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Individual and Combined Effects of Salinity and Nanoparticles (Al_2O_3, TiO_2) on the Activity of Antioxidant Enzymes in Freshwater Fish (*Oreochromis niloticus*)

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Abstract

Salinity increase in freshwaters affects the physiology and metal uptake in fish, though there is no enough evidence on the influence of salinity on metal-oxide nanoparticle (NPs) toxicity. Therefore, the effects of salinity and NPs (Al_2O_3, TiO_2) were tested in acute (2 days and 10 mg NPs/L) and chronic (20 days and 1 mg NP/L) exposures at different salinities (0 and 10 ppt). Following the exposures, the activities of CAT (catalase), SOD (superoxide dismutase), GPX (glutathione peroxidase), GR (glutathione reductase) and GST (glutathione S-transferase) were determined in the liver of *O. niloticus*. Data showed that CAT and SOD activities did not change significantly ($P>0.05$) in acute exposures, though their activities significantly ($P<0.05$) decreased in chronic exposures at both salinities. Similarly, GPX and GR activities did not respond to acute NP exposures, but their activities decreased significantly in chronic exposures. However, GST showed the opposite response in acute and chronic exposures following NP and salinity exposures. Data showed that chronic exposures were more effective than acute exposures in regard to the response of the enzymes. Data also revealed that salinity did not have a predominant effect on the antioxidant enzymes, and also did not influence NPs toxicity.

Introduction

The use of metals or metal-containing products such metal-oxide NPs have increased in recent years following increases in their usage of nanotechnological products. Additionally, anthropogenic activities have also increased the salinity of freshwaters, especially in areas where human settlements and agricultural applications are widespread. The toxic effects of metals have been well studied in freshwater fish (Wood et al. 2012a; 2012b), though studies and debates are still going on NPs toxicity in aquatic organisms. There is no tolerable limit set for metal-oxide NPs, though the standard were set for the maximum tolerable metal levels in the aquatic systems (McLusky, 1989).

Metal-oxide NPs have size ranges between 1-100 nm and represent high surface/volume ratios. The other important characteristics of NPs are their reactivities, surface structures and crystal characteristics which make them unique for nanotechnology (Handy and Shaw, 2007; Hoseini et al., 2016). NPs are used in many technological products such as medical products, moisturizers, suntan creams, toothpaste, textiles, feed, gas sensors, batteries, solar cells, catalysis, rectifiers, antennas and electronics (Jeng and Swanson, 2006; Janrao et al., 2014; Chavali & Nikolova 2019). Laboratory studies demonstrated that NPs can pass across the cell membranes and accumulate in animal tissues and finally cause alterations in biomarkers in the blood and organs (Osborne et al., 2015; Adam et al., 2015; Hoseini et al.,

2016; Canli & Canli, 2019; 2020).

Environmental contaminants can cause free radicals (ROS) and affect the antioxidant defense systems of animals, altering the delicate balance between free radicals and antioxidant defense systems. Once this balance shifts in the direction of ROS as a result of environmental oxidants, then it is likely to cause the oxidative stress (Winston, 1991; Martinez-Alvarez et al., 2005; Kanak et al., 2014; Khan et al., 2016). Therefore, oxidative stress is one of the most investigated biomarkers in environmental studies both in the field and laboratory. There are many enzymatic and non-enzymatic defense mechanisms to combat ROS caused by the metabolisms or due to environmental contaminants. The most famous enzymes of the antioxidant systems are CAT, SOD, GST, GPx and GR. Studies have shown that the enzymes belonging to the antioxidant defense systems are sensitivity NPs exposures, causing significant alterations in their activities (Benavides et al. 2016; Bacchetta et al. 2017; Carmo et al. 2018).

The salinity of rivers and lakes are normally <0.5 ‰, though it can be increased by human activities. The road salting in cold areas may also be an important source of salt input into freshwaters. Only in the USA, nearly 15 million tons of salt (around 200,000 tons in Turkey) are used for road salting in the winter period (Baysoy et al., 2013). Some of these salts eventually enter into freshwater systems such as lakes and rivers, increasing salinity of their ecosystems. Because salinity increase is important factors in the freshwater ecosystem that influence the physiology, survival and growth in fish (Loretz, 1995; Marshall and Grosell, 2005; Blanchard & Grosell, 2006), it should be investigated thoroughly and especially the interaction between salt and metal containing contaminants should be studied to better understand their effects (Handy et al. 2008; Kulac et al., 2012; 2013; Baysoy et al., 2013; Dogan & Canli, 2019). Several studies reported the effects of salinity on the toxic effects of NPs in fish, suggesting the need to carry out more studies to understand better the NPs toxicity under salinity stress (Wang and Wang, 2014; Villarreal et al., 2014; Joo et al., 2018; Banan et al., 2020; Pérez-López et al., 2020).

