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Original Article

Individual and mixture effects of Nano-TiO_2 and microplastics on antioxidant and immunological responses of Nile tilapia juvenile, *Oreochromis niloticus* (L.)

Behzad Nematdoost Haghi, Alireza Mirvaghefi*, Hamid Farahmand

Department of Fisheries, Faculty of Natural Resources, University of Tehran, Karaj, Iran.

Abstract: This study aims to evaluate the effect of nano titanium dioxide and microplastics, alone and in combination, on immune system function and oxidative stress of juvenile Nile tilapia, *Oreochromis niloticus*. For this purpose, fish were randomly divided into 27 100L tanks using a 3×3 factorial design, including three concentrations of Nano titanium dioxide (0, 1, and 5 mg/l) and three concentrations of microplastic (0, 0.5, and 1 mg/l) separately and together each in triplicate. The results showed that immunoglobulin, malondialdehyde (MDA), and glutathione reductase (GR) had significant effects in all groups (P<0.05). Lysozyme, total antioxidant capacity (TAC), and glutathione peroxidase (GPx) showed significant differences between the combined groups and the control (P<0.05). No significant differences were observed in C3 and C4 between groups exposed to MPs and Nano-TiO2 and the control (P>0.05). Superoxide dismutase (SOD) showed significant differences between the group exposed to MPs and Nano-TiO2 alone and the control group (P<0.05). In conclusion, the results showed that MPs could have a synergistic effect on the toxicity of Nano-TiO_2.

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Introduction

Fishes are directly or indirectly exposed to chemical compounds; their effects depend on the concentration of these substances and how they are absorbed through the gills, skin, and gastrointestinal tract (Christophe et al., 2015). Pollutants have various effects on fish, including physiological, immune, and reproductive responses (Lawrence and Hemingway, 2008; Banaee et al., 2015; Derikvandy et al., 2020). Furthermore, since pollutants can produce active oxygen radicals and damage these substances in living organisms (Lackner, 1998), oxidative stress-related damage can be used to evaluate the effects of contaminants on organisms (Sies, 1985; Ibrahim et al., 2021).

High-consumption compounds can harm aquatic organisms such as fish if their residues enter the water. Nanoparticles are used in various industries, and their production and application volume is increasing. These materials also have many applications in aquaculture, including vaccine transfer, food product transport, and water refinement (Shah and Marz, 2020). Despite these applications, entering these materials into aquatic environments harms the environment and living organisms in them (Wang et al., 2008). Nano titanium dioxide (Nano-TiO_2) is one of these materials with excellent environmental dispersion. TiO_2, because of its unique properties, has many applications, including sunscreen production, water purification, and medication delivery (Chen and Selloni, 2014). Due to its wide use, it can enter the aquatic environment and influence aquatic organisms.

Microplastics (MPs), generated from garbage and plastic waste breakdown, are one of aquatic ecosystems' most critical environmental pollutants (Crawford and Quinn, 2016). They can act as potential vectors of various biological and non-biological pollutants (Kinigopoulou et al., 2022;

*Correspondence: Alireza Mirvaghefi
E-mail: avaghefi@ut.ac.ir
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Table 1. Properties of Nano TiO2 based on the Iranian Nanomaterials Pioneer.

Titanium Dioxide	TiO2,80%Anatase20%Rutile
purity	+99%
Average primary particle size (D50)	20 nm
Specific surface area (SSA)	10-45 m2 g^{-1}
Color	White
Bulk density	0.46 g ml^{-1}
pH	5.5-6.0
Loss of weight in drying	0.48%
Loss of weight on ignition	0.99%

Banihashemi et al., 2022). MPs can absorb environmental contaminants and enter them into organisms' bodies (Verla et al., 2019). This process elevates pollutant bioavailability for organisms (Nematdoost Haghi and Banaee, 2017; Banaee et al., 2019a), and pollutant accumulation (Thammatorn and Palic, 2022). Thus, MPs help transfer xenobiotics through the food chain (Carbery et al., 2018). Therefore, investigation of the simultaneous effect of MPs and titanium dioxide nanoparticles on Nile tilapia, *Oreochromis niloticus*, was considered as this study's aim.

