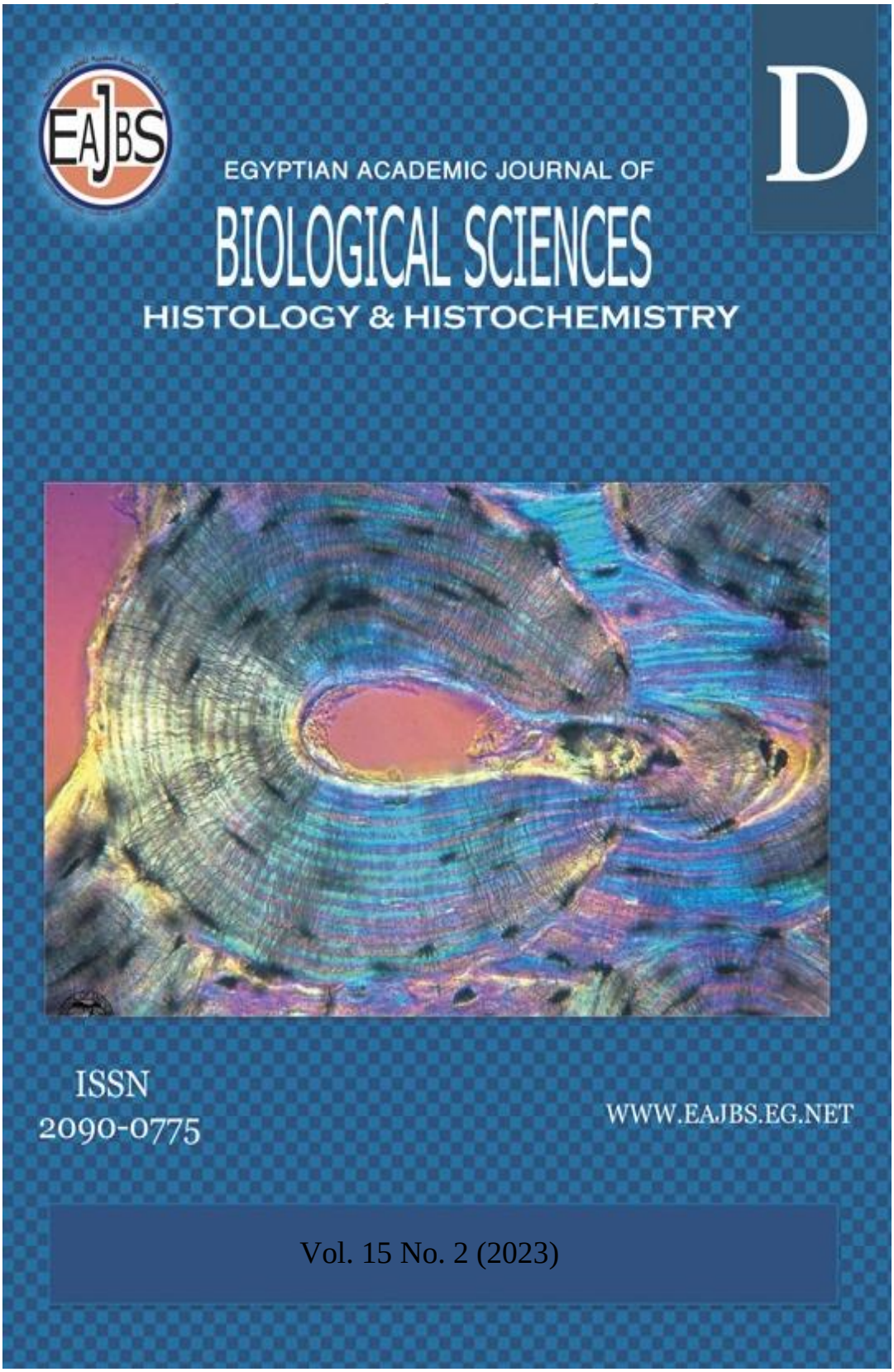

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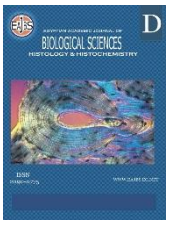

Histological and Histochemical Effects of Microplastics Administration in *Oreochromis niloticus* Fingerlings