The Nile tilapia is known for their high resistance the environmental pollution and salinity increase. Therefore, they were suggested as a bioindicator animal in detecting the effects of environmental contaminations (Cioni et al., 1991; Kamal & Mair, 2005; Dogan & Canli, 2019). There are nearly scarce data on the effects of NPs in waters with increased salinity on the antioxidant enzymes in tilapia. Metal-oxide NPs (Al_2O_3, TiO_2) are widely used in different areas of nanotechnology and consequently released to the environments, having a potential to cause toxicity. Because salinity has significant effects on the physiology of freshwater fish and NPs have the potential to cause alterations on the responses of biomarkers, this study was conducted to investigate the effects of salinity on the toxic effects of Al_2O_3 and TiO_2 NPs.

Materials and Methods

Experimental Protocol

Fish (*O. niloticus*) were obtained from the culturing pools of Çukurova University that reproduce them for about 30 years. Fish were transferred in 5 plastic tanks (50 L capacity) from the pools to the laboratory. Then, they were acclimatised to the new conditions in glass aquariums containing 100 L of water ($40 \times 40 \times 100$ cm) for one month before the experiments. The chemical and physical qualities of water were measured using a multimeter (Orion 5 Star, Thermo Scientific, ABD) and the hardness and alkalinity were measured with a standard titration method (Table 1).

Following the adaptation period, fish were individually exposed to differing salinities (NaCl) alone (0, and 10 ppt) and combination of salinity and NPs (Al_2O_3, TiO_2) for acute (10 mg NPs/L for 2 days) and chronic (1 mg NPs/L for 20 days) durations. Each exposure concentration and control contained 6 fish (72 fish in total) which the length (10.44±0.20 cm) and weight (17.18±1.39 cm) were similar (P>0.05) among exposure groups. Waters in experimental aquaria were changed every second day to keep NPs concentrations close to the nominal values and also clean the exposure media. After each water renewal, NPs were also re-loaded. During adaptation and experiments, animals received fish feed purchased from Pinar Company (Izmir, Turkey). Feeding was done just 1 h before water renewal. After acute and chronic durations, fish were taken from the aquaria, washed up with tap water and killed by transaction of spinal cord, according to the

Table 1. Chemical and physical conditions of experimental waters (N=5). As there was no significant difference between data from both acute and chronic experiments, the mean values (mean±se) of both experiments were given in this table.

	EXPERIMENTAL CONDITIONS			
	0 ppt NaCl	10 ppt NaCl	0 ppt NaCl+NPs	10 ppt NaCl+NPs
Measured salinity	0.20±0.09	10.6±0.14	0.33±0.22	10.9±0.84
Temp. °C	22.8±0.5	23.0±1.3	22.3±0.9	22.6±1.1
pH	7.60±0.91	7.72±0.20	7.68±0.31	7.80±0.43
Oxygen (mg O_2/l)	6.12±0.14	6.95±0.81	6.17±0.77	6.36±0.21
Total hardness(mg Ca_2CO_3/l)	306±20.5	316±18.7	310±11.6	325±30.3
Alkalinity (mg Ca_2CO_3/l)	204 ± 11.7	220±12.2	198±10.1	217±8.87

decision of the Ethic Committee (decision number: 9.3.12.10.2020) of Çukurova University. Samples of liver tissues of fish were carefully dissected out and kept at -80^{0}C (Esco UUS-480A) until the analyses.