Materials and methods

The fish were purchased from a tilapia breeding farm in Qom and kept in a laboratory until they reached about 24 g. Two weeks of acclimatization were performed on these fish before the experiment. A factorial design (3×3) was chosen for the experiment, including 0, 0.5, and 1 mg/l microplastic and 0, 1, and 5 mg/l Nano-TiO_2in triplicate. Each group consisted of 10 fish with an average weight of 23.5±0.6 g in triplicate. 100% daily water exchange was done (50% every 12 hours) to maintain a constant concentration of Nano TiO_2 (Banaee et al., 2019). Water chemical factors, including temperature (25±1°C), oxygen (6±1 mg/l), and pH (7.6±0.2), were kept constant during the experimental period. The photoperiod was 12l:12d during the experiment. Nano-Tio_2 was purchased from Iranian Nanomaterials Pioneers (Table 1). Stock preparation was done every day. After ultrasonication by an ultrasonic bath (10 min, 35 kHz, 100/400 W), the stocks added up to 10 L of dechlorinated water in the tanks to reach nominal concentrations (1 and 5 mg/l).

Sampling: On the 30th day, six fish were randomly caught from each replication of the treatments. After anesthetizing with clove extract (1/5000), blood was drawn from the caudal vein by heparinized syringes. The samples were separated by centrifugation (6000 g for 10 minutes) and stored at -28°C until the measurement of the parameters.

Analysis of oxidative stress and immunological parameters: SOD (McCord and Feridovich, 1969), GPx (Johnson et al., 1999), and GR (Zanetti, 1979) were measured by kits of Kiazist Company. CAT (Góth, 1991), MDA (Placer et al., 1966), and total antioxidants capacity (TAC) (Benzie and Strain, 1996) were assessed. Total immunoglobulin (Panigrahi et al., 2005), Lysozymes (Lange et al., 2001), total complement (AC50), and C3 and C4 (Yano, 1992) were measured using Pars Azmun company kits. Assessment of synergism and antagonism was carried out based on Banaee et al. (2020).

Statistical Analysis: The data was analyzed based on factorial design. Statistical analysis was performed using SPSS22 software. A two-way analysis of variance was used for data analysis, and mean comparisons were performed using the Tukey test. The data normality test was performed using the Shapiro-Wilks test. The confidence level used in this study was 95%. Finally, the results were presented as mean ± standard deviation.

Results

There was no mortality during the experimental

Table 2. The results of two-way ANOVA analysis of immunological and oxidative stress parameters of Nile tilapia juvenile.

parameter	treatment	sig	parameters	treatment	sig
SOD	Nano-Tio$_2$	0.000	Lysozyme	Nano-Tio2	0.000
	microplastic	0.000		microplastic	0.000
	Interaction	0.000		Interaction	0.000
CAT	Nano-Tio$_2$	0.199	ACH50	Nano-Tio2	0.000
	microplastic	0.000		microplastic	0.001
	Interaction	0.000		Interaction	0.086
MDA	Nano-Tio$_2$	0.000	C3	Nano-Tio2	0.000
	microplastic	0.000		microplastic	0.808
	Interaction	0.000		Interaction	0.713
GPX	Nano-Tio$_2$	0.000	C4	Nano-Tio2	0.078
	microplastic	0.000		microplastic	0.072
	Interaction	0.000		Interaction	0.005
GR	Nano-Tio$_2$	0.000	Immunoglobulin	Nano-Tio2	0.000
	microplastic	0.000		microplastic	0.000
	Interaction	0.000		Interaction	0.006
TAO	Nano-Tio$_2$	0.000			
	microplastic	0.000			
	Interaction	0.000			

