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Toxic effects of MPs and Up Grade® herbicide on the Nile tilapia (*Oreochromis niloticus*): Oxidative stress, immune responses, and histopathological investigations

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Abstract

This study explored the combined toxicity of polyethylene microplastics (PE-MPs) and the herbicide Up Grade 46% SL (UPGR) on Nile tilapia (*Oreochromis niloticus*), focusing on antioxidant, immunological, and histopathological responses. Nile tilapias were divided into four groups: control, PE-MPs exposure (2.916 mg/L), UPGR exposure (10 mg/L), and combined PE-MPs and UPGR exposure. Over 15 days, significant changes were observed in various biomarkers. Total antioxidant capacity (TAC) levels significantly decreased in the PE-MPs group compared to the control ($p < 0.05$), while superoxide dismutase (SOD) levels increased in both PE-MPs and UPGR groups ($p < 0.05$). The combination group showed significantly elevated levels of immune biomarkers IL-1β and IL-6 compared to individual exposures ($p < 0.05$). Histopathological examination revealed severe alterations: gills showed degenerated lamellae, malformed secondary lamellae, and congested blood capillaries; intestines had vacuolations, degenerated muscularis, hydropic degeneration, and numerous inflammatory cells; and testes exhibited degraded tubules, pigmentation, few primary spermatogonia, and necrosis of many cysts. These findings indicate that both PE-MPs and UPGR individually and synergistically induce oxidative stress, immune response alterations, and significant tissue damage in *O. niloticus*. The study underscores the urgent need to explore how microplastics interact with other pollutants in aquatic ecosystems, posing combined threats to aquatic life. The observed joint toxicity suggests potential long-term impacts on fish health and calls for comprehensive risk assessments and mitigation strategies to protect aquatic environments from such chemical pollutants.

1. Introduction

Aquatic animals are frequently exposed to a variety of chemical contaminants, including microplastics (MPs), pesticides, pharmaceuticals, organic and inorganic pollutants, and metals, either individually or as complex mixtures (Hamed et al., 2023; Hamed et al., 2019; Horton et al., 2018; Hossain et al., 2022; Said et al., 2023; Said et al., 2021). MPs are tiny solid plastic particles smaller than 5 mm in size (Kim et al., 2021). Today, MPs and pesticides contamination has become a major and a serious issue in aquatic environments and pose a threat to aquatic animals including fish (Wright and Kelly, 2017; Hamed et al., 2019). Due to their large surface area and hydrophobic nature, MPs can adhere to and absorb aquatic toxins (Banaee et al., 2019; Burgos-Aceves et al., 2021).

Pesticides and MPs can pass from aquatic habitats to aquatic animals' bodies through gills, oral and skin (Mohamed et al., 2023; Curi et al., 2024; Zazouli et al., 2022; Sayed et al., 2023a). Once ingested by aquatic animals, MP particles may bring in a series of deleterious effects on fish health, such as blockage and injury of the digestive tract and reducing food intake, triggering morbidity, inhibiting growth and development, and finally contributing to death (Wang et al., 2022; Bhuyan, 2022; Habumugisha et al., 2024). Studies have also indicated that exposure to MPs can induce embryotoxicity, neurotoxicity, oxidative stress, genomic instability, immunotoxicity, reproductive abnormities, hepatic stress, intestinal abnormalities, and endocrine system disruptions in several freshwater and marine fish species (Carlos de Sa et al., 2015; Ferreira et al., 2016; Oliveira et al., 2013; Peda et al., 2016; Hamed et al., 2019;

Rochman et al., 2013; Barboza et al., 2018; Hamed et al., 2022; Bakr et al., 2023). Furthermore, MP particles are capable of absorbing different chemical pollutants (e.g., pesticides, metals and antibiotics), and pathogens from the surrounding aquatic environment into fish and other aquatic organisms, thus threatening health of these organisms as well as human (Haleem et al., 2024; Wang and Secombes, 2009; Wen et al., 2018; Hamed et al., 2024c).

Over the past decade, herbicides such as 2-methyl-4-chlorophenoxyacetic acid (MCPA) and bentazone have become a crucial element in agricultural systems due to their widespread use, particularly in controlling serious weed infestations (Kudsk and Streibig, 2003). The large-scale and continuous application of herbicides such as MCPA and bentazone in agricultural and urban activities has resulted in contamination of fresh water bodies and leading to adverse effects on fish (Saleh et al., 2020; Lutnicka et al., 2018; Lopez-Pineiro et al., 2017; Morton et al., 2020). Many herbicides also have lethal and sub-lethal toxic effects on several freshwater fish and amphibia (Lutnicka et al., 2018; Saleh et al., 2022; Mahmoud et al., 2022; Said et al., 2022; Fathy et al., 2019). Sub-lethal exposure to MPCA (100 µg/L) for 14 days, *Cyprinus carpio* produced a great change in haematological variables and partial to the ultrastructural lesions in different hematopoietic organs (Lutnicka et al. 2018). Lower concentration of bentazone also induced oxidative damage, haematological and biochemical changes, neurotoxicity, and histopathological effects in *O. niloticus* (Fathy et al., 2019; Saleh et al., 2022).

The way microplastic particles interact with other chemical contaminants, like pesticides, when they coexist in aquatic environments, significantly impacts their toxicity and environmental persistence (Atugoda et al., 2021; Yang et al., 2024; Klavins et al., 2022). PE-MP have higher affinity and adsorption capacity to hydrophobic organic compounds including various pesticides than other polymers (Wang et al., 2020) and it influenced on toxicity of these compounds.