Measurement of Antioxidant Enzyme Activity

Homogenization of the liver was performed (Janke & Kunkel Ultra Turrax T25 homogenizer, Germany) on ice for 90 seconds in a homogenization buffer (pH 7.4) containing 100 mM KCl ,100 mM potassium, and 1 mM EDTA. After homogenization, the homogenates were centrifuged (10,000 g) for 30 min using a Hettich Universal 30 (Germany) to obtain the supernatants (+4 °C). All measurements of antioxidant enzyme activities were performed in the supernatants, following the characterizations of the enzymes published earlier (Atli et al., 2016) and using the methods of Lartillot et al. (1988) for CAT activity, Livingstone et al. (1992) for GPX activity, Carlberg & Mannervik (1975) for GR activity, McCord & Fridovich (1969) for SOD activity and Habig et al. (1974) for GST activity. The method of Lowry et al. (1951) was used to measure total protein concentrations in the liver of fish.

NPs Characterization

Characterization of metal oxide nanoparticles (Al_2O_3, TiO_2) was detailed in our previous papers (Canli & Canli, 2019). According to data, Al_2O_3 NPs have an approximate size of ~40 nm and >30 m^2/g surface area, whereas TiO_2 NPs have an approximate size of ~21 nm and >30 m^2/g surface area. Al_2O_3 NPs was gamma form and had a cubic phase, whereas TiO_2 NPs was anatase form and had a tetragonal phase. Both NPs had polycrystalline structure. XRD (X-Ray Diffraction) analysis were done on NPs with an instrument (Rigaku RadB SmartLab diffractometer system). Element composition of NPs was done with an EDX (Energy-Dispersive X-ray) system supplied using a Field Emission Scanning Electron Microscope (Zeiss/Supra 55 VP). Data demonstrated that metal contents of Al_2O_3 NPs and TiO_2 NPs were 38.26 % and 33.83 %, respectively. The remaining percentages only consisted of oxygen atoms. Transmission electron microscope images of NPs (Figure 1) were obtained using a Jeol JEM-1010 with a power of 80kW connected to a camera (GATAN 782 ES500W Erlangshen).

Statistical Analysis

One of the latest version of SPSS (20) program was used to perform statistical analysis on data. First, the One-way-ANOVA test was applied to the data to estimate differences that showed significant ($P<0.05$) variations among control and exposure groups in acute and chronic exposures. Significant data were re-tested by Tukey post-hoc test to determine group differences. Second, the T-test was applied to data to estimate significant differences between acute and chronic exposures at 0 and 10 ppt salinities. Third, the T-test was applied to the data to estimate significant differences between 0 and 10 ppt salinity in acute and chronic exposures. Mean values and associated standard errors of data were presented in figures (Figures 2-6), indicating the results of statistical tests.

Results

The exposure to different concentrations of NPs and salinities did not kill any fish in both acute and chronic durations. Data revealed that CAT and SOD activities did not alter significantly ($P>0.05$) in all acute durations. However, in chronic durations, their activities decreased significantly ($P<0.05$) by Al_2O_3 and TiO_2 exposures at both salinities (Figure 2-3). These changes also caused significant differences between acute and chronic exposure, though there were significant variations in enzyme activities between salinities in acute or chronic exposures. Similarly, GPX (Figure 4) and