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ABSTRACT

Background: Plastics in the environment have provoked increasing worries in recent years, and recurrently, plastic waste is increasingly being discharged into water resources including rivers, where it breaks down into smaller particles. **Objective**: Investigate the histological and histochemical effects of two types of microplastics (MPs) in the gills, liver, and kidney of Nile tilapia (*Oreochromis niloticus*). **Methods**: The study included seven groups of *O. niloticus* fingerlings, the first was set as a control and the remainders were fed on a diet containing three different concentrations of two types of microplastics; namely low-density polyethylene (LD-PE) and polyethylene terephthalate (PET). **Results**: Chronic administration of MPs induced different histopathological lesions in fish gills, liver, and kidney. The prevailing histological changes in fish gills were hypertrophy, hyperplasia, lamellar aneurysm, and fusion of lamellae, while the liver showed hypertrophied hepatocytes, vacuolation, blood vessels congestion, karyolysis and pyknotic nuclear hepatocytes, necrotic cells, and hydropic degeneration. Meanwhile, the renal tissue of MPs-fed fishes delineated lymphocytic infiltration, degenerated renal tubules, obliteration in Bowman's space, and necrotic areas. These lesions were more obvious with high concentrations of MPs, LD-PE, or PET. Histochemically, feeding of diets containing MPs induced significant depletion in each of glycogen and total protein contents of the liver, and kidney of experimental fishes as compared with control.

INTRODUCTION

Plastics are used in a wide range of manufacturing and there is increasing demand due to their relatively low costs. In 2018, global plastic production reached 360 million tons, of which 80,000 are estimated to flow into the aquatic environment (Zhang *et al.*, 2021). Plastic waste as a fraction of municipal solid waste poses a critical environmental threat due to its non-biodegradable nature (Canopoli *et al*., 2020). Plastic debris is broadly classified by size: mega-debris (>100 mm), macro-debris (> 20 mm), meso-debris (20–5 mm) and micro-debris (<5 mm) (Barnes *et al*., 2009).

Microplastics (MPs) in the environment have aroused increasing concerns in recent years (Bakir *et al*., 2012). The term "microplastics" is credible to fragments smaller than 5mm in size (Arthur *et al*., 2009). Microplastics are the products of the degradation of larger plastic items into smaller fragments (Andrady, 2011). In the marine environment, such microplastics can be engendered from primary sources such as the accidental spillage from industry or from the use of cosmetic products (Fendall and Sewell, 2009), or from secondary sources because of the fragmentation of larger debris because of thermal or photo-degradation tied with mechanical degradation (Andrady, 2011).

Among the most used plastics are polyethylene (PE), polyethylene terephthalate (PET), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), and polyurethane (PU) (Gewert *et al*., 2015 and Moharir, and Kumar, 2019). Ingestion of microplastics (MPs) has been assessed in marine species both with an ecological and commercial interest at sea and under experimental conditions (Strungaru *et al*., 2019 and Alomar *et al*., 2021), emphasizing the importance to assess MPs ingestion in commercially and aquaculture important species such as Nile tilapia fish. Goodman *et al*. (2022) suggested that ingesting microplastics may lead to toxicological problems in cell metabolism and cell−cell interactions. The present study explores the histopathological and histochemical effects in the gills, liver, and kidney of *Oreochromis niloticus* fingerlings induced by feeding on a diet containing three concentrations of MPs: namely low-density polyethylene (PE) or polyethylene terephthalate (PET), separately.

MATERIALS AND METHODS

Fish: Mono-sex Nile tilapia (*Oreochromis niloticus*) fingerlings were purchased from a private fish hatchery at Kafr El-Sheikh Governorate, Egypt. The fish were transferred to a private lab of fish breeding and acclimated for two weeks to the lab's condition. Through this period, the fingerlings were fed the basal diet; then, they were divided randomly into seven groups (45 fish/group). During the experimental period of 120 days, fish were fed twice daily (07:00 and 16:00 hr.), with a diet previously formulated at 5% of the body weight. The light period was controlled by a timer to provide a 14h light: 10 darks as a daily photoperiod. The basal diet was prepared according to the standard method of (AOAC, 2012).

Microplastic Samples: Low-density polyethylene (LD-PE) and polyethylene terephthalate (PET) powders with an average particle size of 1-5 mm were obtained from plastic factories for the manufacturing of plastic bags and bottles in Quesna, Menoufia Governorate; the purity of LD-PE was 90.5%, while PET was 97.3%. The particle size of microplastics was measured using Scanning Electron Microscope (SEM) at Mansoura University; the size ranged from 1 to 3 mm.

Experimental Groups: The fish were divided into seven groups; GI served as control and fed on a basal diet only. **GII** fed on basal diet + 1% (LD-PE), G**III** fed on basal diet + 3% (LD-PE), **GIV** fed on basal diet + 5% (LD-PE), **GV** fed on basal diet + 1% (PET), **GVI** fed on basal diet + 3% (PET) and **GVII** fed on basal diet + 5% (PET). MPs concentrations in the present study were within the ranges (0.5–10% of the diet) reported in the previous studies (Santana *et al*., 2016; Jovanovic *et al*., 2018).