Up Grade 46% SL is a selective premix herbicide, containing the active ingredients bentazone sodium-salts and MCPA, registered in Egypt to eliminate sedges and various broad-leaved weeds in rice, where *O. niloticus* fish are frequently farmed. The Nile tilapia (*O. niloticus*) is frequently utilized in toxicology studies due to its suitability as a freshwater fish model (Hamed et al., 2019). The toxicity of co-exposing *C. gariepinus* to lead and microplastics was evaluated by (Soliman et al., 2023). In our previous study, the acute toxicity of UPGR herbicide on *O. niloticus* was done with 96h-LC_{50} of 29.16 mg/L and combination of UPGR and PE-MP caused behavioral and morphological changes, along with alterations in hematological and biochemical parameters in *O. niloticus* (Mohamed et al., 2023). However, sub-lethal effects of combined and individual effects of UPGR and PE-MP on the immune defense systems variables (e.g., interleukins [IL] and tumour necrosis factor [TNF]), and the antioxidant system variables (e.g., CAT, GPx, and SOD enzymes) of *O. niloticus* have never been studied before. Pro-inflammatory cytokines, including IL and TNF were used in the fish immunotoxicity testing the immune genes that were most frequently examined in fish immunotoxicity investigations (Graham et al., 2010; Mendiola and Cardona, 2018). Histopathological studies are also commonly used as an essential indicator in the acute and chronic toxicity investigation to assessed the resultant alternations in fish' tissues of the vital organs resulting from exposure to MPs, pesticides and other pollutants (Saleh et al., 2022; Sayed et al., 2023b;

Hamed et al., 2024c; Hamed et al., 2024b; Hamed et al., 2024a). Thus, this study is pioneering in its assessment of the impact of PE-MPs and Up Grade 46% SL (UPGR), both separately and together, on oxidative stress markers, immune responses, and histopathological changes in the gills, intestines, and testes.

2. Material and methods

2.1. Chemicals

PE-MPs powder was procured from Toxemerge Pty Ltd. in Melbourne, Australia. A fresh stock solution was made by dissolving the powder in Milli-Q purified water as per the manufacturer's guidelines and was kept in the dark at 4 ºC. Before each use, the stock solution (2.5 g MP/L) was sonicated as outlined by Hamed et al. (2019). The study also used a commercial herbicide formulation, Up Grade SL 460 g ae/L, which contains 400 g ae/L bentazone and 60 g ae/L MCPA. This herbicide was acquired from StarChem Industrial Chemicals in Egypt.

2.2. Experimental design

Juvenile *O. niloticus* (weight 35.97 ± 1.95 g and length 13.10 ± 0.96 cm) were obtained from fish Farm, College of Agriculture, Assiut University, Egypt. The fish were carefully transferred to the Fish Biology and Pollution Laboratory, Collage of Sciences, Assiut University and were stoked for 30 days acclimation in 96 L glass aquariums filled with dechlorinated water and under a photoperiod of 12:12h light dark (Mohamed et al., 2023). 120 of the acclimated juvenile *O. niloticus* were chosen to use for the laboratory experiment. Fish were randomly divided into 4 equal treatment groups (30 fish per group) and placed in 12 glass aquariums (10 fish per aquarium [96 L]). The first group was assigned as the control and was kept in clean dechlorinated water without MPs or herbicide. The fish in the second group were exposed to one-tenth of the 96h-LC_{50} of a premix Up Grade 46% SL herbicide (2.916 mg/L) (Mohamed et al., 2023). The fish in the third group were exposed to individual polyethylene MPs (10 mg/L) according to Hamed et al. (2019). In the fourth group, fish were exposed to Up Grade 46% SL (2.916 mg/L) plus polyethylene MPs (10 mg/L). The glass aquariums were cleaned, the water was completely changed, and the exposure solutions of the mentioned treatments were freshly prepared every 48 h. Fish were exposed to the tested materials individual or in combinations for 15 days and no mortality was found in fish. Water quality variables during the period of acclimation and the experiment were measured as follows: ambient temperature, conductivity, pH, and dissolved oxygen of 26 ± 1 ºC, 260.8 mM/cm, 7.4 units, and 6.9 mg/L, respectively. After exposure period (15 days), nine fish were randomly collected from each treatment group (3 fish from each aquarium) and anesthetized with crushed ice (Hamed et al., 2019). Blood samples from fish in the control and treatment groups were carefully collected for antioxidant biomarker and immunological analyses using the caudal severance (tail-cutting) method.

2.3. Measurement of antioxidant and immune parameters biomarkers

Superoxide dismutase (SOD) activity was measured using the method described by (Nishikimi et al., 1972; Hamed et al., 2020). Total antioxidant capacity (TAC) was quantified in serum in triplicate based on methods outlined in (Koracevic et al., 2001; Hamed et al., 2024c). TAC was measured using colorimetric spectrophotometry (Spectronic 21, Moton Roy Co., Madison, WI, USA).

Serum cytokine levels for IL-1β and IL-6 level were quantified as described by (Wang et al., 2009; Hamed et al., 2024b) using an ELISA kit (CSB-E13259 F h) and (Hanington and Belosevic, 2007; Hamed et al., 2024b) using an ELISA kit (CSB-E13258 F h), respectively.

2.4. Histopathological investigations

Gills, intestines, and testes samples were promptly removed after laparotomy and preserved in 10% neutral buffered formalin for at least 24 hours for histological analysis. The samples were then processed using a standard paraffin embedding technique, sectioned to a thickness of 5 µm, and stained with Harris' hematoxylin and eosin (H&E). Examination of the stained sections was carried out with an Olympus BX50F4 microscope (Olympus Optical Co., Ltd., Tokyo, Japan).

2.5. Statistical analysis

Data analysis was carried out using SPSS software (Version 25), with a significance threshold set at 0.05. First, the normality of the data was evaluated using the Shapiro-Wilk test. A one-way analysis of variance (ANOVA) was then performed, and homogeneity of variances was assessed using Levene's test. If the variances were homogeneous, Fisher's LSD post hoc test was used to compare treatment groups with the control group. If variances were not homogeneous, Dunnett's post hoc test was used for comparisons between treatment groups and the control group.

3. Results

3.1. Antioxidants parameters

The findings for TAC and SOD are presented in Table 1. TAC levels were notably reduced after exposure to PE-MPs in comparison to the control group, while exposure to UPGR and UPGR + PE-MPs did not produce significant changes in TAC levels relative to the control. Conversely, SOD levels showed a significant increase following exposure to PE-MPs, UPGR, and UPGR + PE-MPs when compared to the control group.

Table (1): The antioxidant parametersin the serum of *O. niloticus* exposed to PE-MPs, UPGR and their combination.

Parameters	Treatment			
	Control	PE-MPs	UPGR	UPGR + PE-MPs
TAC (μM/L)	1.08 ± 0.00^{a}	0.87 ± 0.04^{b}	1.05 ± 00^{a}	1.07 ± 0.01^{a}
(SOD) (IU/L)	11.5 ± 0.17^{a}	11.9 ± 0.05^{c}	11.7 ± 0.1^{b}	11.6 ± 0.06^{ab}

Data are expressed as means ± SD. Significant differences between values within the same row are indicated by different superscript letters ($P < 0.05$).