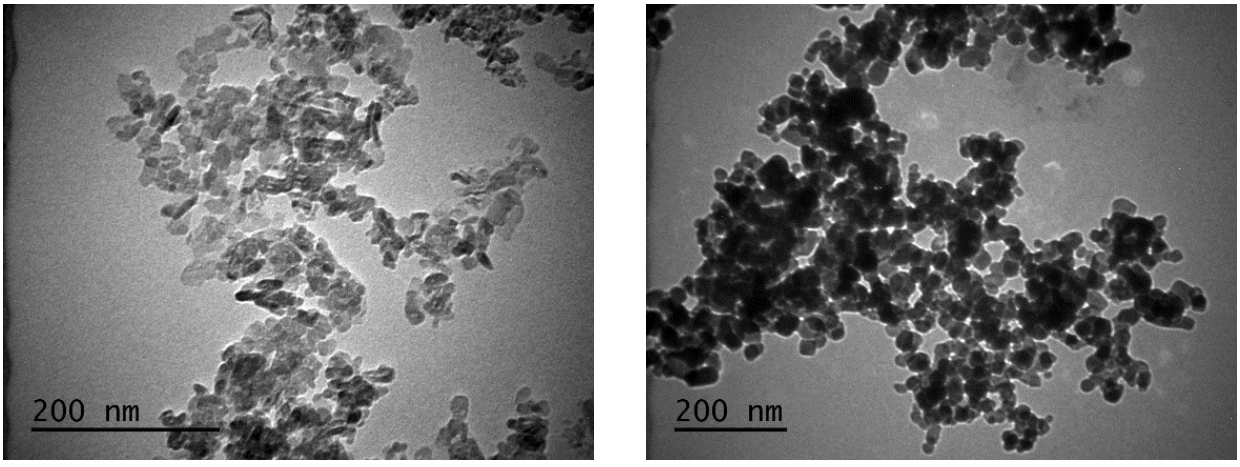

Figure 1. Transmission Electron Microscope images of Al_2O_3 NP (A) and TiO_2 NP (B). These images were published in Canli and Canli (2019).

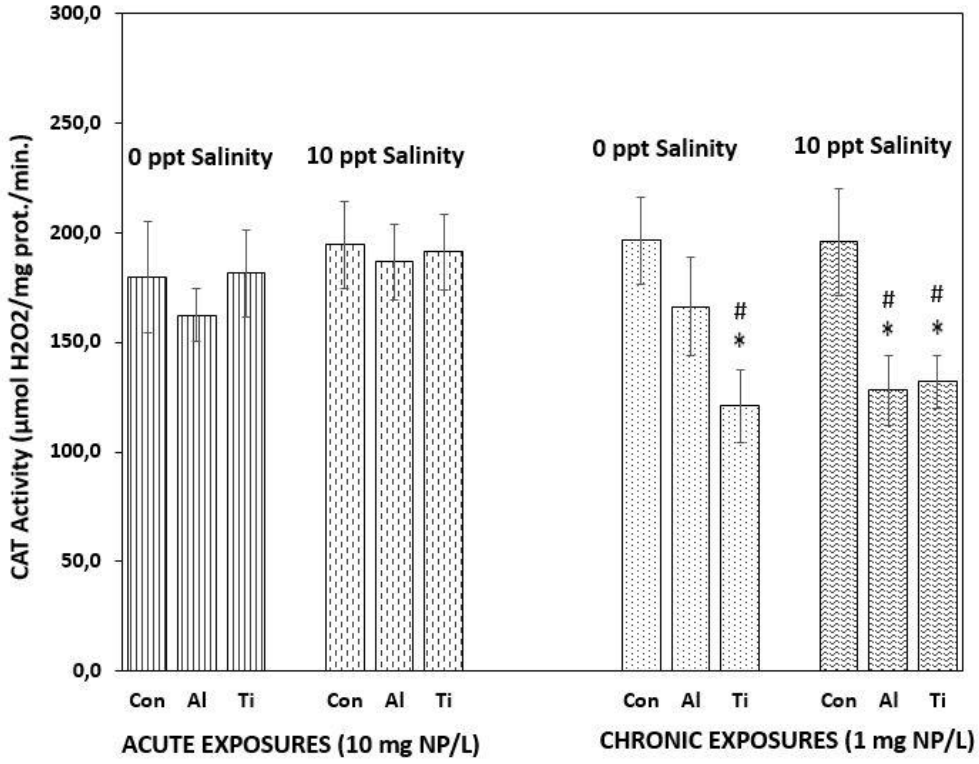

Figure 2. The effects of salinity alone and salinity+NP combinations on the activities of CAT in acute and chronic exposure. Data are the mean of 6 measurements and associated standard errors. * indicates significant (P<0.05) difference among control and NP exposures in acute or chronic exposures. # indicates significant (P<0.05) difference between acute and chronic exposures at 0 or 10 ppt salinities. ∞ indicates significant (P<0.05) difference between 0 and 10 ppt salinities in acute or chronic exposures. "Con" refers to control group.

