L^{-1}$) | 45.33±3.51 $^{c}$ | 53±3.61 $^{a}$ | 116.67±7.51 $^{a}$ |
| T6 (4 $g.L^{-1}$+ 46.77 $mg.L^{-1}$) | 47.67±3.06 $^{c}$ | 54.33±4.51 $^{a}$ | 120.33±7.09 $^{a}$ |
| T7 (8 $g.L^{-1}$+ 120.80 $mg.L^{-1}$) | 31.67±3.06 $^{a}$ | 71.67±4.72 $^{c}$ | 127.67±6.11 $^{a}$ |
| T8 (12 $g.L^{-1}$+ 176.579 $mg.L^{-1}$) | 36.67±4.04 $^{ab}$ | 68.33±3.06 $^{bc}$ | 131±4.58 $^{a}$ |

Different lowercase letters indicate significant differences among reagent treatments at $P<0.05$, and the data are presented as mean±SD. T1-T8: Treatment 1- 8.

to 12 $g.L^{-1}$, the LC50 concentration decreased from 24 hours to 96 hours. The LC50 concentration rose from 37.56 ($mg.L^{-1}$) at a salinity of 0 ($g.L^{-1}$) to 176.57 ($mg.L^{-1}$) at a salinity of 12 $g.L^{-1}$ after 96 hours.

**Serum biochemical indices:** The interaction between salinity and nitrite on GPX factor was not significant) ($P\geq0.05$), whereas the interaction effect was significant in CAT, SOD, ALT, AST, ALP, and LDH ($P\leq0.05$) (Table 3). The results showed that increasing water salinity in the control treatment was associated with significant increases in CAT and SOD ($P\leq0.05$). With increased nitrite concentration and salinity in combined treatments, CAT decreases while SOD increases ($P\leq0.05$). For GPX, no significant differences were observed among treatments ($P\leq0.05$). The highest activity of GPX, SOD, and CAT parameters was observed in T1 (0 $g.L^{-1}$ + 0 $mg.L^{-1}$), T7 (8 $g.L^{-1}$ + 120.80 $mg.L^{-1}$), and T8 (12 $g.L^{-1}$ + 176.579 $mg.L^{-1}$) (Table 4). The results indicated that an increase in nitrite at a salinity of 12 $g.L^{-1}$ resulted in significant increases in ALT and AST levels, whereas ALP and LDH showed significant declines ($P\leq0.05$). The maximum ALT and AST values were recorded in T8, while ALP showed the highest level in T3, and the highest LDH in T5 (Table 5).

**Digestive enzymes activities of intestinal tissues:** The interaction effect was significant in amylase, lipase, and alkaline protease ($P\leq0.05$) (Table 6). According to the results, the digestive enzyme activities had an interaction between nitrite and salinity. According to the results, amylase, lipase, and alkaline protease activities decreased with increasing salinity in Control T1 (0 $g.L^{-1}$ + 0 $mg.L^{-1}$), T2 (4 $g.L^{-1}$ + 0 $mg.L^{-1}$), T3 (8 $g.L^{-1}$ + 0 $mg.L^{-1}$), and T4 (12 $g.L^{-1}$ + 0 $mg.L^{-1}$) ($P\leq0.05$). With the increase in salinity and nitrite in combined treatments (5 to 7), amylase and alkaline protease activities increased, peaking at T8 ($P\leq0.05$). Lipase showed a downward trend in T5 (0 $g.L^{-1}$ + 37.56 $mg.L^{-1}$) to T8 ($P\leq0.05$). The highest amylase, lipase, and alkaline protease were found in T8, T5, and T8, respectively ($P\leq0.05$) (Table 7).

Table 5. Liver enzyme changes in the blood serum of fingerlings of stellate sturgeon, *Acipenser stellatus*, exposed to salinity and 50% of the LC50-96h of sodium nitrite after 4 days.

| Treatments | ALT (U/L) | AST (U/L) | ALP (U/L) | LDH (U/L) |
|---|---|---|---|---|
| Control (Salinity + Nitrite) | | | | |
| T1 (0 $g.L^{-1}$+ 0 $mg.L^{-1}$)* | 16.33±2.08 $^{a}$ | 121.33±4.73 $^{a}$ | 201.67±6.51 $^{cd}$ | 917±15.39 $^{a}$ |
| T2 (4 $g.L^{-1}$+ 0 $mg.L^{-1}$) | 18.33±1.53 $^{ab}$ | 173.67±6.11 $^{c}$ | 187.67±6.81 $^{bc}$ | 884±11.53 $^{a}$ |
| T3 (8 $g.L^{-1}$+ 0 $mg.L^{-1}$) | 13.33±1.53 $^{a}$ | 159.67±4.16 $^{c}$ | 242.67±7.09 $^{e}$ | 1021±15.87 $^{b}$ |
| T4 (12 $g.L^{-1}$+ 0 $mg.L^{-1}$) | 14.67±0.58 $^{a}$ | 137.33±6.66 $^{b}$ | 217.33±3.06 $^{d}$ | 997.33±12.06 $^{b}$ |
| Exposed (Salinity + half LC50-96h of nitrite) | | | | |
| T5 (0 $g.L^{-1}$+ 37.56 $mg.L^{-1}$) | 14.67±1.53 $^{a}$ | 135.33±5.68 $^{ab}$ | 201.33±8.62 $^{cd}$ | 1450.33±38.01 $^{d}$ |
| T6 (4 $g.L^{-1}$+ 46.77 $mg.L^{-1}$) | 15.33±2.08 $^{a}$ | 143.33±5.51 $^{b}$ | 158±6.56 $^{a}$ | 1243±35.04 $^{c}$ |
| T7 (8 $g.L^{-1}$+ 120.80 $mg.L^{-1}$) | 22.67±3.51 $^{b}$ | 190.67±4051 $^{d}$ | 188.33±8.02 $^{bc}$ | 914±26.51 $^{a}$ |
| T8 (12 $g.L^{-1}$+ 176.579 $mg.L^{-1}$) | 23.33±2.08 $^{b}$ | 198±7 $^{d}$ | 177.33±7.02 $^{ab}$ | 880±35.51 $^{a}$ |

Different lowercase letters indicate significant differences among reagent treatments at $P<0.05$, and the data are presented as mean±SD. ***Control** (Salinity + Nitrite): T1-T8: Treatment 1- 8.