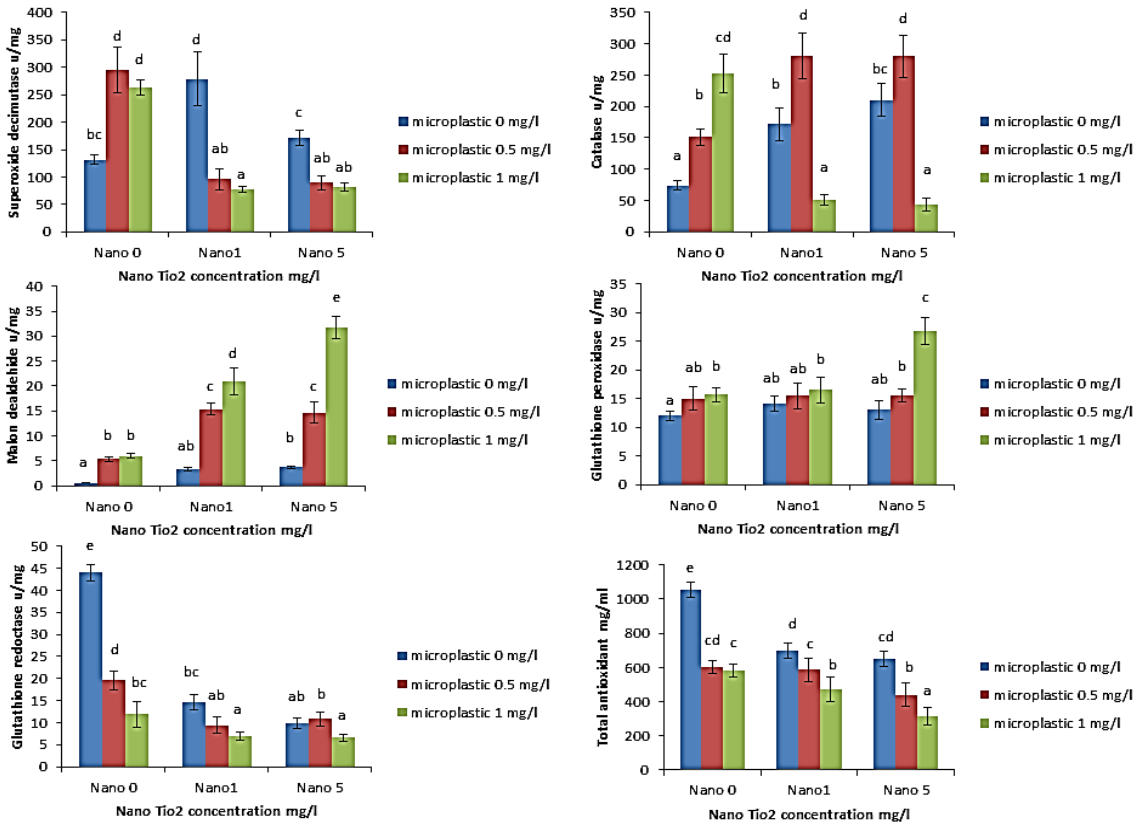

Figure 1. The antioxidative parameters of juvenile Nile tilapia expose to Nano titanium dioxide and moicroplastics. Different letters show significant differences. Data are shown as mean±SD.

period. The results of oxidative stress markers are shown in Table 2. SOD activity showed a significant impact on factor interaction (P<0.05) (Table 2). Nano TiO_2 and MPs significantly impacted SOD activity (P<0.05). Furthermore, SOD activity showed a significant difference between groups of Nano TiO_2 0 mg/l + MPs 0.5 mg/L, Nano-TiO_2 0 mg/l + MPs 1 mg/l, NanoTiO_2 1 mg/l + MPs 1 mg/l, Nano TiO_2 5 mg/l + MPs 0 mg/l and control (P<0.05) (Fig. 1).

