MARINE REPORTS
e-ISSN: 2822-5155
Journal homepage: https://scopesscience.com/index.php/marep/

Received: 09 October 2023; Received in revised form: 10 November 2023
Accepted: 11 November 2023; Available online: 05 December 2023
RESEARCH PAPER

Citation: Acar, Ü., Erden, Y., Günay, S., Kesbiç, O.S., & Yılmaz S. (2023). Evaluation of the impact of different size and shape polyethylene microplastic in Nile tilapia (*Oreochromis niloticus*). *Marine Reports*, *2*(2), 95-104. https://doi.org/10.5281/zenodo.10182777

EVALUATION OF THE IMPACT OF DIFFERENT SIZE AND SHAPE POLYETHYLENE MICROPLASTIC IN NILE TILAPIA (*Oreochromis niloticus*)

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Abstract
Plastic waste that is discharged into the aquatic environment can eventually break down into different size particles known as microplastics (MP). MPs have become a significant concern due to their potential negative effects on fish species. In this study, the effects of sublethal concentrations of various size and shape polyethylene microplastics (PE-MPs) on Nile tilapia were investigated on numerous levels, including hematological and DNA damage in blood. For this purpose, the phsysiological effects of freshwater fish species Nile tilapia (*Oreochromis niloticus*) exposed to PE-MPs at control (0 mg/L), 5 mg/L, 10 mg/L, 25 mg/L and 50 mg/L for 14 days were investigated. In Nile tilapia PE-MPs exposure caused a significant decrease in hematological parameters. DNA damage in blood cells of fish exposed to PE-MPs was significantly higher than in the control group. Consequently, our findings show that PE-MPs affect many physiological parameters by causing oxidative stress-induced DNA damage in adult fish, and MPs should be considered a potent environmental pollutant when the rate in water is more than 10 mg/L for Nile tilapia.

Keywords: Polyethylene, microparticles, hematology, DNA damage, Nile tilapia

Introduction

Global plastic consumption has been increasing due to its common usage in commercial, industrial, and medical purposes. Plastics may reach aquatic environments and be found all over the water. Plastics have a significant share in the total waste found in aquatic ecosystems. However, it is known that plastic pollution is a global problem and its impact on aquatic ecosystems including rivers, lakes and oceans is a matter of concern (Cunha et al., 2020; Gürses, 2023a, b, Ergün et al., 2023). Various size of plastics such as nanoplastics (<1000 nm), small-sized MPs (1–1000 μm), large-sized MPs (1-5 mm), and MPs (Eriksen et al., 2014; Hanvey et al., 2017) can be found in aquatic environments. Fish can mistakenly consume small plastics, including microplastics, that resemble their natural prey. Many fish species rely on visual cues to identify and capture their food, and microplastics can be visually mistaken for plankton or other small organisms that fish typically feed on (Banei et al., 2022). It is also known that the plastics reaching the digestion systems of the fish pose serious threats to fish health (Barría et al., 2020). Moreover, it is anticipated that humans who consume plastic-contaminated fish and other aquatic creatures may have health issues (Smith et al., 2018). Studies conducted on the effects of MPs on fish health report that MPs can reach various tissues of the fish. The study by Jovanović et al. (2018) provides evidence that particles ranging from 5 to 150 μm in size can be detected in the circulatory system of fish, even after passing through the intestine. This finding suggests that microplastic particles can potentially enter the bloodstream of fish and distribute throughout their bodies. The study conducted by Liu et al. (2021) provides evidence that the bioconcentration of polyethylene microplastics (PE-MPs) in Japanese medaka (*Oryzias latipes*) is dependent on the size of the microplastic particles. The toxicity mechanisms of microplastics are not yet fully understood, and there can be variations in findings across different studies. For example, Ivleva et al. (2017) suggested that very small microplastic particles (<15 μm) and nano-sized particles (<1 μm) may pose a higher concern than larger microplastic particles. This is because smaller particles have a larger surface area relative to their volume, which can lead to increased potential for chemical interactions and adsorption of toxic substances. Additionally, smaller particles can more easily be taken up by organisms and potentially cause adverse effects at the cellular and subcellular levels. On the other hand, Avio et al. (2015) observed the presence of relatively larger plastic particles (400 μm) in fish mullet (*Mugil cephalus*) livers. This finding suggests that larger MPs can also be ingested by fish and accumulate in their tissues, potentially causing adverse effects. Research in the literature mostly focuses on the effects of MPs on marine species. Recent reports have highlighted the potential accumulation of MPs in various freshwater fish species, including carp, rainbow trout, catfish, and tilapia. These MPs can pose risks to the health of these fish species (Park et al., 2020; Merga et al., 2020; Savoca et al., 2021). The Nile Tilapia (*Oreochromis niloticus*) is a significant species for the ecosystem both in the natural environment and its intense aquaculture. Besides, it is used as a model in toxicology studies. It has been reported that MPs lead to DNA damage and lipid peroxidation (Hamed et al., 2020), histopathologic damages (Hamed et al., 2021), and changes in hematologic and serum biochemical parameters (Hamed et al., 2019; Fazio, 2019; Guerrera et al., 2021), and biochemical changes in tissues (Ding et al., 2018) on the Nile tilapia. However, such studies on the Nile tilapia have been conducted on juvenile, young fish, and/or fries. The present study, aimed to investigate the effects of MPs on portion-size broodfish, which has a significant place in new generation aquaculture. Furthermore, MPs contamination was identified in freshwater fish from both natural and farmed sources (Garcia et al., 2021). It was recently indicated that almost 70 % of freshwater fish from the previous studies ingested fibers (Sarijan et al., 2021). It has been reported that there are concerns about human health, especially upon consumption of fish reared in net cages, due to the possibility that fish with biting behaviors on the nets in order to eat the biofouling organisms, could be a reason of ingesting some microplastics from nylon net pieces (Ergün et al., 2023).