Table 6. The results of Two-way ANOVA for digestive enzyme activities of fingerling of stellate sturgeon, *Acipenser stellatus*, exposed to salinity and the 50% of the LC50-96h of sodium nitrite.

| Digestive enzyme activities | P-value | | |
|---|---|---|---|
| | Salinity | Nitrite | Salinity + Nitrite |
| Amylase | 0.000 | 0.000 | 0.000 |
| Lipase | 0.000 | 0.000 | 0.000 |
| Alkaline protease | 0.000 | 0.000 | 0.000 |

**Histopathology of gill, liver, kidney, and intestine:** The histopathology of the gill in stellate sturgeon across various treatments shows that alterations increase with rising salinity and nitrite concentrations. Fusion of secondary lamella due to hyperplasia, hyperplasia of the mucous-secreting cells in the epithelium, hemorrhagic lesions on the gill, destroy cartilage in gill filaments, cartilaginous core disruption, lamellar aneurysm, destruction epithelial cells covering secondary lamellae, clubbing of gill lamellae, loss of epithelium completely lost by necrosis in the primary lamellae, detachment the epithelial layers on the two sides of the secondary lamellae, curling of the secondary lamellae, pillar cells degeneration, edema in the primary lamellae, secondary lamellae necrosis, and primary lamellae necrosis were the observed Histopathological changes (Fig. 3). The greatest gill lesions were observed in T8.

The common lesions observed in liver tissue include the melano-macrophage center, sinusoid, cloudy swelling of liver cells, liver hemorrhage, sinusoidal dilatation and congestion with blood, vacuolar degeneration of hepatocytes, red blood cells in the sinusoids, atrophy of hepatocytes, karyolysis, pyknosis, karyorrhexis, and necrosis of hepatocytes. The greatest damage to the kidneys was observed in T4, T5, T6, and T8 (Fig. 4).

The major complications detected in the kidney are as follows: dilation of cellular degeneration of tubules, karyolysis, pyknotic nuclei, karyorrhexis, necrosis of tubular epithelial cells, vacuolar degeneration, occlusion of the tubular lumen, renal tubules atrophy, necrosis of the haematopoietic interstitial, necrosis of bowman's capsule cells, necrosis glomeruli, dilation of bowman's space, haemorrhage, haematopoietic interstitial tissue, and red blood cell (Fig. 5). The alteration across different treatments, with nitrite exposure, was of greater extent. With increasing salinity and nitrite concentrations, damage to kidney tissue increased.

The loss of epithelial cells, distortion of columnar epithelial cells, distortion of the lamina propria, necrosis in the muscularis, damage to the serosa, hypertrophy of goblet cells, and flattening of microvilli were the most commonly observed lesions in the intestinal tissue across different treatments (Fig.