3.2. Immunological parameters

The results for IL-1β and IL-6 were shown in Table 2. IL-1β levels were significantly higher in *O. niloticus* exposed to PE-MPs, UPGR, and UPGR + PE-MPs compared to the control group, which had IL-1β levels of 10.7 ± 0.14 pg/mL. Although IL-6 levels only slightly increased from 51.5 ± 3.0 pg/mL in the control group to 54.9 ± 1.9 pg/mL in the PE-MPs exposed group, they increased markedly in the UPGR (60.0 ± 1.0 pg/mL) and UPGR + PE-MPs (65.9 ± 2.66 pg/mL) treatment groups.

Table (2): The immunological parameters in the serum of *O. niloticus* exposed to PE-MPs, UPGR and their combination.

Parameters	Treatment			
	Control	PE-MPs	UPGR	UPGR + PE-MPs
IL-1β (pg/mL)	10.7 ± 0.14^{a}	12.2 ± 0.24^{b}	14.6 ± 0.34^{c}	17.2 ± 0.33^{d}
IL-6 (pg/mL)	51.5 ± 3.0^{a}	54.9 ± 1.9^{a}	60.0 ± 1.0^{b}	65.9 ± 2.66^{c}

Data are expressed as means ± SD. Significant differences between values within the same row are indicated by different superscript letters ($P < 0.05$).

3.3. Histology

3.3.1. Gills

Like other teleost fish species, the control group of *Oreochromis niloticus* displayed typical gills features, including numerous rows of filaments from which the lamellae emerge. The squamous epithelium that lines the lamellae is followed by lamellar blood sinuses that are divided by pillar cells (PC). Between the lamellae, the filament is bordered with a thick stratified epithelium made up of many cell types, including pavement cells (PVC), erythrocytes (E), and chloride cells (CC) (Fig. 1A). Fish exposed to MPs had severe pathological alterations in their gills. Severe deterioration and ulcerations were seen in the epithelium and cartilage of the primary lamellae. Additionally, blood capillary of the secondary lamellae had aneurysms (a). Secondary lamellae have also shown atrophy, numerous degenerations (DSL), and pillar

cell disorganization (Fig. 1B). Furthermore, The UPGR treated groups showed significant deformation in gill architecture, enlargement of the primary gill core with hemorrhage and deformed cartilage (DC) and necrosis (N) in other regions, disarchitecture in gill morphology, deformed and shorting of (SL) with significant distracted of (PC-BC) system, as well as significant damage in circulatory system with aneurysms (a) like PE-MPs groups. Epithelial lifting and edema were seen at the base of secondary and stratified epithelium (Fig. 1C). The histological characterization of the gills in the joint (UPGR + PE-MPs) treatment group revealed shouter stratification in morphology, thinning of primary gills, and ablation of their core. Furthermore, secondary lamellae appeared to be thinning and elongated, with cartilage distortion. The blood capillary system and pavement cells were severely disrupted, and there were numerous dissolutions and breakdowns of the stratified epithelium (SE) as well as vacuolation (black arrow) between the secondary lamellae (Fig. 1D).

Figure (1): A: The gills of control fish have numerous gill filaments with two rows of secondary lamellae (SL), made up of epithelium sheets bordered by pillar cells (PC). Erythrocytes (E) are recognized within each capillary lumen. Chloride cells (CC) are present at the lamellae base, while mucous cells (MC) are observed in the inter-lamellar epithelium and at the distal tip of the primary lamellar epithelium (PLE). Pavement cells (PVC) are found in the filament epithelium and at the lamellae base. B: PE-MPs groups showed severe gill deformation with deformed (D) cartilage, thinning, degenerated, and lifting of stratified epithelium (SE). Degenerated curved SL, changes in the blood circulatory system with aneurysms (a), atrophy, and degeneration in SL core were noted, along with disorganization of pillar cells and blood capillaries. C: UPGR-treated groups showed severe gill architecture deformation, hemorrhage, deformed cartilage (DC), and necrosis (N). Gill morphology disorganization, shortened SL, severe disruption of pavement cells and blood capillary system, and damage in the circulatory system with aneurysms (a) were noted. Epithelial lifting and edema were observed at the SL base. D: Joint (UPGR + PE-MPs) treatment showed shortened stratification in gill morphology, thinning of primary gills, and core disappearance. SL appeared as one layer with deformed cartilage (DC), thinning, and lengthening. Severe disruption of pavement cells and blood capillary system, few aneurysms (a), many DSL dissolutions, and SE breakdown with vacuolation (black arrow) between SL were observed (stained with Harris' hematoxylin and eosin- 40X).

3.3.2. Intestine

The intestine of *O. niloticus* in the control group displayed normal morphology in all layers, with the mucosal layer being thrown into finger-like villi and composed of many goblet cells (mucous cells) with centrally located nuclei and simple, long columnar cells (Fig. 2A). While, in fish administered PE-MPs, the intestine showed vacuolated cells of longitudinal muscle fibres and destroyed circular muscle fibres (CMF). Additionally, inflammatory cells with various morphologies, cell proliferation in the crypt region, and vacuolated cytoplasm are present in the submucosa layer. Columnar cells with active nuclei, vacuoles, and hyperaemic cytoplasm, as well as a few goblet cells and numerous pyknotic nuclei, can be found on the lateral side of villi (Fig. 2B). The intestine in the fish treated with UPGR showed deteriorated serosa and vacuolation muscle fibres as well as the breakdown of muscle layers cells. Muscle fibres had pyknotic nuclei, and the submucosa layer contained inflammatory and edematous cells (IC). Additionally, the mucosal layers displayed deteriorated cells and vacuolated cytoplasm (D) (Fig. 2C). The intestine of fish that received combined chemical treatment showed confusing borders between the different layers, serosa loss, and widening of the muscle layers. Deformed cells, vacuolation, and hydropic degeneration

(HD). There is also hydropic degeneration in the submucosa, which has a lot of inflammatory cells (IC). Columnar cells with mild acidophilic cytoplasm, vesicular nuclei, and a degenerating brush border were present in the mucosal layer (Fig. 2D).