Evaluating the impact of different sizes and shapes of PE-MPs on Nile tilapia (*Oreochromis niloticus*) is crucial for understanding the potential risks associated with MP pollution in aquatic environments and the implications for the aquaculture industry. Research on the ingestion and egestion of microplastics (MPs), as well as their physiological and biochemical effects, can provide valuable insights for the development of risk assessment and mitigation strategies in the aquaculture industry. Within the scope of the study, the effects of MPs on Nile Tilapia broodfish, *Oreochromis niloticus*, such as DNA damage in the blood and hematological parameters, have been comprehensively investigated.

Material and Method

Microplastics

Microplastics (MPs) were raw powder with irregular-shaped particles (with >90% of microplastics > 100 µm in size) purchased from Global Polimer (İstanbul/Türkiye). The MPs size and morphology were observed using scanning electric microscope (SEM, Hitachi TM3030 Plus, Japan) (Figure 1).

Fish exposure

A total of mix sexed 150 Nile tilapia with an average weight of 259.89±12.39 g was obtained from the Çanakkale Onsekiz Mart University, Marine Science and Technology Faculty Live Resources Laboratory. The fish found clinically healthy and without lesion or injury used for the experiments. During the experiment water quality parameters were measured daily and temperature 28.4±1.50 °C, dissolved oxygen 7.1±0.6 mg L-1 and pH 7.6 was found. Also, 12:12 (light:dark) photoperiod has been applied and aquarium waters are aerated during the experiment. Fish were randomly selected and exposed to one control group without MPs and 5, 10, 25 and 50 mg/L MPs in 180 L glass aquarium for 14 days in triplicate, according to Katzenberger and Thorpe (2015) 10 fish per aquarium. The exposure solution was changed every other day to prevent the deposition of polyethylene MPs during the 14-day experimental period. PE-MPs test solutions were created by adding measured amounts of MPs to dechlorinated water. These solutions were then diluted sequentially into tanks at concentrations of 5, 10, 25, and 50 g/L. These concentrations were chosen based on previous studies of microplastic levels in freshwater environments (Hamed et al., 2019).

Blood sampling and hematological analyses

Blood was collected from the fish at the end of the 14-day trial from a total of 9 fish per group, 3 from each tank. The fish were randomly selected from the experimental tanks and in-stantly fainted in a bucket containing clove oil (20 mg L^{-1}) as an anesthesia (Iversen et al., 2003). A 2.5 ml plastic syringe was used to sampling blood from the caudal vein. Blood samples were divided into K3EDTA tubes for hematological analyses. Erythrocytes count (RBC's; × 106 per mm3), blood hemoglobin concentration (Hgb; g/dL) and hematocrit (Hct; %) were determined by using the method of Blaxhall and Daisley (1973). RBC was counted with a Thoma hemocytometer with the usage of Dacie's diluting fluid. The haematocrit was determined by using a capillary hematocrit tube. The haemoglobin concentration was determined with spectrophotometry (540 nm) by using the cyanomethahemoglobin method.