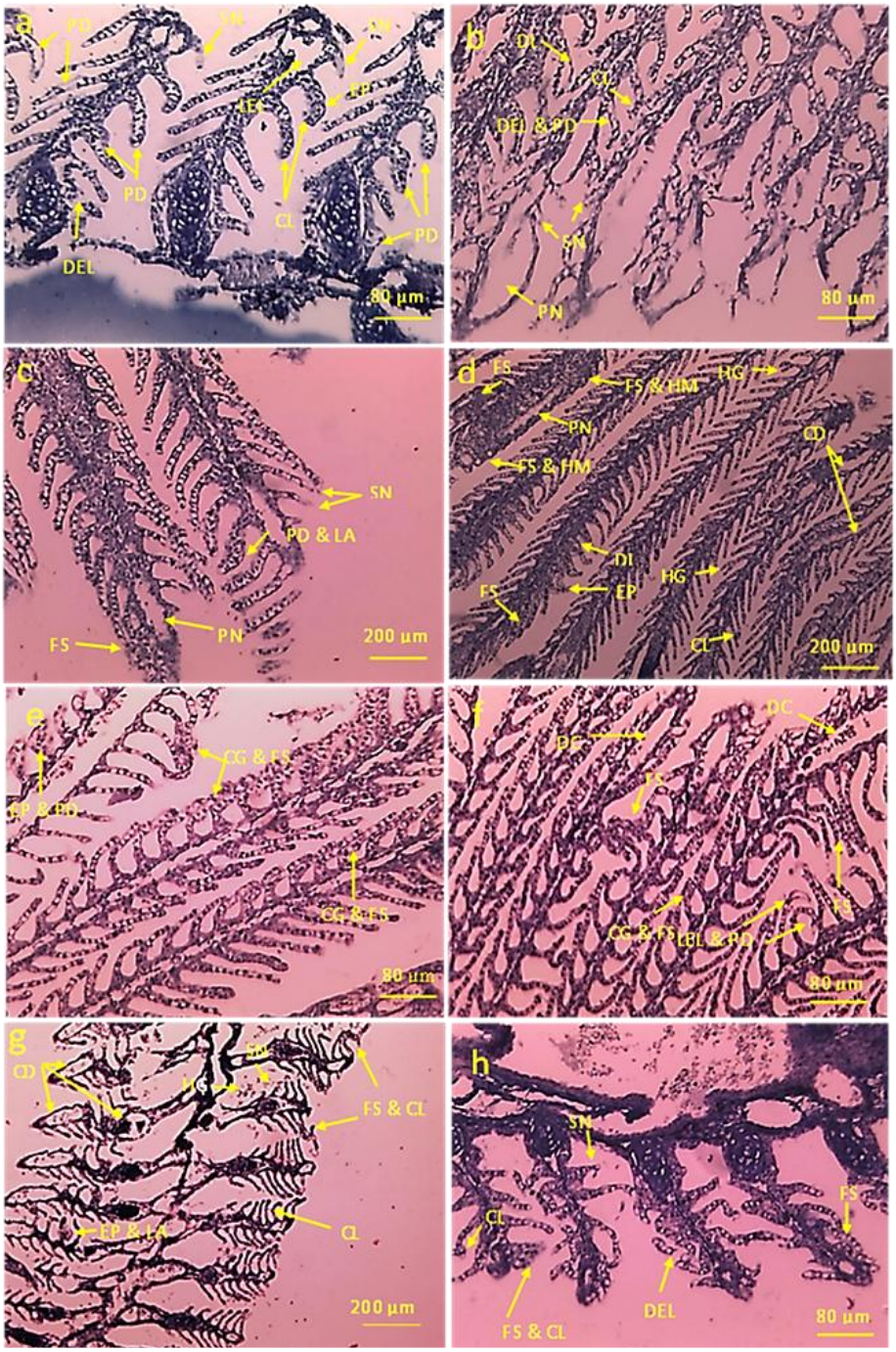


Figure 3. Changes in the gill tissue of fingerling stellate sturgeon, *Acipender stellatus* exposed to salinity and 50% of the LC50-96h of sodium nitrite of $NaNO_2$ after 4 days (H&E). **Abbreviations:** Hemorrhagic lesions on the gill (HG). Destroy cartilage in gill filaments (DC). Cartilaginous core disruption (CD). lamellar aneurysm (LA). Destruction epithelial cells covering secondary lamellae (DEL). Clubbing of gill lamellae (CG). Loss of epithelium completely lost by necrosis in the primary lamellae (LEL). detachment the epithelial layers on the two sides of the secondary lamellae (DL). Curling of the secondary lamellae (CL). Pillar cells degeneration (PD). Edema in the primary lamellae (EP). Secondary lamellae necrosis (SN). Primary lamellae necrosis (PN). Control: (Salinity + Nitrite): a (T1); b (T2); c (T3); d (T4), Exposed: (Salinity + 50% LC50-96h of nitrite): e (T5); f (T6); g (T7); h (T8). (see Figure 2 for treatments).

6). Most of the intestinal lesions were observed in combination treatments the intensity of changes was dependent upon nitrite concentration and salinity. The greatest damage to the intestine was observed associated with increased nitrite concentration in T8. The results indicated that damage to the gill, kidney, liver, and intestinal tissues in nitrite-treated treatments increased with increasing salinity levels. Nevertheless, such damage from nitrite exposure was shown to be much greater than that in the control.