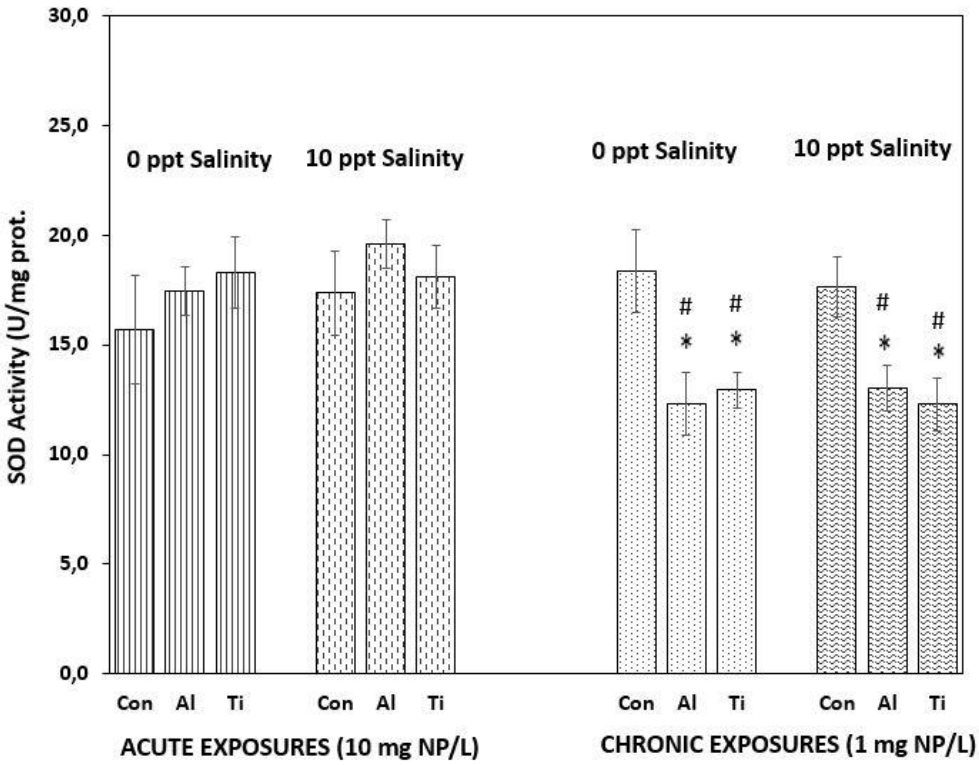

Figure 3. The effects of salinity alone and salinity+NP combinations on the activities of SOD in acute and chronic exposure. See Fig. 2 for details.

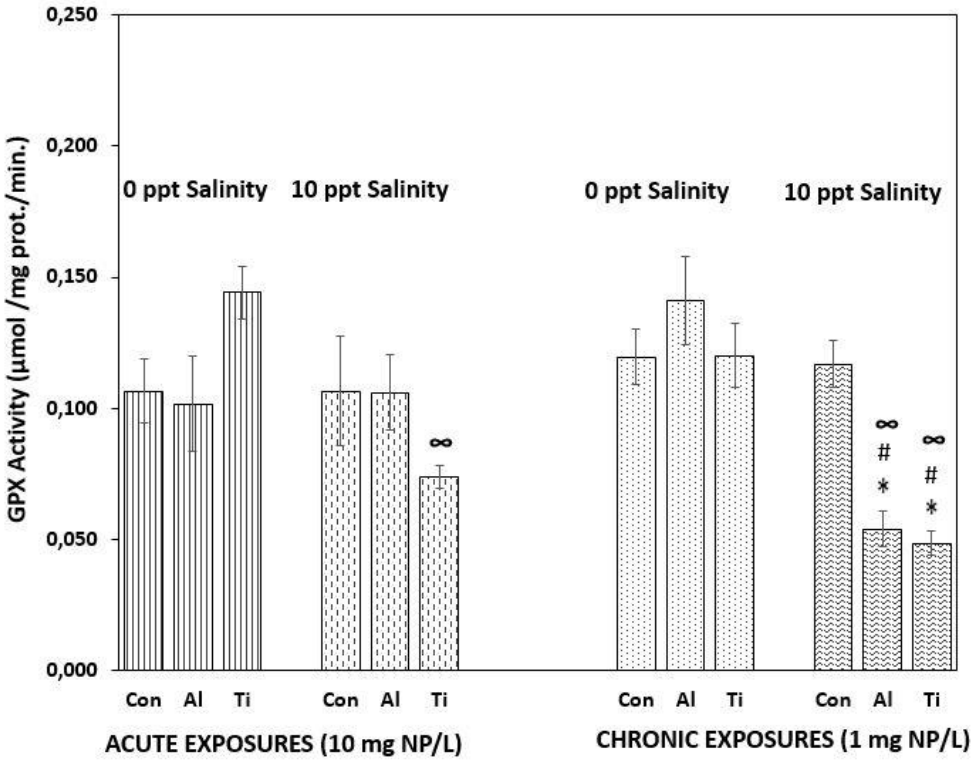

Figure 4. The effects of salinity alone and salinity+NP combinations on the activities of GPX in acute and chronic exposure. See Fig. 2 for details.