Histological and Histochemical Procedures: Six random fish from each group were selected for the histological examination. The fish were dissected to obtain the gills, liver, and kidney. The tissues were fixed in 10% neutral buffered formalin for 24h, then dehydrated in ethanol, cleared in xylene, embedded in paraffin blocks, and

sectioned at 5µ thick. The sections were then deparaffinized with xylene and rehydrated in ethanol. For histopathological alterations, Hematoxylin / Eosin stain was used according to the method of Bancroft *et al*. (2012). The mounted sections were investigated under the microscope using different objectives some cases were selected and photographed with the use of a photomicroscope. Histochemically, glycogen content, Periodic Acid Schaff's technique (PAS) according to the method of Pearse, (1985), and total protein contents, mercury- bromophenol blue (BPB) according to the method of Pearse, (1985) were applied. For quantification analysis of glycogen and total proteins, ten sections of tissue from each organ were examined by a light microscope and then processed by ImageJ Java free Software developed by Wayne Rasband at the National Institute of Health (Bethesda, MD, USA). The assessment of the results was made using SPSS ver. 20.0 software (SPSS Inc., Chicago, IL) program. The results were considered statistically significant when $P < 0.05$.

RESULTS

Histopathological Analysis:

The histological structures of the gills of *O. niloticus* of control and microplastic feeding are illustrated in Figure (1). The gill is made up of double rows of filaments that arise perpendicularly to the lamellae. The lamellae are lined by a squamous epithelium composed of pavement and non-differentiated cells. Below that epithelium are lamellar blood sinuses separated by pillar cells. Between the lamellae, the filament is lined by a thick stratified epithelium constituted by several cell types, such as chloride, mucous and pavement cells. The feeding on diet contained MPs induced different histopathological lesions, that included hypertrophy and hyperplasia in the lamellar epithelia, fusion of some lamellae, proliferation and hyperplasia of the mucous cells, dilatation, and congestion in the blood vessels, as well as focal necrosis in the lamellar epithelium. These lesions were mostly pronounced with Low-density polyethylene (LD-PE) (Fig.1; B-D) than those fed on polyethylene terephthalate (PET) microplastic (Fig.1; E-F). Also, these histological alterations were more obvious with the diet containing high concentrations of microplastics.

The histological structures of the liver of *O. niloticus* of control and MPs fed are displayed in Figure (2). The liver sections of control fish exhibited normal polygonal hepatocytes with clear boundaries, arranged in branched laminae and separated by sinusoids. Hepatocytes constitute hepatic cords and show homogenous eosinophilic cytoplasm with a central or subcentral rounded nucleus with a deeply stained basophilic nucleolus. Branches of the hepatic portal vein and hepatic artery through the sinusoids to central veins that empty into the hepatic vein. Phagocytic cells are sporadically observed in the sinusoids. The pancreatic structure was observed in combination with hepatic tissue.

The histopathological investigation of the liver of fish fed on MPs revealed prevailing adverse effects in the hepatic architecture. The liver sections of fish fed on a diet containing a low concentration (1%) of LD-PE exhibited hypertrophied hepatocytes, cytoplasmic vacuolation, an increase of Kupffer cells and macrophages, congested blood vessels, and pyknosis in the hepatocyte nuclei (Fig. 1, B); whereas slight histological changes were recorded in the liver of fish fed on diet contained 1% of PET microplastics (GV). These alterations were clearly increased with middle and high concentrations of MPs; lymphocytic infiltration, karyolitic hepatocytes nuclei, foci of necrosis, and hydropic degeneration, as well as dilatation in blood vessels and sinusoids, were ratified (Fig.1; C-F). Investigated liver sections of fish from GIV (diet containing 5% of LD-PE) revealed severe effects among other groups (Fig.1; D). The posterior trunk kidney of control *O. niloticus* revealed numerous nephrons

that consist of Malpighian corpuscles composed of well-vascularized glomeruli enclosed by Bowman's capsule, proximal and distal tubules, and the collecting duct system (Fig. 3, A). The recorded histological changes in the renal tissue of *O. niloticus* due to feeding on a diet containing MPs revealed that the severity of these changes corresponded to the concentration of MPs, and LD-PE mostly induced apparent effects than PET (Fig. 3, B-F). The feeding on diet contained low concentrations of MPs (GII and GV) resulted in cellular swelling, lymphocytic infiltration, focal area of hemorrhage, desquamated renal tubular epithelia and lobulation in some glomerular tuft. Moderated histopathological changes were noticed in the renal tissues of *O. niloticus* after being fed diets containing middle concentrations of microplastics (GIII and GVI). While diets contained high concentrations of MPs (GIV and GVII), proceeded in severe deformation in the renal architecture (Fig. 3, D and F). Where degenerated renal tubular epithelia, obliterated Bowman's space, elliptical glomeruli, and necrotic area were perceptive.