Table 3. The combined effects of contaminants on enzyme activity.

parameters	groups	Predicted effect	Observed effect	Synergy ratio	Combined effects
SOD	1nano+0.5 microplastic	4.78	0.73	6.54	S
	1nano+1 microplastic	4.24	0.58	7.31	S
	5nano+0.5 microplastic	2.91	0.68	4.27	S
	5nano+1 microplastic	2.6	0.62	4.19	S
CAT	1nano+0.5 microplastic	4.64	3.78	1.22	S
	1nano+1 microplastic	7.82	0.67	11.67	S
	5nano+0.5 microplastic	5.71	3.77	1.51	S
	5nano+1 microplastic	9.62	0.58	16.58	S
MDA	1nano+0.5 microplastic	53.78	26.84	2	S
	1nano+1 microplastic	60.91	36.66	1.66	S
	5nano+0.5 microplastic	60.29	25.78	2.33	S
	5nano+1 microplastic	68.27	55.61	1.22	S
GPX	1nano+0.5 microplastic	1.47	1.2	1.22	S
	1nano+1 microplastic	1.53	1.37	1.11	S
	5nano+0.5 microplastic	1.36	1.3	1.04	S
	5nano+1 microplastic	1.41	2.24	0.62	A
GR	1nano+0.5 microplastic	0.14	0.21	0.66	A
	1nano+1 microplastic	0.03	0.15	0.2	A
	5nano+0.5 microplastic	0.09	0.24	0.37	A
	5nano+1 microplastic	0.05	0.15	0.33	A
TAO	1nano+0.5 microplastic	0.37	0.55	0.67	A
	1nano+1 microplastic	0.35	0.44	0.79	A
	5nano+0.5 microplastic	0.34	0.42	0.8	A
	5nano+1 microplastic	0.33	0.29	1.13	S

The impact of the two factors' interaction and MPs on CAT activity was significant (P<0.05), but the effect of Nano TiO_2 showed no significant difference (P>0.05) (Table 2). CAT activity indicated significant changes in all groups with control (P<0.05) except NanoTiO_2 1 mg/l + MPs 1 mg/L and Nano-TiO_2 5 mg/l + MPs 1 mg/l (Fig. 1). MDA activity revealed significant impacts of the interaction of two factors and each of them separately (P<0.05) (Table 2). MDA activity showed a significant increase in all groups (P<0.05) (Fig. 1).

The interaction of Nano-TiO_2, and MPs showed a significant effect on GPx activity (P<0.05) (Table 2). GPX activity increased in all groups compared to control but only significant in Nano-TiO_2 0 mg/l + MPs 1 mg/l, Nano-TiO_2 1 mg/l + MPs 1 mg/l, Nano-TiO_2 5 mg/l +MPs 0.5 mg/l and Nano-TiO_2 5 mg/l + MPs 1 mg/l in groups (P<0.05) (Fig. 1). The effect of each parameter on GR activity was similar (P<0.05) (Table 2). GR activity decreased significantly in all groups (P<0.05) (Fig. 1). TAC showed a significant impact of two factors and their interaction on the results (P<0.05) (Table 2). TAC showed a significant decrease in all groups (P<0.05) (Fig. 1). Table 3 shows that Nano-TiO_2 and MPs had a synergistic effect on SOD, CAT, MDA, and GPx activities.

The results of ACH50 revealed no significant effects of factor interaction. The impact of Nano-TiO_2 and MPs were also significant (P<0.05) (Table 2). Total complement's results revealed a significant decrease in Nano-TiO_2 1 mg/l + MPs 0 mg/l, Nano-TiO_2 1 mg/l + MPs 1 mg/l, Nano-TiO_2 5 mg/l + MPs 0.5mg/l, and Nano-TiO_2 5 mg/l + MPs 1m g/l with control (P<0.05) (Fig. 2). No significant effect of interaction observed in the C3 (P>0.05). Among the two factors, only Nano-TiO_2 showed a significant impact on the results of C3 (P<0.05) (Table 2). C4 showed a significant effect of Nano-TiO_2 and MPs interaction (P<0.05). None of the contaminants alone showed a significant impact on results (P>0.05) (Table 2). C4 result showed no significant