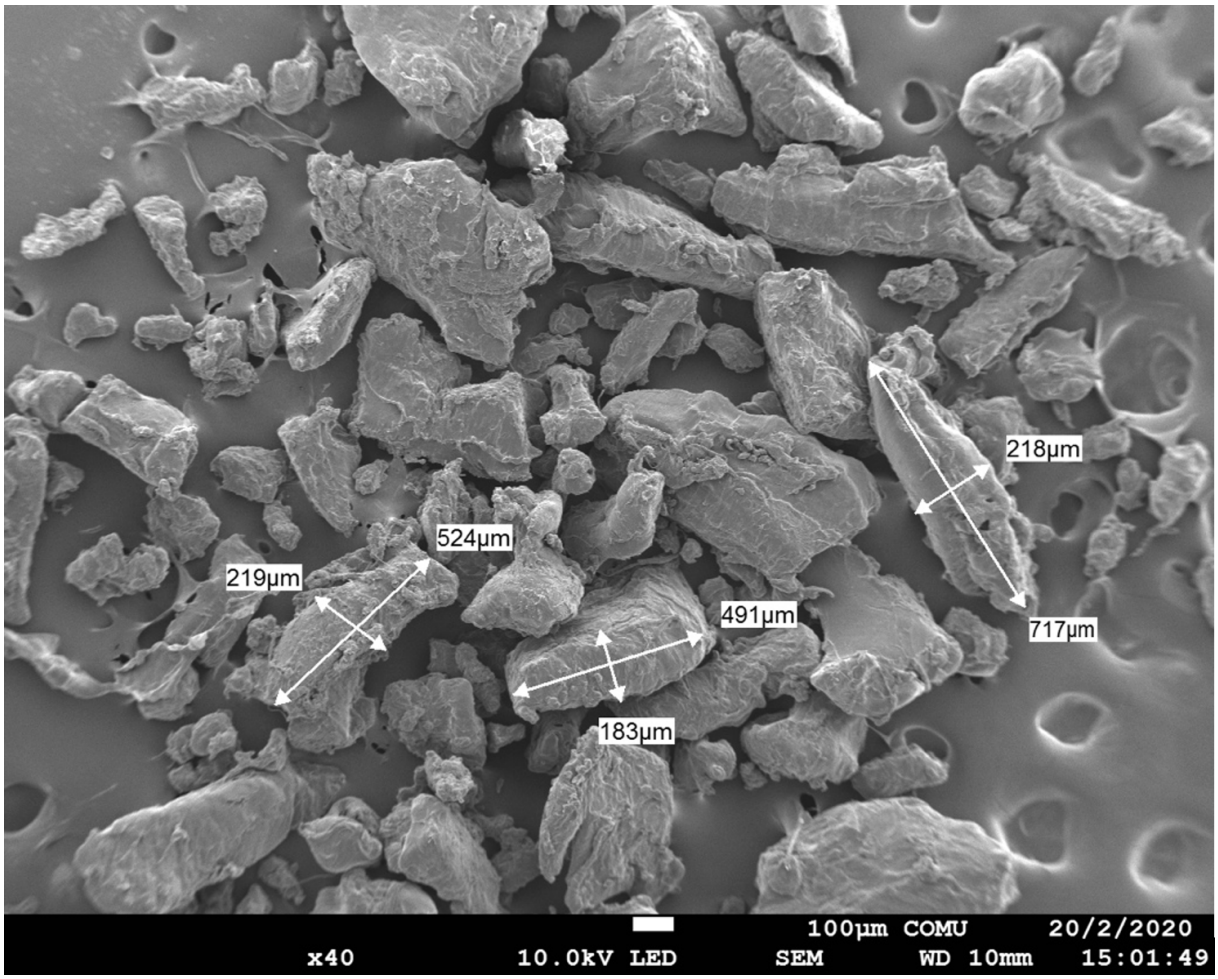

Figure 1. Scanning Electron Microscope (SEM) images of the investigated microplastics (MPs)