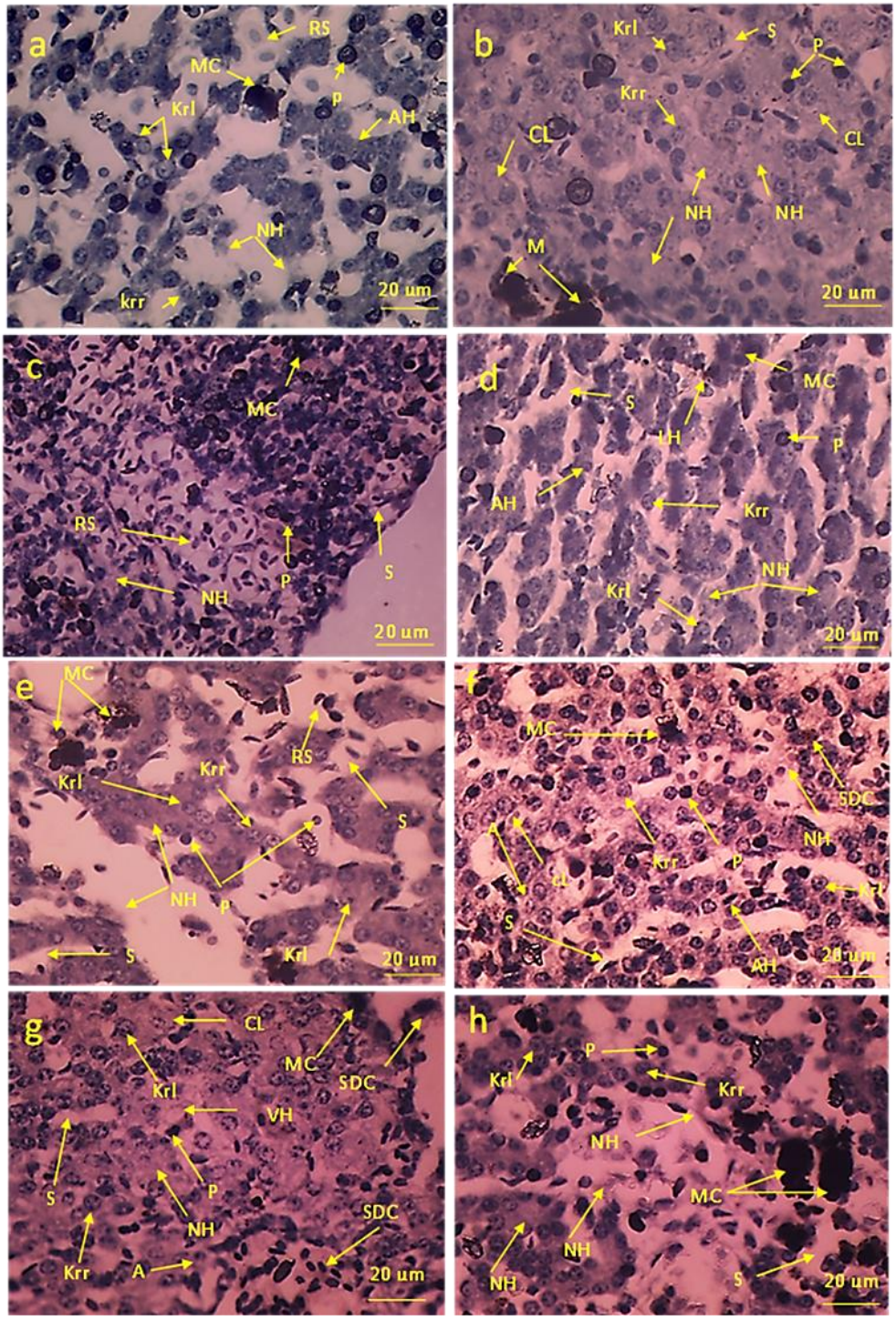


Figure 4. Changes of the liver tissue of stellate sturgeon, *Acipenser stellatus*, exposed to salinity and 50% of the LC50-96h of sodium nitrite after 4 days (H&E). **Abbreviations:** Melano macrophage center (MC), Sinusoid (S), Cloudy swelling in liver cells (CL), Liver hemorrhage (LH), Sinusoidal dilatation and congestion with blood (SDC), Vacuolar degeneration of hepatocytes (VH), Red blood cells in sinusoids (RS), Atrophy in hepatocytes (AH), karyolysis (Krl), Piknosis (P), karyorrhexis (Krr), and Necrosis in the hepatocytes cells (NH). Control: (Salinity + Nitrite): a (T1); b (T2); c (T3); d (T4), Exposed: (Salinity + 50% LC50-96h of nitrite): e (T5); f (T6); g (T7); h (T8). (see Figure 2 for treatments).

**Gene expression cytochrome P450:** With the increasing salinity in the treatments exposed to nitrite at 50% of LC50-96h, gene expression showed a significant increase in *A. stellatus*. In the exposed treatments (salinity + the 50% of LC50-96h of nitrite), a significant difference was observed in terms of the interaction (Two-way Anova: salinity (0), nitrite (0), salinity*nitrite (0)) effects of salinity and nitrite stress

Table 7. Digestive enzyme activities change in the intestines of fingerling stellate sturgeon, *Acipenser stellatus*, exposed to salinity and the 50% of LC50-96h (median lethal concentration) of sodium nitrite after 4 days.

| Treatments | Amylase (U/mg protein/min) | Lipase (U/mg protein/min) | Alkaline protease (U/mg protein/min) |
|---|---|---|---|
| Control (Salinity + Nitrite) | | | |
| T1 (0 $g.L^{-1}$+ 0 $mg.L^{-1}$)* | 11.50 ±0.182 $^{c}$ | 1.81±0.065 $^{a}$ | 3.88±0.075 $^{b}$ |
| T2 (4 $g.L^{-1}$+ 0 $mg.L^{-1}$) | 10.05±0.128 $^{a}$ | 1.93±0.031 $^{a}$ | 4.64±0.081 $^{c}$ |
| T3 (8 $g.L^{-1}$+ 0 $mg.L^{-1}$) | 10.87 ±0.143 $^{b}$ | 2.28±0.035 $^{b}$ | 4.75±0.046 $^{c}$ |
| T4 (12 $g.L^{-1}$+ 0 $mg.L^{-1}$) | 13.27±0.156 $^{d}$ | 1.91±0.065 $^{a}$ | 3.17±0.042 $^{a}$ |
| Exposed (Salinity + 50% LC50-96h of nitrite) | | | |
| T5 (0 $g.L^{-1}$+ 37.56 $mg.L^{-1}$) | 11.50 ±0.182 $^{c}$ | 2.48±0.070 $^{c}$ | 5.39±0.090 $^{d}$ |
| T6 (4 $g.L^{-1}$+ 46.77 $mg.L^{-1}$) | 10.05±0.128 $^{a}$ | 1.90±0.060 $^{a}$ | 3.99±0.167 $^{b}$ |
| T7 (8 $g.L^{-1}$+ 120.80 $mg.L^{-1}$) | 10.87 ±0.143 $^{b}$ | 1.91±0.071 $^{a}$ | 4.09±0.100 $^{b}$ |
| T8 (12 $g.L^{-1}$+ 176.579 $mg.L^{-1}$) | 13.27±0.156 $^{d}$ | 1.86±0.060 $^{a}$ | 5.58±0.086 $^{d}$ |

Different lowercase letters indicate significant differences among reagent treatments at P<0.05, and the data are presented as mean±SD. T1-T8: Treatment 1-8.

on gene expression ($P \leq 0.05$). P450 gene expression did not differ significantly as salinity increased from 0 to 12 $g.L^{-1}$ at a constant nitrite level ($P > 0.05$). But a significant increasing trend was observed with simultaneous increases in salinity and nitrite, rising from 5.09-fold of the control at salinity 0 ($g.L^{-1}$) + Nitrite 37.56 ($mg.L^{-1}$) to 20.92 at salinity 12 ($g.L^{-1}$) + Nitrite 176.57 ($mg.L^{-1}$) (Fig. 7).

## Discussions

**$LC_{50}$ of nitrite ($NaNO_2$):** The $LC_{50}$-96h for nitrite, which is the concentration of an aqueous chemical in the environment that causes 50% mortality in an exposed population (Boudou and Ribeyre, 1997). The fish in the control treatments survived for 96 hours without mortality. With increasing time from 24 to 96 hours and decreasing salinity, the $LC_{50}$ in exposed fingerling of stellate sturgeon was increased. On the fourth day, as salinity increased from 0 to 12 $g.L^{-1}$ and $LC_{50}$ increased, the tolerance of fingerlings to nitrite toxicity increased. Similar results have been reported in Rachycentron canadum (Barbieri and Doi, 2012), Sparus aurata (Kir and Sunar, 2018), and *Dicentrarchus labrax* (Kir et al., 2019).