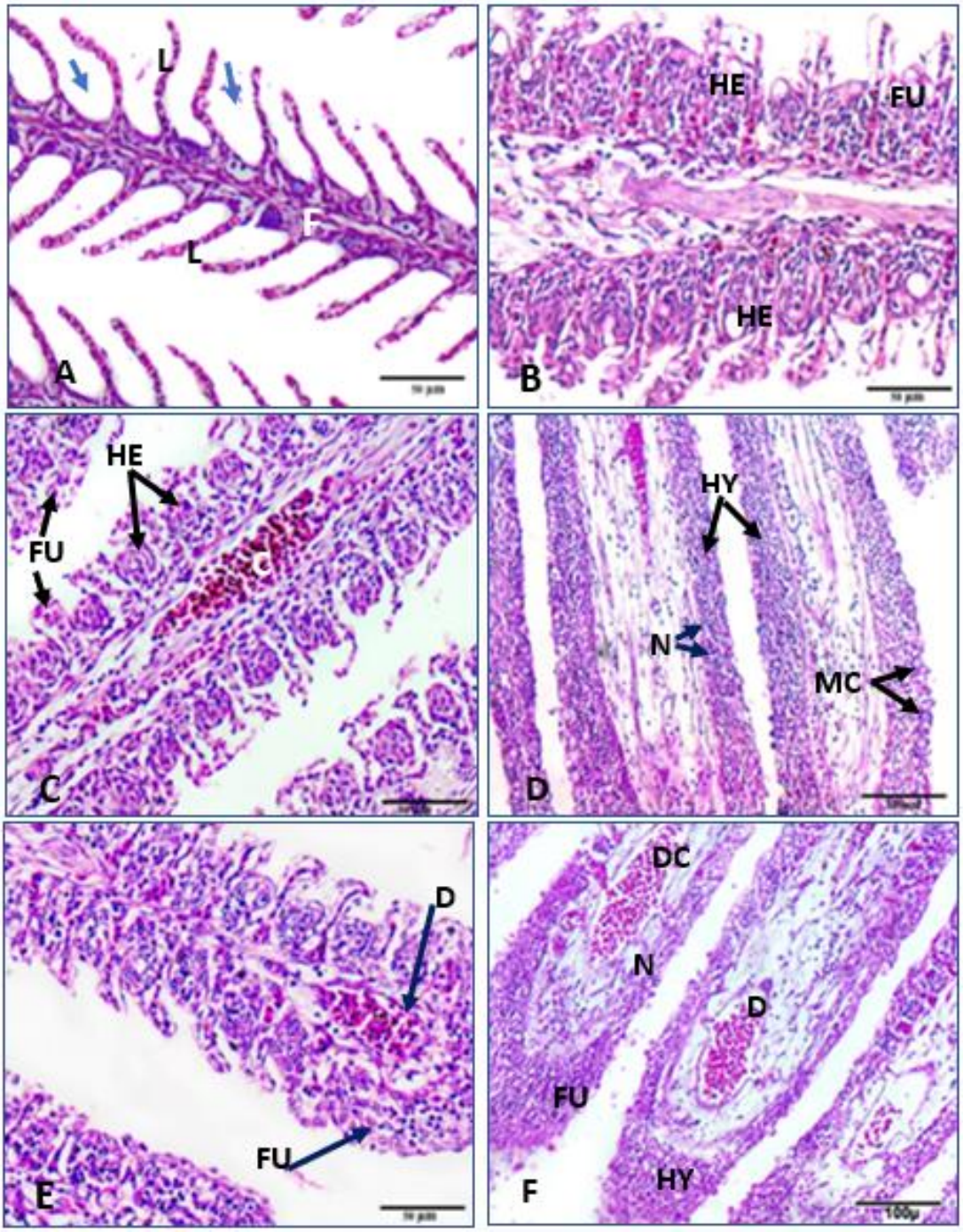

Fig. 1: Photomicrographs of control and MPs fed *O. niloticus* gills; A: **GI** (control); B: (**GII**); C: (**GIII**); D: (**GIV**); E: (**GVI**) and F: (**GVII**), where filament (F), lamellae (L), water channel (arrow), hypertrophy of the epithelia (HE) and fusion of some secondary lamellae (FU), hyperplasia of lamellae (HY), fusion of secondary lamellae (FU), congested blood vessel (C), hyperplasia of filament epithelia (HY), focal necrosis in primary lamellae (N), proliferation of mucous cells (MC), dilation and congested blood vessel (DC). (H/E stain).