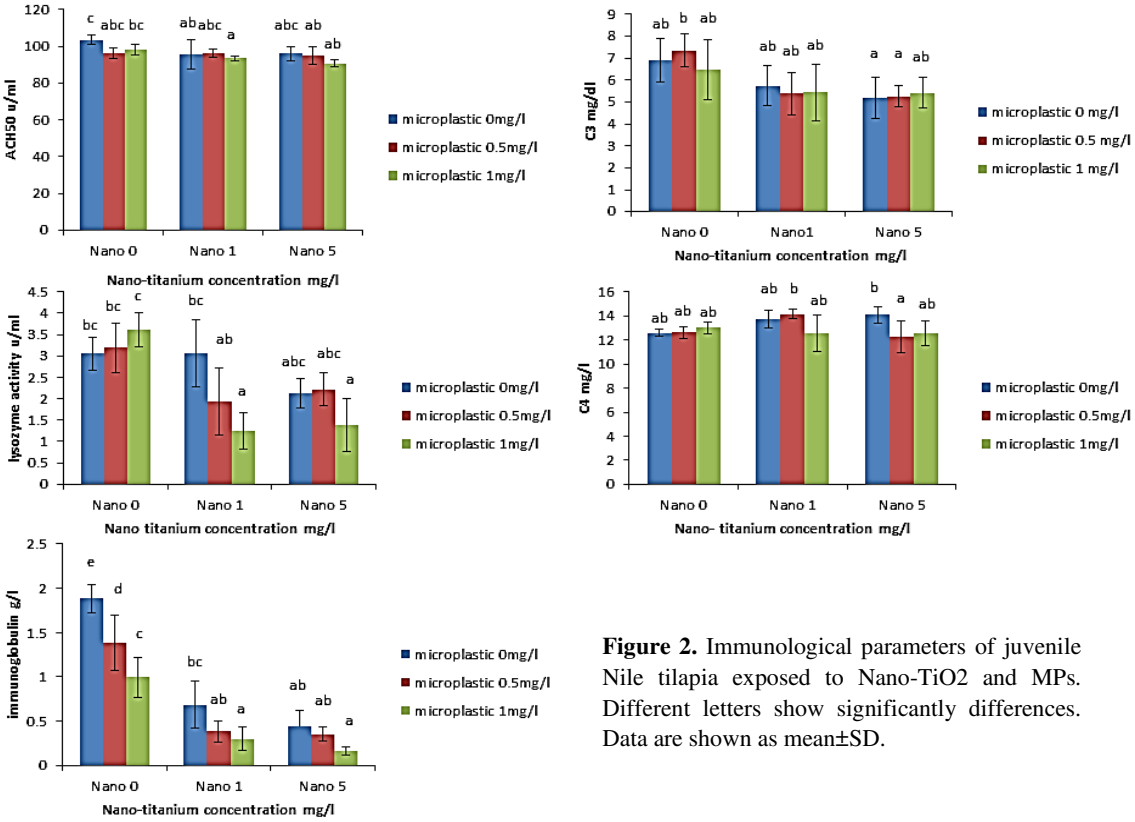

Figure 2. Immunological parameters of juvenile Nile tilapia exposed to Nano-TiO2 and MPs. Different letters show significantly differences. Data are shown as mean±SD.

Table 4. The combined effects of contaminants on immunological parameters.

parameters	groups	Predicted effect	Observed effect	Synergy ratio	Combined effects
ACH50	1nano+0.5 microplastic	0.85	0.93	0.91	A
	1nano+1 microplastic	0.87	0.9	0.96	A
	5nano+0.5 microplastic	0.86	0.92	0.93	A
	5nano+1 microplastic	0.88	0.87	1.01	S
C3	1nano+0.5 microplastic	0.88	0.78	1.13	S
	1nano+1 microplastic	0.78	0.79	0.98	A
	5nano+0.5 microplastic	0.79	0.76	1.03	S
	5nano+1 microplastic	0.7	0.78	0.89	A
C4	1nano+0.5 microplastic	1.09	1.12	0.97	A
	1nano+1 microplastic	1.12	0.99	1.13	S
	5nano+0.5 microplastic	1.12	0.97	1.15	S
	5nano+1 microplastic	1.15	1	1.15	S
Lysozyme	1nano+0.5 microplastic	1.04	0.63	1.65	S
	1nano+1 microplastic	1.18	0.4	2.95	S
	5nano+0.5 microplastic	0.71	0.72	0.98	A
	5nano+1 microplastic	0.81	0.45	1.8	S
Immunoglobulin	1nano+0.5 microplastic	0.26	0.2	1.3	S
	1nano+1 microplastic	0.19	0.15	1.26	S
	5nano+0.5 microplastic	0.16	0.18	0.89	A
	5nano+1 microplastic	0.12	0.08	1.5	S