**Serum biochemical indices:** Fish possess an effective antioxidant system to counteract the toxic effects of free radicals. Superoxide dismutase (SOD), catalase (CAT), and peroxidase (GSH-Px) are crucial detoxification enzymes of ROS (Reactive Oxygen Species) in fish. SOD enzymatically breaks down $H_2O_2$ into $O_{2-}$, while CAT converts $H_2O_2$ into $O_2^-$ and water. SOD and CAT act as primary antioxidant defense enzymes (Abele et al., 2011). These enzymes play vital roles in preventing the generation of hydroxyl ions by reducing $O_2^-$ and $H_2O_2$ levels. Changes in oxidative stress enzyme levels can indicate the fish's condition (Faheem and Lone, 2018; Santana et al., 2018). Although antioxidant defense is strong, overproduction of ROS and disruption of this defense (e.g., inhibition of enzyme activity) can lead to the oxidation of biological molecules (Hellou et al., 2012). In the current work, SOD and CAT in the serum of *A. stellatus* significantly increased under combined treatments (Exposed: T5-T8). This increase in enzyme activity likely results from the elimination of excess free radicals under nitrite stress (Jensen et al., 2002). Zhang et al. (2020) showed that nitrite toxicity in yellow catfish (*Pelteobagrus fulvidraco*) caused a marked decrease in superoxide, catalase, and glutathione peroxidase enzyme activities. Kim et al. (2020) noted that exposure of *Paralichthys olivaceus* to nitrite significantly raised SOD activity. The nitrite exposure resulted in a significant increase in SOD activity in *Paralichthys olivaceus* (Kim et al., 2019) and hybrid grouper (*Epinephelus lanceolatus* ♂ × *Epinephelus fuscoguttatus* ♀) (Kim et al., 2022). Molayemraftar et al. (2022) reported that the serum CAT activity in *Cyprinus carpio* was significantly higher in the exposed groups with nitrite than in the control group after 96 h. Similar results were also found by Xu et al. (2011) with *Larimichthys crocea*.

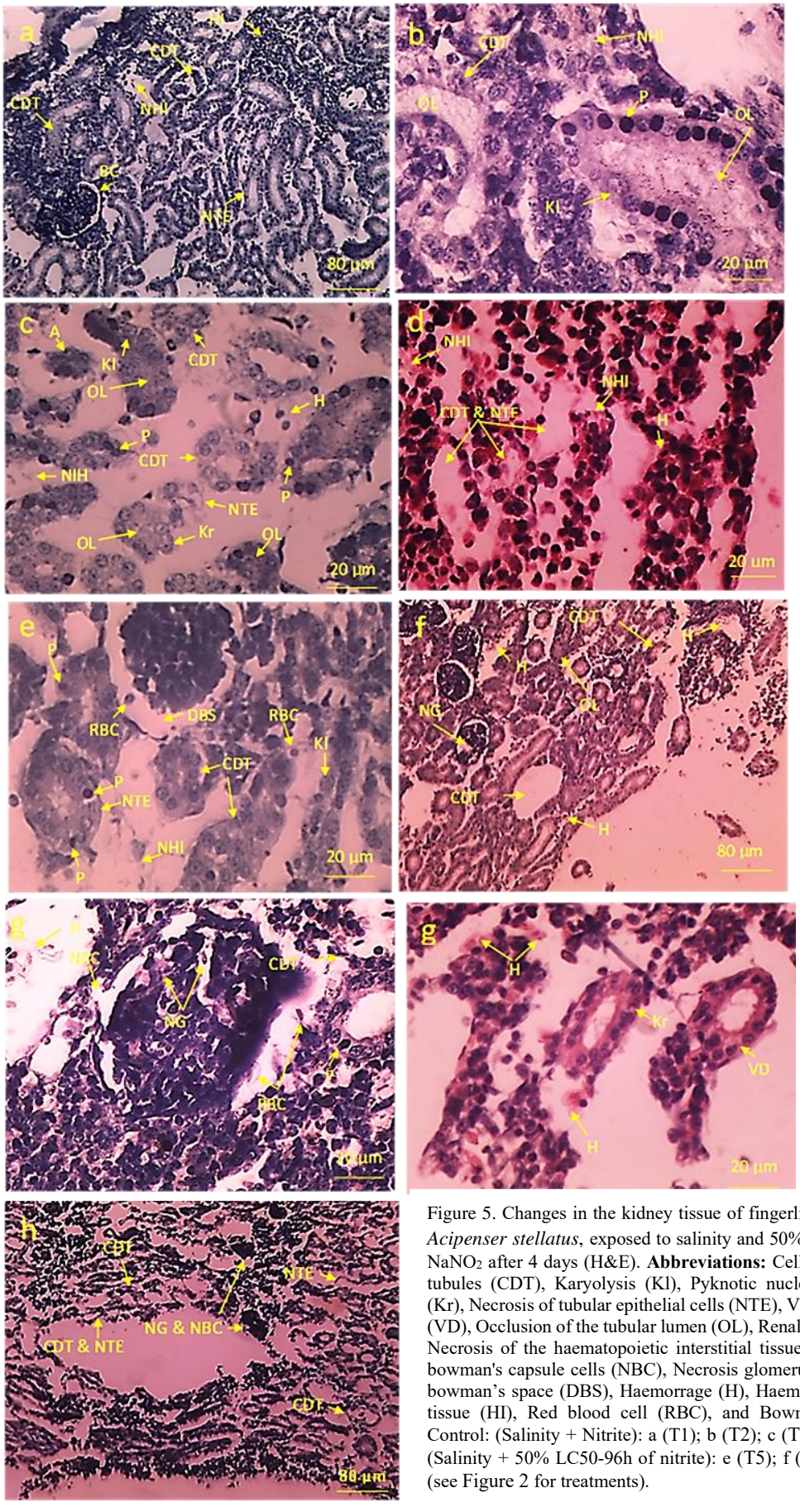


Figure 5. Changes in the kidney tissue of fingerling stellate sturgeon, *Acipenser stellatus*, exposed to salinity and 50% of the LC50-96h of $NaNO_2$ after 4 days (H&E). **Abbreviations:** Cellular degeneration of tubules (CDT), Karyolysis (Kl), Pyknotic nuclei (P), Karyorrhexis (Kr), Necrosis of tubular epithelial cells (NTE), Vacuolar degeneration (VD), Occlusion of the tubular lumen (OL), Renal tubules atrophy (A), Necrosis of the haematopoietic interstitial tissue (NHI), Necrosis of bowman's capsule cells (NBC), Necrosis glomeruli (NG), Dilation of bowman's space (DBS), Haemorrage (H), Haematopoietic interstitial tissue (HI), Red blood cell (RBC), and Bowman's capsule (BC). Control: (Salinity + Nitrite): a (T1); b (T2); c (T3); d (T4), Exposed: (Salinity + 50% LC50-96h of nitrite): e (T5); f (T6); g (T7); h (T8). (see Figure 2 for treatments).