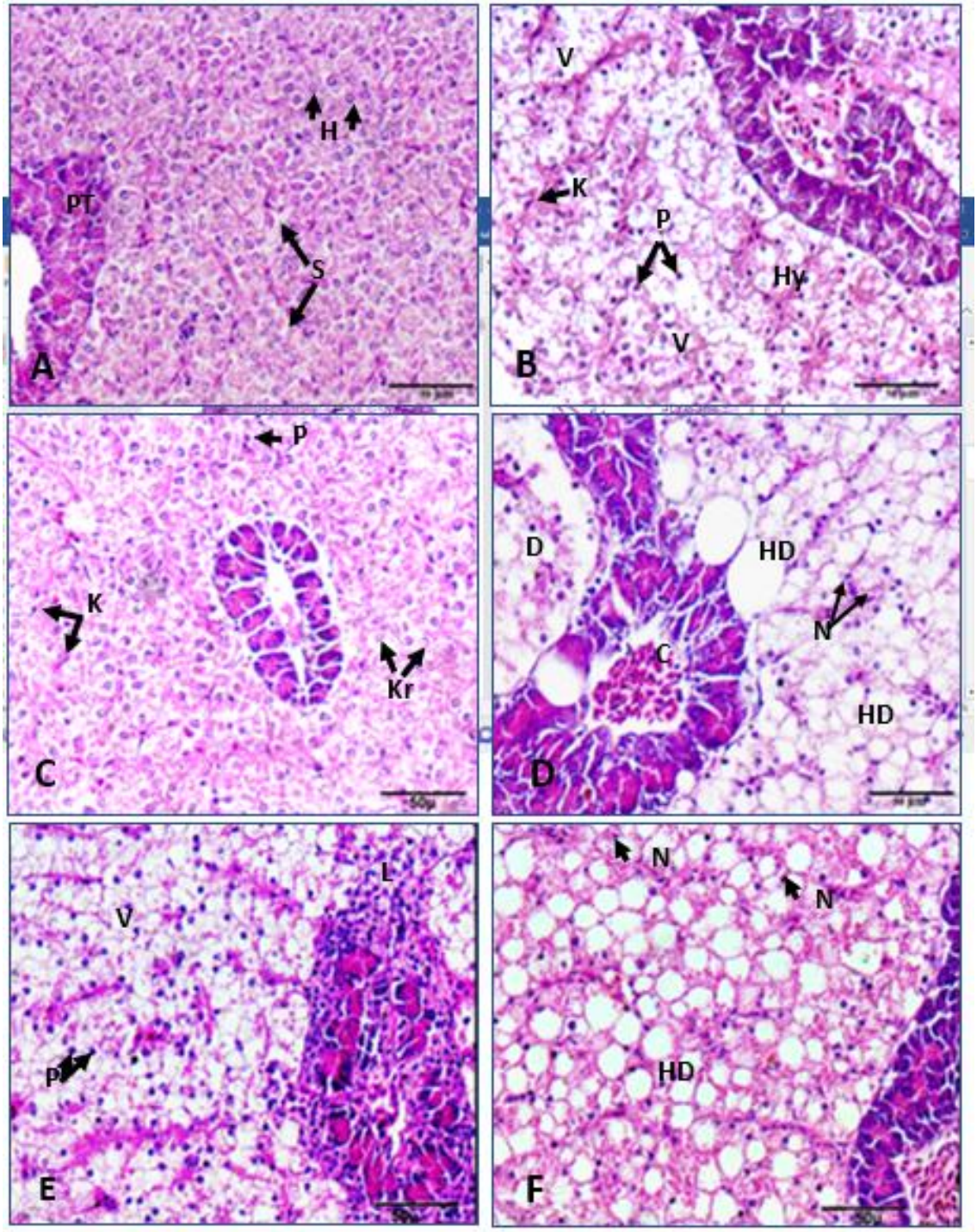

Fig. 2: Photomicrographs of control and MPs fed *O. niloticus* liver; A: (**GI)**; B: (**GII**); C: (**GIII**); D: (**GIV**); E: (**GVI**) and F: (**GVII**), where normal hepatocytes (H), sinusoids (S), pancreatic tissue (PT), hypertrophy of hepatocyte (Hy) cytoplasmic vacuolation (V), pyknosis (P), dilation of sinusoids (D), increased Kupffer cells (K), karyloysis (Kr), necrotic cells (N), lymphocytic infiltration (L), congestion of portal vein (C) and hydropic degeneration (HD). (H/E stain).