Figure (2): A: The control group showed normal intestine morphology with finger-like villi, simple long columnar cells, goblet cells (GC), thin submucosa (SM) projecting into mucosal folds (F), loose connective tissue with blood cells in the lamina propria (LP), and defined circular (CML) and longitudinal muscle layers (LML) with a peritoneal serosa (S). B: PE-MPs exposure resulted in thin serosa (orange arrow), vacuolated longitudinal muscle fibers (LMF), and disintegrated circular muscle fibers (CMF) with deeply stained nuclei (black arrow). The submucosa had inflammatory cells (IC) and vacuolated crypt cells (green arrows). Lateral villi showed vacuoles, hyperemic cytoplasm, and fewer goblet cells with a damaged brush border (BB) (blue arrows). The villi trough exhibited vacuolated cells (V) with many pyknotic nuclei (PN). C: UPGR-treated fish had degenerated serosa (orange arrow), vacuolated muscle fibers (V), disintegrated muscle cells, pyknotic nuclei (PN) in muscle fibers (blue arrow), and edematous submucosa (E) with inflammatory cells (IC). Mucosal layers showed vacuolated cytoplasm, active nuclei in columnar cells, and a damaged brush border (BB). D: Fish treated with UPGR and PE-MPs showed indistinct layer boundaries, serosa loss, widened muscle layers, hydropic degeneration (HD), vacuolated cells (V), and deformed cells. Submucosa had extensive inflammatory cells (IC), and the mucosal layer rested on an undulating basement membrane (dark arrow) with vacuolated columnar cells, increased goblet cells, and a degenerated brush border (BB) (stained with Harris' hematoxylin and eosin- 40X).

3.3.3. Testes

In the testes of the *O. niloticus* control group, seminiferous tubules (ST) seem healthy with all stages of spermatogenesis and clearly visible. Primary spermatogonia cysts are large, spherical cells with pale, acidophilic cytoplasm and vesicular and central nuclei. There was an accumulation of microscopic spermatozoa inside the lumen of lobules that appeared spherical and were heavily colored (Fig. 3A). On the other hand, fish that had their testes exposed to PE-MPs showed seminiferous tubules that were encircled by thick basement membrane (BM). It was clear to see fully empty tubules, degenerated cysts (DC), necrotic stages inside tubules, and vacuoles. Only secondary spermatocytes or spermatids were found. Edema in interstitial tissues was seen as a drop in ledge cell number (Fig. 3B). The testes *O. niloticus* from the UPGR exposed group show enlargement of the seminiferous tubule, a few early stages of primary spermatogonia, and Sertoli cells (SC). Only a small number of tubules have degraded phases, while additional tubules have secondary necrotic and vacuolated spermatocytes. Between tubules, there were a few ledge cells that were interstitial. Additionally observed were brown pigments (P) (Fig. 3C). *O. niloticus* testes from the jointly treated group with chemicals showed deformation (D) of seminiferous tubule that was bordered by thick basement membrane (BM). Necrosis (N) of many cysts and borderline degeneration of cysts in tubules. Both secondary spermatocytes' main cysts contain sperm and spermatozoa in the lumen. With few ledge cells, the interstitial space is reduced (Fig. 3D).

Figure (3): A: Control testes of O. niloticus showed normal seminiferous tubules (ST) with all stages of spermatogenesis, including primary spermatogonia (PSG = 1), secondary spermatogonia (SSG = 2), primary spermatocytes (PSC = 3), secondary spermatocytes (SSC = 4), spermatids (SP = 5), and spermatozoa (SZ = 6), with Sertoli cells (S = 7) and Leydig cells in the interstitial tissue (CT). B: PE-MPs exposure caused tubule degeneration (D), necrosis (N), and vacuolation (V), with only secondary spermatocytes and reduced interstitial space. C: UPGR treatment resulted in widened tubules with thick basement membranes (BM), degeneration (D), necrosis (N), and vacuolation (V), with spermatozoa in the lumen and brown pigments (P). D: Combined UPGR + PE-MPs treatment showed severe tubule deformation, necrosis (N), and vacuolation (V), with reduced interstitial space and few Leydig cells (stained with Harris' hematoxylin and eosin- 40X).

4. Discussion

The current study on *O. niloticus* after exposure to a combination of PE-MPs and UPGRs is a sequel of our earlier research on the ecotoxicology of emerging contaminants, namely PE-MPs and Up Grade 46% SL (UPGR) (Mohamed et al., 2023). PE is the most common type of plastic polymer in freshwater ecosystems and is the source of PE-MPs due to its buoyancy in surface currents, and sensitivity to the sun's UV radiation (Corcoran et al., 2015; Cole et al., 2011), and are easily ingested by fish via many routes (Sayed et al., 2023b). The co-existence with MPs has a negative impact on the bioaccumulation processes of numerous contaminants. However, the most frequently observed harms include histological, genetic, and physiological changes, and the severity of the injury appears to be related to MP concentration, size, and pollutant type (Rios-Fuster et al., 2023). The bioavailability of co-contaminants may be influenced by MPs since other chemicals might adsorb on them (Sleight et al., 2017). As an illustration by (Zhang et al., 2019), the inclusion of MPs can alter the toxicity and accumulation of related toxic compounds in *O. niloticus* as compared to exposure to the chemical alone. These mechanisms, though, are still poorly understood. Nevertheless, it has recently been shown that microplastics can adhere to lipid membranes and stretch the lipid bilayer without the need for oxidative processes (Fleury and Baulin, 2021). The cell machinery may become seriously dysfunctional because of this mechanical stretching. This may be accounted for the current results in term of deterioration in the antioxidant markers, immune indices and histopathological observations. In study on *Oreochromis niloticus* after exposed to microplastics, found alterations in antioxidants enzymes (Hamed et al., 2020). They suggested that MPs cause generation of ROS, which alter antioxidants.

In this regard, ROS generation could be increased by pesticides (Es Ruiz de Arcaute et al., 2019). Similarly, the herbicide butachlor was reported to cause acute poisoning in *C. gariepinus* as a result of a suppressed glutathione detoxification pathway, due to oxidative damage (Farombi et al., 2008). Following exposure to 10 and 100 mg/ L of 2,4-D herbicide, respectively (Atamaniuk et al., 2013), the activities of SOD in the gills of *Carassius auratus* increased by 29 and 35% in comparison to controls. Microplastic-exposed zebrafish displayed symptoms of oxidative stress, which altered their metabolism (Lu et al., 2016).