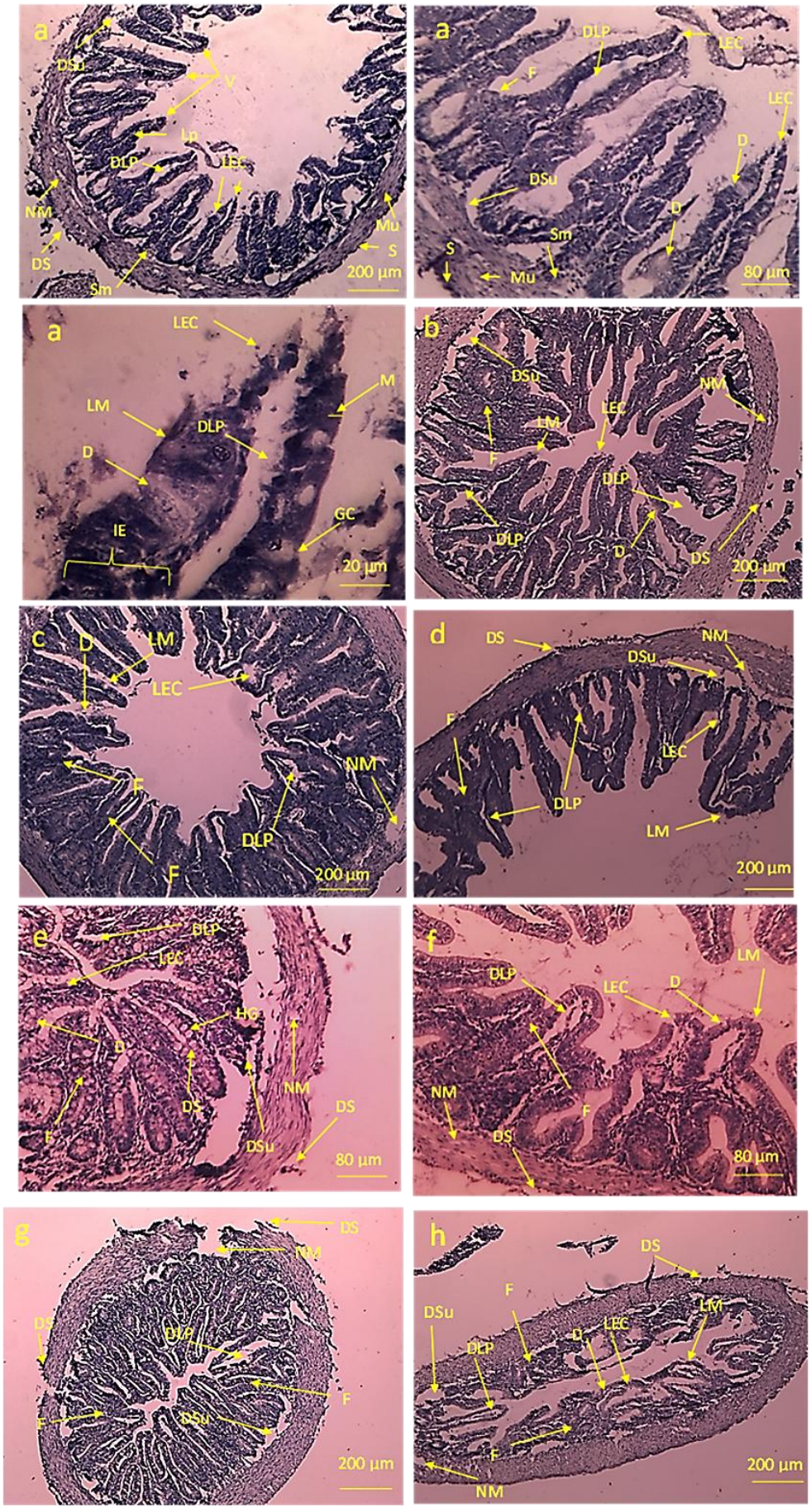
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Figure 6. Changes in the intestinal tissue of fingerling stellate sturgeon, Acipenser stellatus, exposed to salinity and 50% of the LC50-96 h of $NaNO_2$ after 4 days (H&E). **Abbreviations:** Serosa (S), Muscularis (Mu), Submocosa (Sm), Villi (V), lamina propria mucosa (Lp), Intestinal epithelial (IE), Goblet cell (GC), Microvilli (M), Loss of epithelial cell (LEC), Distortion of CEC (D), Distortion of lamina propria (DLP), Loss of microvilli (LM), Fusion of villies (F), Degeneration of submucosa (Dsu), Necrosis in muscularis (NM), Damage of serose(DS), and Hypertrophy of goblet cells (HG). Control: (Salinity + Nitrite): a (T1); b (T2); c (T3); d (T4), Exposed: (Salinity + 50% LC50-96h of nitrite): e (T5); f (T6); g (T7); h (T8). (see Figure 2 for treatments).

In the current study, there was no significant difference in GPX across the treatment.

In the present study, the activities of AST, ALP, and LDH in the serum of stellate sturgeon fingerlings were significantly reduced in the control treatments (salinity without nitrite), whereas ALT was not. AST and ALT activities increased in the exposed treatments, whereas ALP and LDH activities decreased significantly. ALP reduction can probably be attributed to a decrease in the substrate's intracellular pH due to nitrite buildup in the exposed fish's blood (Huang and Chen, 2002). Increased AST and ALT activity in serum probably resulted from increased synthesis of aminotransferases (Rajyasree and Neeraja, 1989) in the liver and subsequent leakage into the circulation due to nitrite-induced tissue damage. The increase in ALT and AST activities in serum in our study supports earlier findings and serves as an indicator of tissue damage (Das et al., 2004; Cheng, 2020). Decreases in hepatic LDH levels may result from liver damage and necrosis induced by toxicants (Bernet et al., 2001). The variations in liver enzyme activity observed in this study may stem from species differences, toxin type, exposure duration, nitrite dose, and other unidentified factors (Rahbar et al., 2019). Changes in these enzymes (AST, ALT, LDH, and ALP) may be due to fish exposure to nitrite (Das et al., 2004; Cheng, 2020).