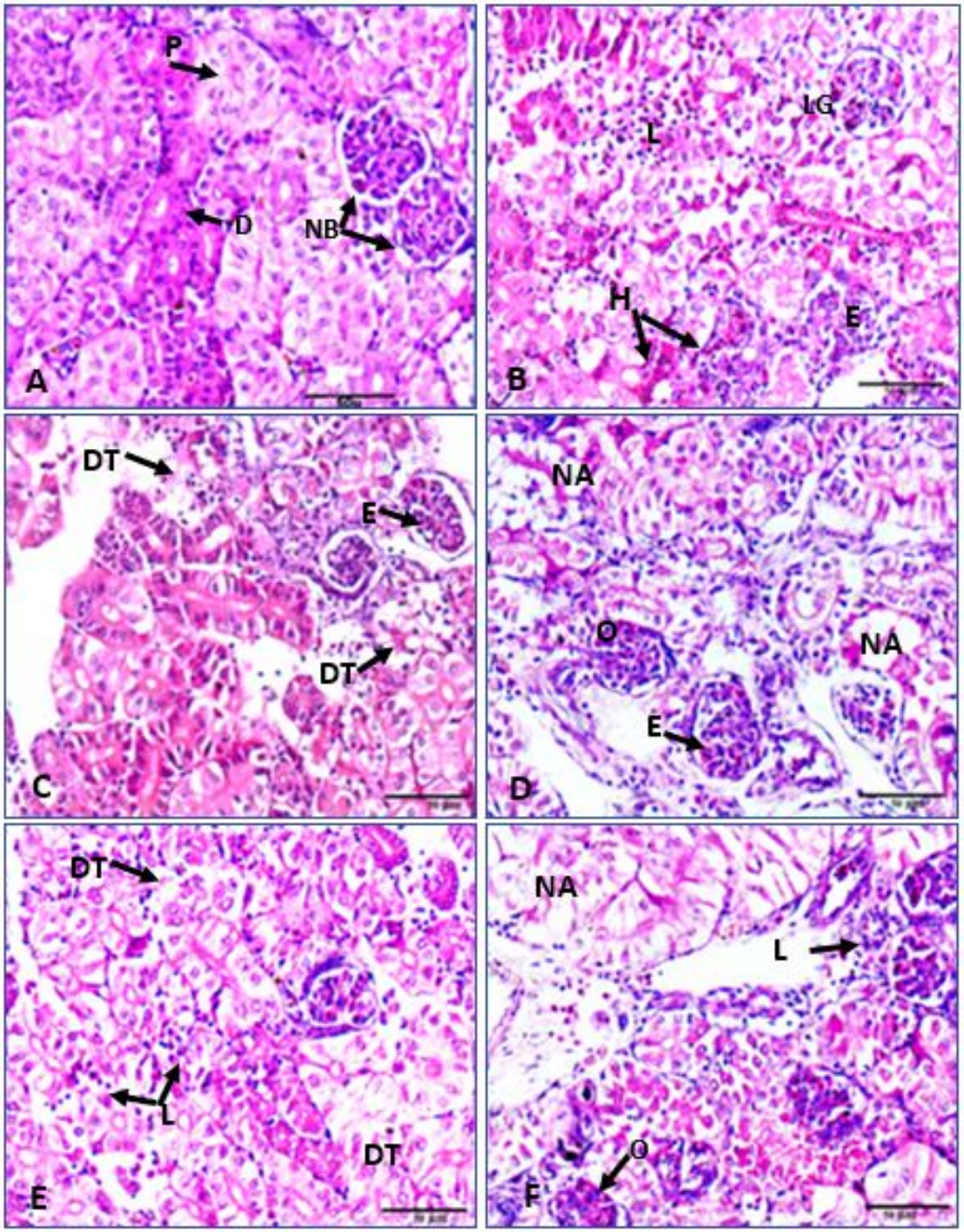

Fig. 3: Photomicrographs of control and MPs fed *O. niloticus* kidney; A: **GI** (control); B: (**GII**); C: (**GIII**); D: (**GIV**); E: (**GVI**) and F: (**GVII**), where; normal Bowman's corpuscles (NB), proximal tubule (P), distal tubule (D), lymphocytic infiltration (L), lobulated glomerular tuft (LG), haemorrhage (H), degenerated renal tubules (DT), Obliterated Bowman's space (O), necrotic area (NA) and elliptical glomerulus (E). (H/E stain).