These observation, therefore could be due to microplastics exhibited efficacy of adsorption when combined with POPs, such as oil hydrocarbons, endosulfan, chlorpyrifos, musk xylene, musk ketone,

musk moskene, galaxolide, tonalide, and celestolide (Concha-Grana et al., 2022; Gonzalez-Soto et al., 2022). However, Horton et al. (2018) suggested that PE-MPs are unlikely to act as a vector for enhanced pesticide uptake in aquatic organisms. While, in the current study, *O. niloticus* exposed to PE-MPs coupled UPGR showed considerably different immunological and oxidative parameters as compared to control and separately treated groups, indicating notable alterations in physiological and biochemical responses. This is in accordance with Araujo et al. (2023) who resulted multiple alterations including mutagenicity, cytotoxicity, antioxidant and cholinesterase responses in the amphibian tadpole *Physalaemus* cuvieri exposed to mixture of microplastics and a mix of pollutants. A study by Karami et al. (2016) on the biomarker responses in the African catfish as a result of exposure to microplastic-loaded phenanthrene matches our findings. Their findings showed that PE-MPs synergistically with phenanthrene significantly increased the degree of tissue changes.

Antioxidants and related enzymes are recognized as responsive and sensitive indicators of environmental stressors. The current study's observation of increased SOD may be a compensatory response to an imbalance between the production of ROS and the total antioxidant capability TAC as indicated by Kim et al. (2021). Another indication is that SOD and CAT are frequently used to prevent lipid oxidation, as they stabilize free radicals through direct interactions and mitigate the production of ROS in response to environmental stressors (Hermund, 2018; Pandey et al., 2003), and their alteration is a signal for stress. Similar research by (Ding et al., 2018), concluded altered enzyme activity and oxidative damage. The study by Wen et al. (2018), exposure of *Symphysodon aequifasciatus* to MPs and Cd demonstrated that the combined impact of these two stressors influenced the activity of CAT and SOD. Also, Hamed et al. (2020) tabulated similar alterations in SOD and TAC from *O. niloticus* exposed to MPs.

Additionally, Wang et al. (2022) assessed the combined toxic effects of polyvinyl chloride microplastics and di(2-ethylhexyl) phthalate on *Danio rerio*, revealing that this combination led to the production of ROS and the activation of antioxidant defenses. Measuring TAC alone does not fully capture the roles of crucial enzymes such as SOD, GPx, and CAT (Fraga et al., 2014). Therefore, caution is needed when interpreting data, as plasma antioxidant capacity may oversimplify the complex in vivo state (Costantini, 2011).

To regulate the immune response and inflammation during stressful conditions, immune cells known as macrophages produce pro-inflammatory cytokines (TNF-α, IL-16 and IL-1β) (Silvestre, 2020). According to Tuñón et al. (2019), inflammation is frequently linked to anomalies in lipid metabolism, and the two interact and control one another. Following treatment with 100 nm – 5 µm MPs, quantitative examination of serum revealed a rise in the serum levels of TNF-α and IL-1β in both healthy and diabetic mice (Liu et al., 2022). IL-6 and IL-1βserves a variety of physiological purposes, and fish retain its ability to regulate the inflammatory response (Secombes et al., 2011). This supports the current findings, where (UPGR + PE-MPs) exposed *O. niloticus* exhibited synergistic and remarkable increase in IL-6 andIL-1β compared to individual exposures or control.Such inflammatory responses were reported to be caused by PS-MPs after they interfered with the immune systems of various fish, including *Oryzias melastigma* (Chen et al., 2020)d *rerio* (Limonta et al., 2019). Following 15-day exposure to pyrogallol, our previous search (Hamed

et al., 2024a; Hamed et al., 2024c; Hamed et al., 2024b) have revealed similar findings including oxidative stress, oxidants /antioxidants changes, endocrine disruption, immunotoxicity, and histopathology in *C. gariepinus*.

Depending on the type of toxin and the delivery mechanism, either separately or jointly, the pattern and severity of the histopathological abnormalities were obviously different as mentioned in the result part. Research has shown that exposure to MPs can lead to oxidative damage, inflammation, loss of gut epithelial integrity, a decrease in the mucus layer, microbial imbalances, and toxicity to immune cells (Huang et al., 2021). According to Lu et al. (2016), the distribution and accumulation of toxic and xenobiotic chemicals within tissues may result in the impairment of detoxifying mechanisms that are responsible for the removal and excretion of these toxins. Confirmedly, the author demonstrated the aggregations of MPs particles in gills intestines, and liver of *D. rerio* exposed to MPs, accompanied with severe inflammation. Consistently, Kim et al. (2022), revealed behavioural and gastrointestinal damage in *D. rerio* as consequence for MPs exposure. Fat vacuoles, inflammatory cell infiltration, hepatocyte enlargement, and apoptosis were the most notable changes in tissues of mice exposed to MPs (Liu et al., 2022). Not only MPS, but also, the degree of the histopathological abnormalities caused by pesticides was linked with the types of tissues and the route of pesticide entry (Salvo et al., 2015a). In *D. rerio* subjected to combination pesticide the histological investigation of liver showed significant cytoplasmic vacuolation, spreading sinusoidal congestion, steatosis, pyknotic, and karyorrhectic nuclei, and total breakdown of necrotic hepatocytes (Rajini et al., 2015).

Histological abnormalities were found in *O. niloticus* exposed to sublethal levels of various commercial herbicides, including bentazon (Saleh et al., 2022) included loss of liver architecture, the appearance of fatty liver cells, necrotic regions, foci of leukocytic infiltration, and a significant number of apoptotic cells were observed. The hematopoietic system of common carp subjected to 100 µg/l of MCPA (phenoxy acid herbicide) displayed granulocytes with many granules, vacuoles with varying electron densities and sizes, melanomacrophage, and myelin-like structures(Lutnicka et al., 2018). Also Salvo et al. (2015b)revealed the potential effects of MCPA (0.275, 2.75 and 27.5 µg/ L) as potent uncouplers of oxidative phosphorylation in fish *Metynnisroosevelti*.