The results of our study showed that although increased salinity may reduce nitrite toxicity on stellate sturgeon fingerlings, due to the fact that $NO_2$ is thought to compete for the same transport site as chloride ions in the $HCO_3^-/Cl^-$ exchanger (located in the apical side of the gill cells). Then, increased salinity prevents the direct effect of $NO_2$ on *A. stellatus* (Waisberg et al., 2033), but salinity had no effect on reducing the effects of nitrite on the activity of biochemicals in the serum of fingerling stellate sturgeon.

**Digestive enzyme activities of intestinal tissues:** The present research showed that in the control treatments (salinity + no nitrite), elevated salinity markedly reduced digestive enzyme activity. At first, the activities of amylase and alkaline protease decreased with increasing nitrite concentration and salinity, but then increased in the last treatment (12 $g.L^{-1}$ + 176.579 $mg.L^{-1}$), which had the highest salinity and nitrite concentration. The initial reduction of digestive enzymes might be due to oxidative stress, induced by nitrite exposure. This can disrupt the integration of intestinal villi and cause intestinal inflammation (Ip and Chew, 2010). The increased protease and amylase levels detected in the last treatment could result from hormesis triggered by nitrite toxicity in the fish gut (Schram et al., 2010). Another cause of the increase in such enzymes in fish guts might be the energy-compensation processes that occur during environmental stress to meet the energy demands of stress metabolism (Cao et al., 2021). Lipase, whose main duty is in digesting fats and lipids and maintaining the healthy functioning of the gallbladder, showed a significant decrease with increased salinity and nitrite concentration. This means that long-term exposure to nitrite might have an impact on carbohydrate absorption in stellate sturgeon fingerlings. Digestive enzymes play an important role in fish resistance to nitrite toxicity and tend to offset stress effects by adjusting protean activity or abundance (Zhang et al., 2011; Xu et al., 2011). Long et al. (2015) reported that nitrite decreased digestive enzyme activities in Nile tilapia. Xu (2013) noted depressed digestive enzyme activity in *Charybdis Japonica* induced by lower nitrite concentrations (3.0 and 1.5 $mg.L^{-1}$).

**Histopathology of gill, liver, kidney, and intestine:** Tissue damage due to salinity alone and with nitrite on gill tissue was numerous, but it was more in nitrite treatments. In the present study, secondary lamellae necrosis, fusion of secondary lamella due to hyperplasia, curling of the secondary lamellae, and

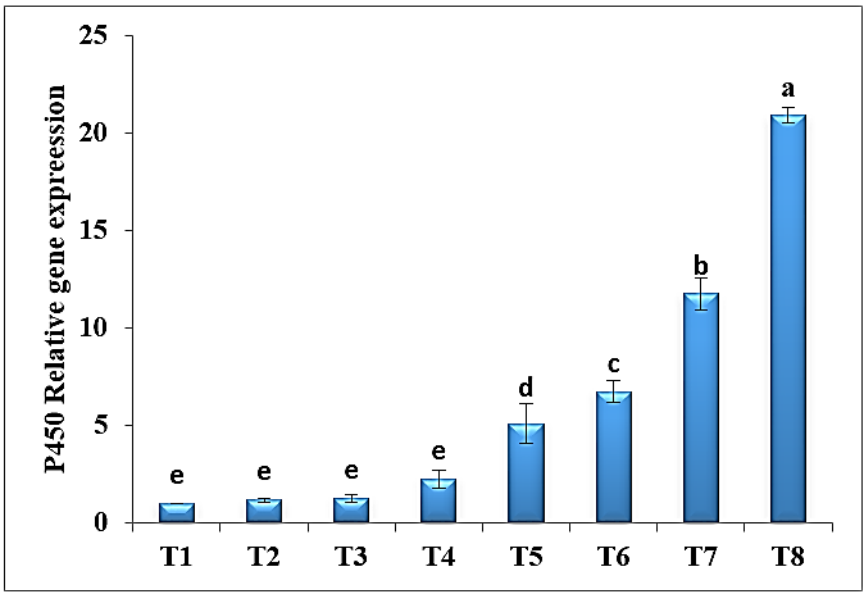


Figure 7. The cytochrome P450 gene expression in the liver tissue of *Acipenser stellatus* exposed to salinity and 50% of LC50-96h of $NaNO_2$ after 4 days. Different lowercase letters indicate significant differences among reagent treatments at $P<0.05$ (see Figure 2 for treatments).

cartilaginous core disruption were more evident. Similar results were also reported by Michael et al. (1987) in *Clarias lazera*, Baskar (2014) in *Cirrhinus Mrigala*, Gholipour et al. (2017) in *A. stellatus*, and Grynevych et al. (2018) in *Oncorhynchus mykiss*.

The present work showed that the liver of stellate sturgeon fingerlings had the greatest alternations in T4 and in T5, T6, and T8. The most notable symptoms included the melanomacrophage center, necrosis in the hepatocytes, sinusoids, and karyorrhexis. Gholipour et al. (2017) reported that the liver of juvenile *A. stellatus* gave indications of hyperplasia, cholestatic hepatitis, lipid degeneration, cellular necrosis, and cellular atrophy following 96 hrs of exposure to nitrite. Similar results were also found by Grynevych et al. (2018) in juvenile *O. mykiss* and Ajani et al. (2011) in *Clarias gariepinus*. The kidney is among the organs most severely affected by toxic chemicals (Grynevych et al., 2018). The study also indicated damage to kidney tubule cells in all treatments, except for treatment 1, where the damage was acute. Meanwhile, necrosis of tubular epithelial cells, karyolysis, necrosis of glomeruli, and necrosis of Bowman's capsule cells were more prevalent and more salient in T8. Grynevych et al. (2018) report that acute nitrite exposure has led to histopathological alterations in the kidney of exposed juvenile rainbow trout. Gholipour et al. (2017) showed that juvenile *A. stellatus* experienced hyperplasia and cellular necrosis in the kidneys after 96 hrs.