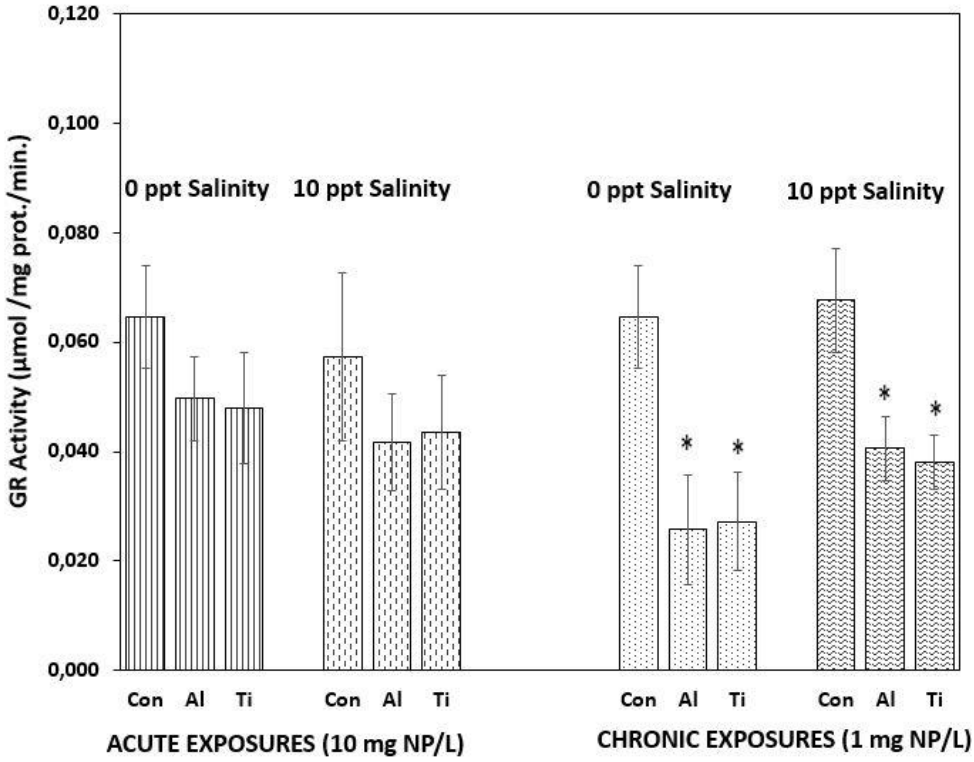

Figure 5. The effects of salinity alone and salinity+NP combinations on the activities of GR in acute and chronic exposure. See Fig. 2 for details.

GR (Figure 5) activities did not alter significantly following acute NP exposures at both salinities. However, the activities GPX and GR decreased significantly in chronic exposures at 10 ppt salinity. Both enzyme activities were significantly different between acute and chronic exposures. Likewise, there was also a significant variation in GPX activity between 0 and 10 ppt salinities at both acute and chronic exposures. However, GST activity increased in acute exposure at both salinities (Figure 6). Contrary to the other enzymes, there was no significant alteration in GST activity in chronic exposures at both salinities. Nevertheless, no significant alteration in GST activity was detected when compared the acute and chronic exposures and also between salinities.