Comet assay

The "Comet Assay", also known as single cell gel electrophoresis, is a widely used method to determine DNA damage (Genotoxicity) in eukaryotic organisms (Nandhakumar et al., 2011; Olive & Banáth, 2006). The 2 µL blood samples taken were mixed with 80 µL low melting agarose. This mixture was pipetted onto slides covered with 1% high melting agarose and a coverslip was covered. The slides were left in the dark at +4 °C for 10-15 minutes to solidify the agar. After the coverslips were discarded, the slides were transferred from the stock lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH: 10) into freshly prepared working solution (prepared by adding 1% Triton X-100 and 10% DMSO to the stock lysis solution). After the lysis process, the slides were placed in the horizontal electrophoresis tank (Cleaver Scientific, UK) in the same direction and the voltage of the power supply was fixed at 25 V and the ampere at 300 mA, and electrophoresis was performed for 30 minutes. After electrophoresis, the slides were washed with neutralization buffer (0.4 M Tris, pH 7.5) 3 times for 5 minutes at +4 °C. Finally, slides stained with 50 µL of ethidium bromide (20 µg/mL) were photographed under a fluorescence microscope (Carl Zeiss / Scope A1, Germany) by closing the coverslip. The analysis of the comets was done through visual scoring and image analysis software (Tritek's CometScore™ Version 2.0). (REF: 10.1089/rej.2009.0931; 0.1074/jbc.M414391200). At least 100 cells were analyzed in each control and treated group. Tail DNA %, Tail Intensity, Tail Length (px), and Tail Moment parameters were used to detect increased DNA damage.

Statistical analysis

To determine the significance differences DNA damage in blood of experimental fish, the data were analyzed using the Sigma Plot 12 (for Windows version) program. The normality test of the groups was done by Shapiro-Wilk analysis. Kruskal-Wallis H test was used to determine the differences between groups, and Dunn's test was used for multiple comparisons. Data were summarized as the Median (25%-75%) and a $p<0.05$ value was considered significant. The statistical analysis was using the SPSS 21.0 package program. ANOVA with Tukey post-test (one-way ANOVA for comparison between the exposure groups and the control group) was used. The significance level was considered to be 0.05.

Results

Throughout the trial, mortality rates were evaluated on a daily basis by keeping an eye on any changes in fish behavior. There were no fatalities or behavioral changes in *O. niloticus* as a result of exposure treatment, showing that MPs did not produce severe acute toxicity.

Hematological parameters

Table 1 showed that, the hematological indices; erythrocytes count (RBC's), blood hemoglobin concentration (Hgb) and hematocrit (Hct) showed significant ($P < 0.05$) decrease after exposure to 5, 10 and 50 mg/L of MPs for 14 days in comparison with the control group.

Table 1. Effect of MPs exposure for 14 days on the hematological parameters of the Nile Tilapia (*Oreochromis niloticus*)

Groups	RBC($\times 10^6$ per mm3)	Hct (%)	Hgb (g/dL)
Control	2.30±0.15^{a}	22.96±1.06^{a}	7.74±0.45^{a}
5 mg/L	2.19±0.19^{a}	22.42±1.19^{a}	8.12±0.26^{a}
10 mg/L	2.06±0.19^{a}	20.04±1.31^{b}	6.90±0.22^{b}
25 mg/L	1.64±0.04^{b}	17.08±0.24^{ac}	6.32±0.15^{c}
50 mg/L	1.56±0.07^{b}	16.02±0.41^{c}	5.78±0.13^{d}

Values are mean (n = 9). (Mean ± SD). Values within the same column having different superscripts are significantly different ($P < 0.05$). RBC, red blood cells; Hbg, haemoglobin concentration; Hct, haematocrit

DNA damage

Table 2 summarizes the findings of comet analysis of blood samples collected from Nile tilapia fish after the application.

Table 2. Genotoxic effect of MPs in different doses on Nile tilapia blood

	Control	5 mg/L	10 mg/L	25 mg/L	50 mg/L
Tail Moment (TM)	114.45 (8.28-288.22)	96.69 (82.4-1179.8)	103.98 (81.86-136.75)	94.18 (68.47-118.91)	101.14 (62.90-155.186)
Tail Intensity (TI)	1136.50 (16.75-12878.75)	5569.0 (1523.5-266135.0)	67817.0 (1180.75-293856.0)*	124822 (450.0-304306.0)*	112528.0 (2252.25-332092.75)*
Tail Length (px, TL)	0.000 (0.00-1.50)	0.00 (0.00-40.00)	19.50 (0.00-41.50)*	29.00 (0.00-49.00)*	22.50 (0.00-41.75)*
% DNA tail	0.209 (0.001-6.105)	0.876 (0.154-18.940)	6.895 (0.148-25.939)	14.279 (0.116-33.669)	15.776 (0.228-30.339)*

Values are expressed as median (25% - 75%). *$p<0.05$ value was considered significant.