In this study, the intestinal tissues were primarily subjected to the combined effects of salinity and nitrite, which were more pronounced in T8, with observed epithelial loss; distortion of columnar epithelial cells and the lamina; loss of microvilli and the lamina propria; fusion of villi; and degeneration of the submucosa. Pereira et al. (2017) reported that higher nitrite doses were associated with greater tissue damage, including goblet cell hyperplasia in protogaster and enterocyte hypertrophy in the hindgut of *Danio rerio*. In another study on white shrimp (*Litopenaeus vannamei*), 72 hours of nitrite exposure damaged intestinal mucus tissues, causing the epithelium to lose its basement membrane and adhere to one another (Duan et al., 2018). Similar results were also reported by Cheng et al. (2020) for the mud crab (*Scylla paramamosain*).

**Gene expression cytochrome P450:** At the molecular level, changes in genetic and protein levels can also be considered as an indicator of exposure to pollution, especially if the molecule under investigation is part of the defense, repair, or detoxification mechanism of the cell, among which we can mention cytochrome P450 (Colombo et al., 2003; Safari et al., 2014). The

cytochrome P450 enzyme is a hemoprotein present at high levels in the liver and has also been reported in other tissues, including gills, kidneys, and intestines (Huang and Chen, 2002; Siroka and Drastichova, 2004; Safari et al., 2014). This protein is associated with the endoplasmic reticulum membrane and mitochondria and is one of the oxidant enzymes produced in the first phase of pollutant metabolism (Safari et al., 2014). Its amount is affected by the species investigated, gender, type, concentration, and duration of exposure to the pollutant, as well as the tissue examined (Huang et al., 2014; Safari et al., 2014).

In the current study, CYP450 expression is significantly induced after 96 h of exposure to high nitrite and salinity. The cytochrome P450 gene expression in stellate sturgeon liver is the result of exposure to nitrite stress, which is most likely due to the accumulation of nitrite in the *A. stellatus* liver tissue. Andleeb et al. (2022) reported that, with the increase in nitrate content, CYP1A levels in *Carassius carassius* increased considerably. Guo et al. (2016) reported that CYP450 expression is significantly induced after nitrite exposure for 4, 8, and 12h, suggesting that CYP450 is involved in the detoxification of nitrite, an environmental toxicant, by up-regulating mRNA expression levels. Lavado et al. (2014) investigated four orthologous cytochrome P450 (CYP1A, CYP2K1, CYP2M1, and CYP3A27) in the liver, gill, and olfactory tissues under various salinities. Except for CYP1A, which decreased in the liver, the protein expression of three other enzymes was induced at salinities higher than 32 $g.L^{-1}$, with the highest increase of CYP2M1observed in olfactory tissues. There are many reports on P450 gene expression in fish in response to various pollutants, including difenoconazole in *Oryzias melastigma* (Zhang et al., 2017). Endosulfan on Persian sturgeons (*Acipenser persicus*) (Safari et al., 2016), cadmium chloride ($CdCl_2$) on *A. persicus* (Safari et al., 2014), and oil on *A. persicus* (Aboutalebi et al., 2020).

Many pollutants produce toxic ROSs that have toxic effects on cells and destroy biological molecules, such as proteins, lipids, and DNA. The first reaction of cells to such stressful conditions is the production of several oxidative enzymes, such as P450 enzymes, and antioxidants, which can disrupt normal metabolism and, if the stressful conditions persist, lead to cell death. It seems that the transcription factor of the aryl hydrocarbon receptor (AHR) is responsible for changes in the P450 expression in exposure to such pollutants. Since these pollutants prevent extracellular calcium from entering cells due to their similar charge, organelles such as mitochondria and the endoplasmic reticulum respond to this cytoplasmic calcium deficiency by releasing calcium. Therefore, intracellular calcium activates proteinase K, which phosphorylates transcription factors at their promoters, thereby increasing their expression (Wang et al., 2018). The results of the present study showed significant changes in gene expression in fish exposed to salinity and nitrite, and a direct relationship between gene expression and increased salinity and nitrite was observed.

## Conclusion

After 96 hours of exposure of *A. stellatus* to sodium nitrite in different salinities for the determination of LC50-96 concentration, with increasing salinity from 0 to 12 $g.L^{-1}$, nitrite concentration increased from 75.11 to 353.31 $mg.L^{-1}$, which indicates an increase in *A. stellatus* tolerance against nitrite toxicity. In the second stage of our experiment, when the fish were exposed to 50% of the LC50 of sodium nitrite across different salinities, no mortality was observed after 4 days. In this stage, blood indices (antioxidant activity and liver enzymes in blood serum) of *A. stellatus* exposed to the 50% of the LC50-96h nitrite showed the greatest changes compared to the control group. The activity of intestinal tissue digestive enzymes [amylase, lipase, and alkaline protease] in the group exposed to half of the LC50-96 h nitrite, compared to the control group, decreased as nitrite concentration increased at a salinity of 12 $g.L^{-1}$. The most damage to the gill, kidney, liver, and intestine tissues was observed at 12 $g.L^{-1}$ salinity and 176.579 $mg.L^{-1}$ nitrite in the group exposed to the 50% of the LC50-96h nitrite. The results showed significant changes in

gene expression in fish exposed to salinity and nitrite, with a direct relationship between gene expression and increased salinity and nitrite levels. Although nitrite toxicity in *A. stellatus* decreased with increasing salinity, as indicated by the higher LC50-96 values, the exposed group showed changes in blood serum biochemical indices, intestinal tissue digestive enzymes, tissue damage, and increased P450 gene expression with increasing salinity. Since *A. stellatus* can tolerate the salinity of Caspian Sea water (12 $g.L^{-1}$) and chloride ions are predicted to compete with nitrite, we hypothesize that salinity reduces nitrite damage in *A. stellatus*.

## Ethical approval

All applicable guidelines for the care and use of animals were followed in accordance with the national ethical framework for animal research in Iran. All experiments were performed in accordance with the protocol approved by the Committee of Ethics of the Faculty of Sciences, University of Tehran (357; November 8, 2000).

## Acknowledgements

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