differences (P<0.05) (Fig. 2). Lysozyme activity significantly affected Nano Tio_2 and MPs interaction (P<0.05). The impact of Nano-TiO_2 and MPs on lysozyme activity was significant (P<0.05) (Table 2). Lysozyme activity showed a significant decrease in Nano TiO_2 1 mg/l + MPs 1 mg/l and Nano-TiO_2 5 mg/l + MPs 1 mg/l group (P<0.05) (Fig. 2). Immunoglobulin showed a significant effect of each

contaminant and their interaction (P<0.05) (Table 2). The result of immunoglobulin showed a significant decrease in all groups (P<0.05) (Fig. 2). Table 4 shows Nano-TiO_2 and MPs synergistic effects on C4, immunoglobulin, and lysozyme activity.

Discussion

Biomarkers of oxidative stress: Simultaneous exposure of organisms to different contaminants in the aquatic environment is inevitable, but awareness of their co-exposure impact can help deal with their effects. One of the critical impacts of Nano-titanium and MPs on organisms is oxidative stress (Kim et al., 2021; Mahboob et al., 2017) and the generation of reactive oxygen species (ROS). Oxidative stress occurs when an imbalance of oxidants/antioxidants happens in the cells (Birben et al., 2012). Organisms can counteract ROS's impact using an antioxidant defense system that includes enzymatic and non-enzymatic parts. The enzymatic part contains a set of enzymes that prevent the formation of free radicals (Li et al., 2010). The prominent role of these enzymes is to neutralize active oxygen radicals. SOD, CAT, and GPx act as the primary line of this system (Ighodaro and Akinloye, 2018; Derikvandy et al., 2020). The first part of these enzymes, when organisms are exposed to xenobiotics, is SOD (Ighodaro and Akinloye, 2018). When SOD is exposed to ROS, it converts superoxide radicals to hydrogen peroxide (Sakamoto and Imai, 2017; Ibrahim et al., 2021), and afterward, the other enzymes act. Thus, higher SOD activity can counteract the organism with higher production of ROS. However, with the elevation of ROS concentration, due to the inability of the body to respond to ROS increase, the system collapses and loses the ability to return to normal conditions. SOD declines after fish exposure to contaminants such as pesticides (Kaur and Jindal, 2017).

Catalase plays an essential role in the antioxidant defense system of organisms by breaking down hydrogen peroxide (Catalan et al., 2018), preventing oxidative stress and protecting cells. Catalase elevation after exposure to Nano-TiO_2 and MPs can be due to the body's response to the increasing hydrogen radicals. Also, the decrease in catalase activity in combination groups can be due to producing catalase to reduce the ROS levels. Le Thu et al. (2021) reported catalase decline after exposure of Nile tilapia to cadmium. Similar results were presented by Chitra and Maiby (2014) and Sayed et al. (2003).

MDA is one of the final products of lipid peroxidation that can form non-enzymatically by ROS inducement or enzymatically by lipoxygenase activity (Farmer and Muller, 2013). The level of MDA shows induced oxidative stress (Karadag et al., 2014) and injuries to cells, tissues, and organs (Ayala et al., 2014). Elevation of lipid peroxidation is a sign of ROS increase (Faheem and Lone, 2018) and probably the incapability of antioxidant defense to cope with ROS generation.