Gills histological analysis is a promising method for monitoring aquatic stress because pathologists and researchers may not be as familiar with gill tissue as they are with mammalian lungs. On the other hand, gills are regarded to be an important pathway for the absorption, bioconcentration, and excretion of toxicants because of the size of the surface area in contact with the external medium and the close proximity of the internal and external media (Ameur et al., 2015). The current results of severe and widespread gill degenerations imply that the used chemicals, specifically PE-MPs and Up Grade, have toxic potential, supporting gills' injuries as a primary signal for pollutants. The gills of *D. rerio* experienced severe structural damages following exposure to a combined chlorpyrifos (8.4 µg/L) and cypermethrin (4.2 µg/L); these damages included inflammatory cell infiltration, congestion, and fusion in lamellae, diffused epithelial hyperplasia, and multifocal mucus (Rajini et al., 2015).

The observed changes in the intestine are consistent with several studies that examined the effects of various contaminants on the digestive tract of fish. For example, Younis et al. (2013) found necrotic changes in the intestinal mucosa and submucosa, atrophy in the muscularis and submucosa and aggregations of inflammatory cells in the mucosa and submucosa in the intestinal section of *O. niloticus* exposed to Cd. Following exposure to Lindane, grass carps had a histological alteration in their intestines comprising vacuolation, hemorrhage, epithelial degeneration, intestinal villi destruction, epithelial cell deformation, and epithelial cell death were described (Vajargah et al., 2021). Additionally Usman et al. (2022) noted energy metabolism disorders in the gut of *Oryzias javanicus* to be associated to polystyrene microplastics. In line with Hossam Mahmoud et al. (2020), the histopathological changes in intestine could be attribute to protein degradation brought on by the induced gut enzyme breakdown. In the gut tissues of the *Gambusia affinis*, deltamethrin had similar histopathological effects that included epithelial hypertrophy, lifting of the lamellar epithelium, oedema, dilatation of the primary capillary lamellae, aneurism, epithelial hyperplasia, and fusion of the secondary lamellae(Cengiz and Unlu, 2006). In accordance, Clark et al. (2022) demonstrated that fish gut lumen-bound nanoplastics could penetrate to tissue layers in the mucosa, muscularis, and serosal saline fluid. Furthermore, In wild caught fish, microplastics were detected in mean amounts of 0.51, 0.25, and 1.26 particles per gramme of fish carcass tissue (Zitouni et al., 2020).

The results of the current study are matching other earlier studies on the effects of contaminants on fish gonads. The study by Hayati et al. (2022) has revealed that exposure to plastic particles had an impact on spermatogenic development and metabolic disorders that could block the mitotic process, resulting in fewer spermatocytes in fish compared to controls. In the male *Catla catla* from polluted water, Bashir et al. (2022) resulted in various degeneration gonads revealed marked histological abnormalities such as testes degeneration, generalized tissue degeneration induced by fragmentation and detachment of basement membrane, necrosis, and fibrosis in the testis. Further investigations demonstrated histological changes, such those for carp *Cyprinus carpio* exposed to arsenic (Talas et al., 2014), D. *rerio* exposure to tris (2-chloroethyl) phosphate (Sutha et al., 2022). Similarly, the tilapia (*Coptodon zillii*), exposed to copper nanoparticles and the fungicide penconazole, where Farrag et al. (2022) described a variety of testicular abnormalities, including degenerative changes in the seminiferous tubules, a reduction in the number of spermatozoa, hemorrhage, congestion in the seminiferous tubules, spermatogonia, and spermatocytes with leucocytes infiltration. When dangerous compounds are our results are in line with those of Islam et al. (2017) on the histomorphology findings in male freshwater fish *Cyprinion watsoni* subjected to endosulfan, as they showed various testicular modification. Lastly, the current findings are significantly supported by our earlier investigation into the co-toxicity of MPs and UPGR on hematobiochemical and histological damage from *O. niloticus* (Mohamed et al., 2023). Totally, the integrated combinations of the studied chemicals show a more harmful effect on histopathology, oxidation variables, and immunotoxicity than their individual effects, which may be due to the potentially jointed effect that boosted by MPs.

Conclusion

This research is a comprehensive addition to our previous study on the impact of PE-MPs and UPGR on the aquatic ecosystem, with a focus on fish, which are vital as a source of meat. the findings elucidated the deteriorating impacts of PE-MPs and UPGR on antioxidant defence, immune systems and tissues histology, which can be fatal to *O. niloticus*. The intestine, gill, and testes all had degenerative and necrotic alterations, along with oxidative stress and immune suppression. The current work, therefore, indicates that toxicity of UPGR to *O. niloticus*, was boosted by PE-MPs. Consequently, additional restrictions on usage should be considered in conjunction with routine monitoring for terrestrial and aquatic ecosystems.

Declarations

Conflicts of Interest

The authors declare no conflicts of interest related to the publication of this study.

Ethical Approval

The studies received approval from the Research Ethics Committee of the Molecular Biology Research and Studies Institute (MB-2024-33-S) at Assiut University, Assiut, Egypt. All methods were conducted in accordance with applicable regulations and the ARRIVE guidelines.

Funding Declaration

No funding was received for this work.

Author Contribution

Rashad E.M. Said: Visualization, Validation, Data curation, Formal analysis, Writing original draft, review, and editing. Ahmed E. A. Badrey, Ibrahim A. Mohamed and Hamdy A.M. Soliman: Visualization, Writing original draft, review, and editing. Mohamed Hamed: Conceptualization, Methodology, Visualization, Validation, Investigation, Data curation, Writing original draft, review, and editing. Alaa Sayed: Conceptualization, Methodology, Visualization, Investigation, Data curation, Review, and editing. All authors have read and approved the final manuscript.

Data Availability

The data supporting the findings of this study are available on request from the corresponding author.