Histochemical Analysis:

The results of the histochemical analysis revealed that the glycogen content was slightly increased in the gills of *O. niloticus* fed on diets containing MPs (Table 1). On the other hand, significant decreases in the glycogen contents in each of the hepatic and renal tissues of fish fed on LD-PE or PET. This reduction was significant with diets either containing LD-PE or PET and corresponded to the concentration of

these MPs (Table1). Marked a similar reduction in the total protein contents in the gills of fish fed on either LD-PE or PET was noticed. The quantification of the total protein content in the liver of *O. niloticus* fed on LD-PE or PET showed a similar significant reduction in this content and the degree of reduction was linked to concentration (Table 2). The renal tissue of fish fed on a diet containing LD-PE microplastics exhibited a slight diminution in the total protein contents. Whereas feeding on diets containing middle and high concentrations of LD-PE and PET (GIII, GIV, and GVI, GVII, respectively) displayed a prominent significant reduction as compared with control (Table 2).

Table 1: Measurements of glycogen content in the gills, liver, and kidney of *o. niloticus* control and MPs fed groups.

Organ / Group	Gills	Liver	Kidney
(GI) Control	192.27±1.88 [f]	206.12±1.88[a]	204.33±2.39[a]
GII	197.98 ±2.10[e]	195.74±2.96[b]	193.26±2.62 [b]
GIII	212.04 ±1.81[c]	188.64±1.92[c]	192.14±2.15[b]
GIV	219.36±1.43[b]	182.17±3.18[d]	179.69±5.07[d]
GV	202.28 ±1.89[d]	192.53±1.28[b]	190.39±0.42[bc]
GVI	217.03±1.69[b]	182.62 ±1.51[d]	186.42±0.68[c]
GVII	224.92 ±1.65[a]	175.51±1.69[e]	163.14±2.52[e]

The data are expressed as the means optical density± SD, within each column, the mean superscript with different letters is significantly different ($P < 0.05$).

Table 2: Measurements of total protein content in the gills, liver, and kidney of *o. niloticus* control and MPs fed groups.

Organ / Group	Gills	Liver	Kidney
(GI) Control	114.68±1.33[a]	130.96±5.04[a]	128.12±2.42[a]
GII	113.80±1.06[a]	121.54±1.25[a]	124.93±1. 65[a]
GIII	106.38±3.87[a]	117.85±2.43[b]	119.99±0.83[b]
GIV	98.33±2.35[c]	108.95±1.68[c]	115.05 ±3.71[c]
GV	107.56 ±2.35[ab]	120.08±1.94[b]	124.07±2.67[ab]
GVI	107.88±2.78[b]	112.73±2.31[b]	113.21±2.22[c]
GVII	94.53 ±2.42[d]	104.86 ±5.15[c]	93.07±2.39[d]

The data are expressed as the means optical density± SD, within each column, the mean superscript with different letters is significantly different ($P < 0.05$).

DISCUSSION

The long-term implications of microplastics are still uncertain compared to better-studied chemical pollutants (Horton *et al*., 2017). The present study investigated the histopathological and histochemical alterations induced in *O. niloticus* chronically fed on diets containing three concentrations of MPs: LD-PE and PET separately.

Histological changes have been used to assess the health condition of fish exposed to contaminants and to depict the effects of exposure to a series of anthropogenic pollutants (Zagatto and Bertoletti 2008 and Nascimento *et al*., 2012). The emerging results showed that the histological lesions in each of the gills, liver, and kidney of *O. niloticus* were correlated with the concentration of MPs in the diet. Where diets containing 5% of LD-PE or PET resulted in severe

injuries as compared with slight and moderate effects of 1% and 3% of these MPs. Hypertrophy, hyperplasia, hyperemia, and fusion of gill lamellae observed in the present study as results of MPs feeding could serve as a defensive mechanism, but these responses may take place at the expense of the respiratory efficiency of the gills. In agreement, Banaei *et al*. (2022) showed that aquatic ecosystems have become a place for accumulating microplastics (MPs), that can directly or indirectly damage organisms, also Da Costa Araújo *et al*. (2020) proved that polyethylene microplastics were found in the gills, liver, and brain of guppy fry fish (*Poecilia reticulata*) at an upper trophic level. Lamella fusion noticed in the present study is often a non-specific response to chronic inflammation that causes proliferation of a mixed population of the pavement, mucous and chloride cells and/or leukocyte infiltration, according to Wolf *et al*. (2015).