After 14 days of treatment, MPs were shown to enhance DNA damage in blood cells. Exposure to MPs of 10 mg/L and above resulted in high DNA damage in fish blood cells ($p<0.05$). Furthermore, compared to the control, the 50 mg/L doses of MPs given resulted in a considerable increase in %DNA tail ($p<0.05$). After the application, however, all groups' TM values were at a similar level. These findings indicate that the MPs compound has a genotoxic effect in Nile tilapia blood cells.

Discussion

To the best of our knowledge, MPs are an important type of pollution in aquatic environments. Nevertheless a few studies have reported toxicological effects of them in aquatic organism. In the current study, the potential adverse effects of MPs in blood exposed for 14 days with varying level and shape of waterborne MPs was examined.

Hematological parameters in fish are good indicators to determine the health status of fish (Thummabancha et al., 2016). In the current study, the hematological parameters such as RBC, Hct and Hgb showed significant reduction after exposed to MPs. In the study by Hamed et al. (2019) decreased in Rbc, Hct and Hgb value in *O. niloticus* was observed after exposed to MPs. Another study on *Etroplus suratensis* Vijayaraghavan et al. (2022) reported decrease in RBC count after exposed PVC microplastics. It is thought that 1-4% of MP particles absorbed in the intestines of fish pass into the bloodstream and that micro- or nano-sized plastics in the bloodstream may cause local inflammation or tissue allergic reactions (Hwang et al., 2020). Iheanacho and Odo (2020) suggested that changes to erythrocyte content, hematocrit values, and hemoglobin concentrations reflect the defense mechanisms of fish to stress caused by exposure to environmental toxicity. MP, which accumulates in the digestive and circulatory systems after MP exposure, may have a toxic effect and cause a decrease in the hematological characteristics of the fish such as RBC, Ht and Hb. The anemia observed in this study may be caused by the lysis of red blood cells (RBCs) or the suppression of erythropoiesis due to damage to the hematopoietic tissue (Wintrobe, 1978). This damage could potentially result from an increased mechanical fragility of the RBC membrane (Rosenberg et al., 1998). The current study also indicates alterations in erythrocytes, supporting this hypothesis.

DNA damage is an important biomarker in ecotoxicological studies in the aquatic environment (Kaloyianni et al., 2020). Table 3 presented the DNA damage in treated Nile tilapia blood with the examined different size and shape of MPs. The TM, TI, TL and %DNA tail measured on 14st day of fish exposed to MPs, was higher in More than 10 mg/L administered compared to control. Supporting our work, exposure of zebra fish to polystyrene microplastics induced increased DNA damage in hearth (Dimitriadi et al., 2021). In another study, exposure of mussels' hemocytes to MPs resulted in an increase of DNA strand breaks (Gómez-Mendikute et al., 2002). Typically, these alterations are attributed to the generation of free radicals that interfere with DNA integrity, repair mechanisms, and the formation of the product of aneuploidy and/or disruption of cytokinesis, which leads to a rise in the formation of erythrocytic nuclear abnormalities (da CostaAraújo et al., 2022).

Conclusion

To be concluded, our study confirms the toxicological potential associated with exposure of Nile tilapia adults to different size and shape MPs. However, the hypothesis that the association of MPs could induce more serious adverse effects in fish was confirmed based on the evaluated toxicity biomarkers. Our findings add to our understanding of the influence of varied size and shape MPs on the physiological response of Nile tilapia *O. niloticus* in the hemaotological

parameters and blood DNA damage following a short-term exposure. These findings show that certain MP particles or fragments produced during digestion may pass through enterocyte cells, enter the circulatory system, and reach the tissues, resulting in a stressful scenario. Additional research is needed, however, to validate the route of entrance and subsequent spread.

Ethical approval
This study was approved by the Animal Ethical Committee of Çanakkale Onsekiz Mart University (Çanakkale, Turkey, Approval Number: 2021/05-05 and 05.03.2021).

Informed consent
Not available.

Data availability statement
The authors declare that data can be provided by corresponding author upon reasonable request.

Conflicts of interest
There is no conflict of interests for publishing this study.

Funding organizations
No funding available for this study.

Contribution of authors
Ümit Acar: conceptualization, supervision, writing review and editing
Yavuz Erden: investigation, writing-review and editing, visualization
Sevilay Günay: formal analysis
Osman Sabri Kesbiç : investigation, writing
Sevdan Yılmaz: Investigation, formal analysis
All authors have read and agreed to the published version of the manuscript.

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