GPx is an enzyme that finds in mitochondria and peroxisome compartments and reduces H_2O_2 to H_2O. This reaction inhibits the harmful impacts of hydrogen peroxide; therefore, the elevation of GPx activity leads to the diminishment of oxidative stress (Lubos et al., 2011). GR is another member of the glutathione family that produces GSH from GSSG through utilizing NADPH and maintains the reduced glutathione (GSH) content (Couto et al., 2016). It plays an essential role in cellular antioxidant protection and regulates metabolic pathways (Cazenave et al., 2006). Elevation of GPx can show the reaction of the antioxidant system of fish against oxidative stress, as reported by Hanachi et al. (2020) in the exposure to pesticides and microplastic in zebrafish. Cellular total antioxidant capacity includes the enzymatic and non-enzymatic antioxidants that regulate the balance between peroxidants and antioxidants in cells. Therefore, a significant decrease in the total antioxidant levels may indicate the collapse of cellular antioxidant defense systems (Fraga et al., 2014). A significant decrease in TAC in the present study is similar to other studies (Banaee and Taheri, 2019; Taheri et al., 2017). TAC decrease can signify oxidative stress in the organism exposed to MPs and Nano-TiO_2

(Hamed et al., 2020). Moreover, it can be due to the consumption of antioxidants to cope with stress.

Immunity parameters: Magnadottir (2010) opined that the immune system of fish is diverse, and its response rate can differ in different fish species exposed to different conditions. Immunoglobulins are used as a marker of the immune system function when the organism is exposed to environmental stressors. Immunoglobulins are essential in maintaining mucosal homeostasis (Angeles Esteban, 2012). As mature B lymphocytes synthesize immunoglobulin, the factors that affect the number of lymphocytes and the tissues that generate them can affect immunoglobulin levels (Banaee et al., 2019b). Immunoglobulin reduction in this study indicates debilitation of the immune system, as reported by Hong et al. (2018). Immunoglobulin decline due to a decrease in total Ig synthesis and destruction and apoptosis of liver cells exposed to contaminants reported previously by Banaee (2013).

Lysozyme, a mucolytic enzyme, is one of the best indicators of the innate immune system (Saurabh and Sahoo, 2008; Li et al., 2021). Exposure to Nano-TiO_2 and MPs decreased the lysozyme activity, which can signify immune system impairment (Ahmadi et al., 2014). Li et al. (2013) reported that a significant reduction of lysozyme activity after exposure to environmental contaminants could be due to a decrease in this enzyme biosynthesis by phagocytic cells and, or a reduction of monocyte and granulocyte counts. Furthermore, its increase after exposure to low concentrations of xenobiotics can be due to the effort of the immune system to neutralize changes in body condition.

The complement system, as a part of the fish defense system, comprises a group of proteins produced by different organ cells, including the liver and intestinal epithelium (Boshra et al., 2006). A decrease in ACH50 can be another sign of the immune system weakening. Such a decline in ACH50 can also be due to hepatocyte and mononuclear phagocyte damage (Bitsayah et al., 2018; Banaee et al., 2019a, b). C3 is a protein made by liver cells and monocytes. Activation of this component of the complement system makes the classical and alternative systems active (Janssen et al., 2005). C4 is a protein made by monocytes and macrophages. It plays a vital role in the immune response (Mortensen et al., 2015), and its cooperation after identifying the infectious agents with other immune system proteins help to destroy them (Pushpa et al., 2014). High levels of C3 and C4 in the serum indicate fish health (Yano, 1992). A significant reduction in the C3 and C4 levels can signify stressors' impact on weakening the immune system. These proteins did not show significant changes when exposed to the selected concentration of Nano-TiO_2 and MPs alone and in combination.

Conclusion

MPs and Nano-TiO_2 can cause an imbalance in reactive oxygen production and antioxidant capacity, leading to oxidative stress. They also can affect the immune response. Co-exposure to these contaminants can cause synergistic and accumulative impacts on organisms. The results indicated that even low concentrations of MPs and Nano-TiO_2 could impact oxidative parameters in juvenile Nile tilapia. Simultaneously exposure to MPs and Nano-TiO_2 showed a more significant impact and synergistic effect on the assessed indicators. Although some immunological parameters did not show significant changes (probably due to low concentration of contaminant or low experimental period), the results showed that MPs and Nano-TiO_2 could synergistically impact on fish health parameters.

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