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Figures

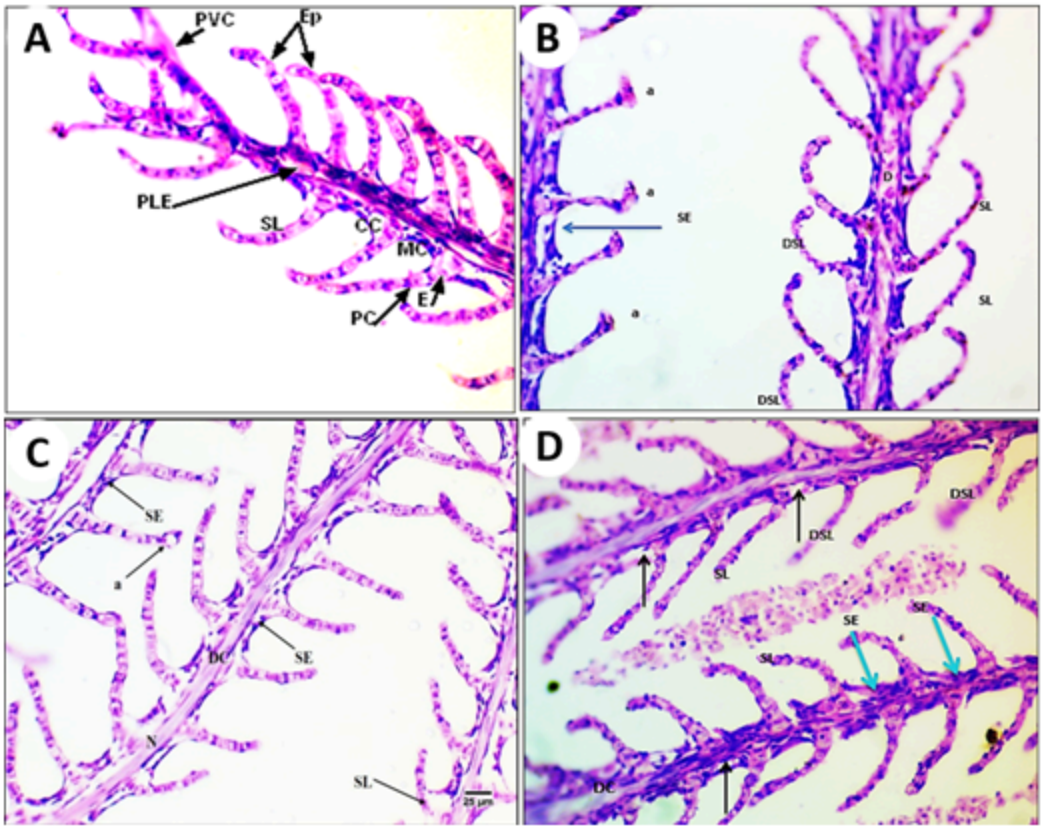


Figure 1

A: The gills of control fish have numerous gill filaments with two rows of secondary lamellae (SL), made up of epithelium sheets bordered by pillar cells (PC). Erythrocytes (E) are recognized within each capillary lumen. Chloride cells (CC) are present at the lamellae base, while mucous cells (MC) are observed in the inter-lamellar epithelium and at the distal tip of the primary lamellar epithelium (PLE). Pavement cells (PVC) are found in the filament epithelium and at the lamellae base. B: PE-MPs groups showed severe gill deformation with deformed (D) cartilage, thinning, degenerated, and lifting of

stratified epithelium (SE). Degenerated curved SL, changes in the blood circulatory system with aneurysms (a), atrophy, and degeneration in SL core were noted, along with disorganization of pillar cells and blood capillaries. C: UPGR-treated groups showed severe gill architecture deformation, hemorrhage, deformed cartilage (DC), and necrosis (N). Gill morphology disorganization, shortened SL, severe disruption of pavement cells and blood capillary system, and damage in the circulatory system with aneurysms (a) were noted. Epithelial lifting and edema were observed at the SL base. D: Joint (UPGR + PE-MPs) treatment showed shortened stratification in gill morphology, thinning of primary gills, and core disappearance. SL appeared as one layer with deformed cartilage (DC), thinning, and lengthening. Severe disruption of pavement cells and blood capillary system, few aneurysms (a), many DSL dissolutions, and SE breakdown with vacuolation (black arrow) between SL were observed (stained with Harris' hematoxylin and eosin- 40X).