Discussion

Tilapia species are known for their relatively high resistance to salt and contaminants (Cioni et al., 1991; Kamal & Mair, 2005). It is perhaps for this reason that no fish died during both acute and chronic exposures following exposure to salinity and NPs, even there was no apparent loss of appetite and swimming performance. Cioni et al. (1991) suggested that tilapia species are suitable biological models to monitor the freshwater environments due to their high capacity to adapt varying salinities. As the size of fish is important in toxicity studies (Kanak et al, 2014), fish belonging to different exposure groups had similar size range to prevent the differences in the metabolic activities in the present study. Despite this, NPs mostly caused a decline in antioxidant enzyme (except GST) activities in liver in chronic exposures, but not in acute durations. Interestingly, there was no significant change in the activities due to salinity increase. It seems that salinity increase up to 10 ppt alone does not cause any stress for tilapia, when antioxidant enzymes are in concern. Dogan & Canli (2019) also demonstrated that salinity increase alone did not affect the osmoregulation system of tilapia up to 30 days. Jun et al. (2012) also exposed tilapia to increased salinities (0-18 ppt) to determine the response of CAT and SOD in liver tissues. Data demonstrated that salinity did not affect the antioxidant enzyme activities and also the growth of fish, suggesting high resistance of *O. niloticus* to increased salinities. Although *O. niloticus* can have high resistance to environmental salinity, they still can show the symptoms of the oxidative stress at higher (21 ppt) salinity (Caxico Vieira et al., 2018).

Our previous data showed that *O. niloticus* take up NPs from exposure water and accumulated in different tissues (Canli et al., 2018; Canli & Canli, 2019; 2020). Similarly, NPs accumulations in tissues of fish were also reported (Jang et al., 2014; Zhang et al., 2015; Mansouri et al., 2016). Additionally, the uptake route of NPs seems an important factor to take into account for the evaluation of occurred toxicity, as accumulated NPs amounts could differ (Kleiven et al., 2018). Studies showed the effects of different NPs on the activities of antioxidant enzymes in freshwater fish (Connolly et al., 2016; Bacchetta et al., 2017; Carmo et al., 2018). There were both increases and decreases in the enzyme

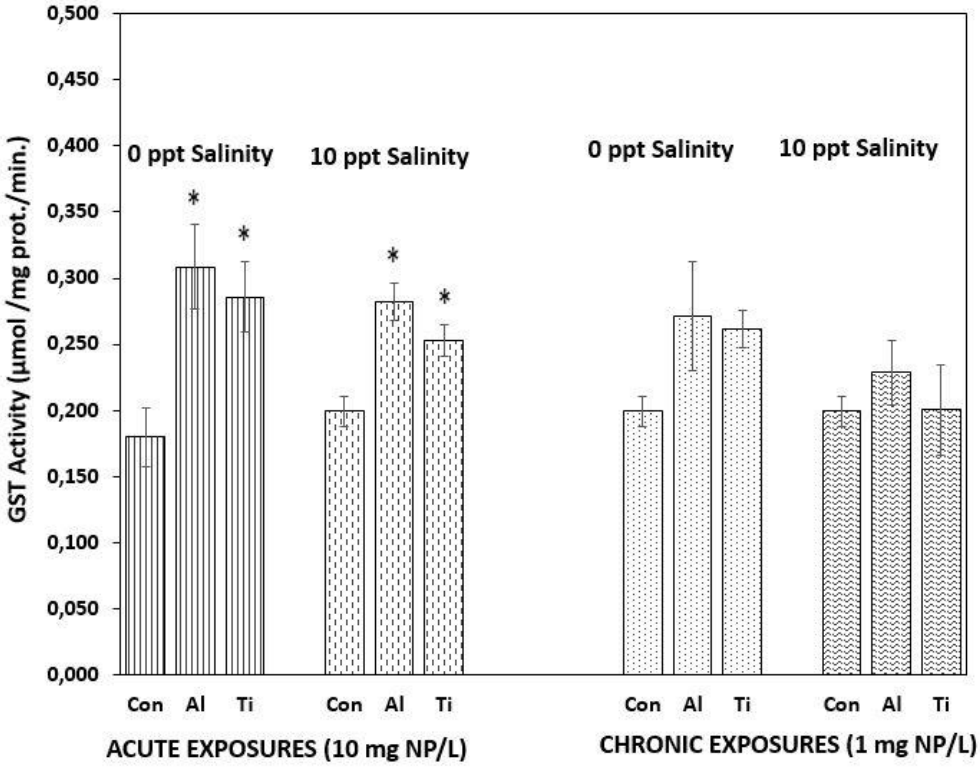

Figure 6. The effects of salinity alone and salinity+NP combinations on the activities of GST in acute and chronic exposure. See Fig. 2 for details.