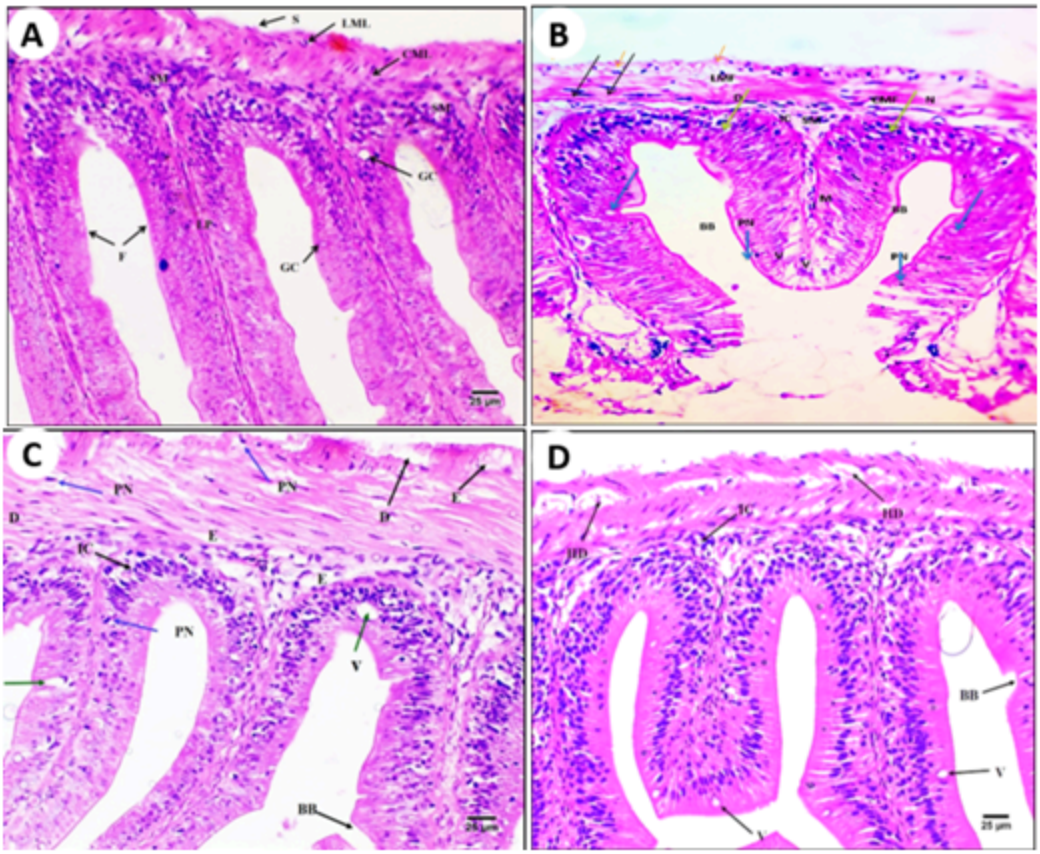


Figure 2

A: The control group showed normal intestine morphology with finger-like villi, simple long columnar cells, goblet cells (GC), thin submucosa (SM) projecting into mucosal folds (F), loose connective tissue with blood cells in the lamina propria (LP), and defined circular (CML) and longitudinal muscle layers (LML) with a peritoneal serosa (S). B: PE-MPs exposure resulted in thin serosa (orange arrow), vacuolated longitudinal muscle fibers (LMF), and disintegrated circular muscle fibers (CMF) with deeply stained nuclei (black arrow). The submucosa had inflammatory cells (IC) and vacuolated crypt cells (green arrows). Lateral villi showed vacuoles, hyperemic cytoplasm, and fewer goblet cells with a

damaged brush border (BB) (blue arrows). The villi trough exhibited vacuolated cells (V) with many pyknotic nuclei (PN). C: UPGR-treated fish had degenerated serosa (orange arrow), vacuolated muscle fibers (V), disintegrated muscle cells, pyknotic nuclei (PN) in muscle fibers (blue arrow), and edematous submucosa (E) with inflammatory cells (IC). Mucosal layers showed vacuolated cytoplasm, active nuclei in columnar cells, and a damaged brush border (BB). D: Fish treated with UPGR and PE-MPs showed indistinct layer boundaries, serosa loss, widened muscle layers, hydropic degeneration (HD), vacuolated cells (V), and deformed cells. Submucosa had extensive inflammatory cells (IC), and the mucosal layer rested on an undulating basement membrane (dark arrow) with vacuolated columnar cells, increased goblet cells, and a degenerated brush border (BB) (stained with Harris' hematoxylin and eosin- 40X).

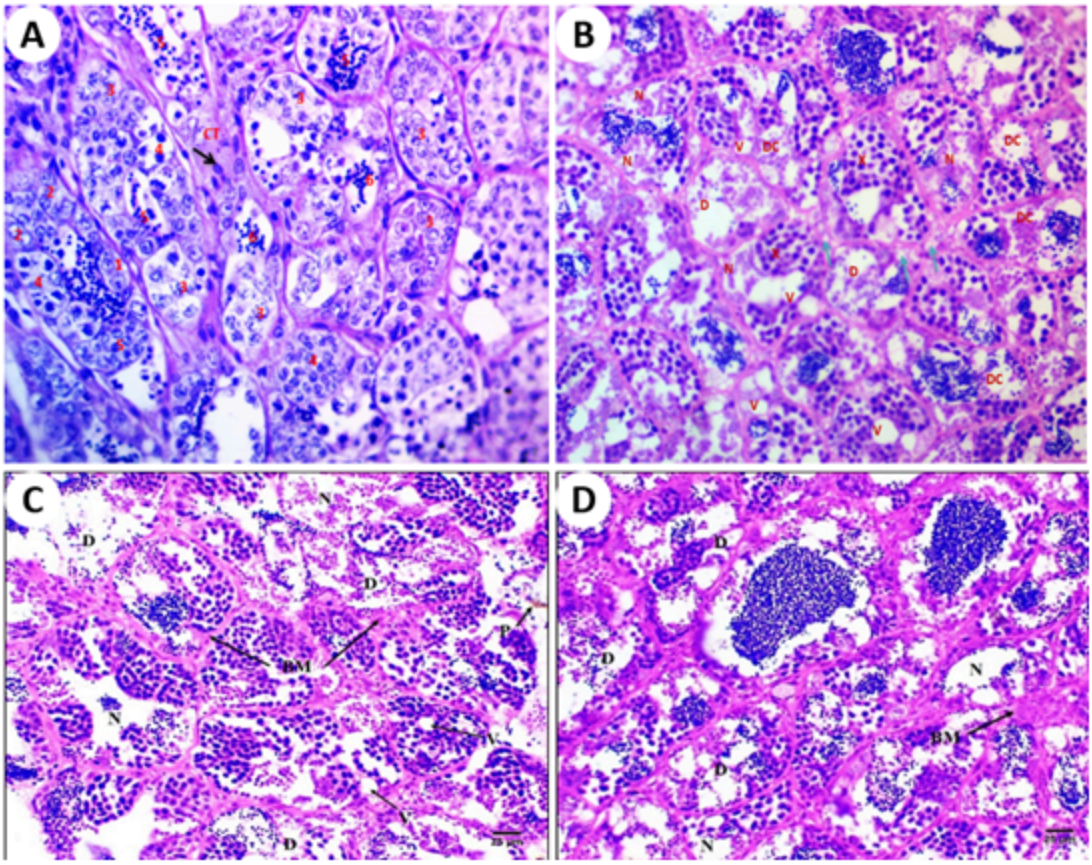


Figure 3

A: Control testes of *O. niloticus* showed normal seminiferous tubules (ST) with all stages of spermatogenesis, including primary spermatogonia (PSG=1), secondary spermatogonia (SSG=2), primary spermatocytes (PSC=3), secondary spermatocytes (SSC=4), spermatids (SP=5), and spermatozoa (SZ=6), with Sertoli cells (S=7) and Leydig cells in the interstitial tissue (CT). B: PE-MPs exposure caused tubule degeneration (D), necrosis (N), and vacuolation (V), with only secondary spermatocytes and reduced interstitial space. C: UPGR treatment resulted in widened tubules with thick basement membranes (BM), degeneration (D), necrosis (N), and vacuolation (V), with spermatozoa in the lumen and brown pigments (P). D: Combined UPGR + PE-MPs treatment showed

severe tubule deformation, necrosis (N), and vacuolation (V), with reduced interstitial space and few Leydig cells (stained with Harris' hematoxylin and eosin- 40X).