activities following NPs exposures of fish. As it occurs in ionic metal toxicity, there are also a few factors (e.g. size and metal content of NP) which play significant roles in NPs toxicity (Wang et al., 2013; Vinardell et al., 2015; Benavides et al., 2016; Bacchetta et al., 2017; Kleiven et al., 2017). Although studies indicate that NPs have relatively lower effects than dissolved forms of metals (Bahadar et al., 2016; Ema et al., 2017), some data demonstrated the opposite as NPs showed higher toxicity comparing to ionic metals (Kiranmai and Reddy, 2013). It is well known that once NPs used in different industries, most of them inevitably enter into the aquatic systems. Therefore, recent studies have investigated the remediation of NPs from the aquatic system using different chelates. For example, Abdel-Khalek et al. (2020) used the rice husk to eliminate Fe_2O_3 NPs and Al_2O_3 NPs from water and concluded that rice husk was an effective adsorber for NPs.

The literature review revealed that the effects of NPs under increased salinity conditions are less studied compared to NPs toxicity alone in freshwater fish. Because NPs toxicity studies relatively newer and also salinity and NPs toxicity combination studies are scarce, there are not enough comparative data in the literature. This might also partly be due to the variations in experimental conditions and biology of studied fish species. Although the present data do not suggest the direct effects of salt on the activity of antioxidant enzymes, several studies demonstrated various effects of NPs and salt for freshwater fish. Joo et al. (2013) studied the bioaccumulation of Ag NPs in the tissues of rainbow trout and found that the order of tissues for NPs accumulation was liver > kidneys ≈ gills > white muscles. They pointed out that Ag levels in all the tissues were higher in increased salinities than relatively low salinities. Further studies on rainbow trout were carried out by Joo et al. (2018) and they found that some leucocyte types counts, blood plasma total protein, blood ion levels and histology of tissues were all affected from AgO NPs treatments and salinity increase was found to ease the occurred effects of AgO NPs. Villarreal et al. (2014) exposed *O. mossambicus* to CuO NPs (0.5, 5 mg CuO/L) in normal freshwater and salinity increased water. Fish exposed to 5 ppm CuO in increased salinity showed an increase in opercular ventilation compared to fish lived in normal freshwater. Activities of CAT, SOD and GR fluctuated in the liver of fish regardless of salinity. Although different forms of glutathione increased in the liver of fish, GSH/GSSG did not change significantly, suggesting that the oxidative stress did not occur. Banan et al. (2020) studied the effect of salinity on the toxicity of silver NPs in Persian sturgeon at different salinity levels. They found that values of lethal concentration (96 h) differed depending on salinity, despite no significant alterations in the plasma parameters. Likewise, the histological deformations occurred following by silver NPs in tissues of Persian sturgeon. Differences in the toxic effects of metal ion and metal-oxide NPs at different salinities were examined by Perez-Lopez et al. (2020). They studied the effects of Zn NPs and $ZnCl_2$ on fish *Gambusia sexradiata* for a 96-h exposure at two salinities (0 and 15 ppt). They found that Zn toxicity increased with increases in NPs concentrations, but it was reduced depending on salinity increases. They also pointed out that although ionic Zn is more toxic than NPs form of Zn, it can be increased if the NPs forms do not agglomerate rapidly and suspend in solution for longer periods. They also suggested to carry out further studies especially on the antioxidant system parameters.

Conclusion

The present data showed that both NPs generally decreased the activities of antioxidant enzymes in chronic durations, but not in acute durations suggesting the necessity for a long exposure period to observe NPs toxicity in fish. Salinity increase did not affect significantly the activities of antioxidant enzymes and also did not influence the toxicity of NPs. This may mean that there are different toxicity mechanisms between NPs form and ionic form of metals. Fish species belonging to seas and freshwaters may also show different uptake capacities of NPs, especially through the intestine. Despite many published papers regarding NPs toxicity, mechanisms of NPs toxicity are still to be investigated thoroughly. Thus, the present data emphasized that further studies should be done to determine the influence of environmental factors (e.g. hardness, salinity, pH, etc.) to understand better the mechanisms of NPs toxicity.

Ethical Statement

This study was conducted following the ethical protocol (9.3.12.10.2020) of Cukurova University (Turkey).

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Author Contribution

This is single author's manuscript.

Conflict of Interest

The author declares that there is no conflict of interest on this manuscript.

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