Although gills also act as filtering organs, they conserve particles/pollutants that would enter the animal's bodies (Powell *et al*., 1992). Like other studies (Lu *et al*., 2018 and Barboza *et al*., 2020), the MPs identified in the gills are the ones retained by the mucus found on lamellar surfaces. Also, the ingestion of particles in the water, along with the offered food, likely enhanced pollutant absorption, as already evidenced in previous studies (Horton *et al*., 2018 Bellas and Gil, 2020 and Xia *et al*., 2020).

The histopathological evaluation of the liver of *O. niloticus* fed on diets containing LD-PE or PET microplastics displayed increased macrophages including Kupffer cells, vacuolated hepatocytes, hydropic degeneration, and necrotic areas, especially with high concentrations of MPs. In coincidence with the present results Robbins and Cotran (1984) reported that morphologic changes in an injured cell become apparent after alteration in the biochemical system of the cells. In addition, Da Costa Araújo *et al*. (2020) pointed out that MPs enter the vertebrate organs, change their behavior, and induce mutagenic and cytotoxic processes in animals, which can cause significant ecological consequences in freshwater ecosystems. Moreover, MPs are found in comparatively high concentrations in marine and estuarine ecosystems and may accumulate in the bodies of freshwater and marine fish species (Strungaru *et al*., 2019). The vacuolation in the hepatocytes and hydropic degeneration could be attributed to alterations of metabolic profiles in fish liver and disturbed lipid and energy metabolism. Similarly, Lu *et al*. (2016) indicated that PS-MPs caused inflammation and lipid accumulation in fish liver and induced significantly increased activities of superoxide dismutase and catalase, indicating oxidative stress due to MPs treatment.

Histological assessments of the gills, liver, and kidneys, the main organs exposed to environmental pollutants, are important biomarkers that make it possible to detect the harmful environmental factors that an organism has been subjected to for a long time (Shuman *et al*., 2019). The present investigation revealed that feeding diets containing a low concentration of MPs (LD-PE or PET) resulted in lymphocytic infiltration, hyperemia, and increased cellularity within the glomeruli in the kidney of *O. niloticus*. Whereas severe effects were recorded with middle and high concentrations of MPs; that mostly represented obliteration in Bowman's space, areas of necrosis, and degeneration in the renal tubular epithelia. In agreement with Cengiz (2006) who reported that the kidney of fish receives the largest proportion of post branchial blood, and therefore renal lesions might be expected to be good indicators of environmental pollution. Necrosis corresponds to a severe and irreparable change, signs of degeneration in addition to focal necrosis indicate that the histological changes are more severe and are associated with exposure of fish

to water contaminated such as polychlorinated biphenyls (Chang *et al*. 2001). Moreover, Wang *et al*. (2021) declared that microplastics (MPs) contaminate our food chain and accumulate in the gut, liver, kidney, muscles, and so on. The latter authors proved that PS-MPs accumulated, and the treated mice had more histopathological lesions in the kidneys and higher levels of ER stress, inflammatory markers, and autophagy-related proteins in the kidneys after PS-MPs treatment by oral gavage. Moreover, Goodman *et al*. (2022) declared that exposing human kidney and liver cells to microplastics results in morphological, metabolic, proliferative changes and cellular stress, that indicate the potential undesirable effects of microplastics on human health.

However, the present data verified that the liver of fish is a more vulnerable organ attributed to its vital role in the biotransformation of xenobiotics in fish, in coincidence, Abbaszadeh and Sisman (2021) found that the frequencies of the histological lesions were higher in the liver in comparison the gills and kidney of *Leuciscus aspius* fish inhabiting polluted river, the authors suggested that hepatocytes are a biological marker of aquatic contamination.

Histochemically, the present study indicated a prevailing significant reduction in the contents of glycogen and total protein in all organs under the study of *O. niloticus*. Toxicants interfere with the process of a glycoprotein for mucus formation in gills Abbaszadeh and Sisman (2021), thus, it disrupts the negative ion charge of the gill epithelium by fusion and lamellae hyperplasia, is quite compatible with the present study. In coincidence with the present results, the liver of *Clarias gariepinus* showed mild to severe levels of glycogen depletion, fatty vacuolation and degeneration, and hepatocellular necrosis in PVC microplastic-treated groups with reference to the control (Iheanacho and Odo, 2020). Also, Goodman *et al*. (2022) showed that polystyrene microplastics (PS-MPs) caused inhibition of cell proliferation, altered metabolism, cellular stress, and morphological changes together can cause various alterations in cellular activity and affect the overall function of the cells, preventing cells from performing their normal functions. Additionally, freshwater and soil systems are subject to both point and diffuse inputs of plastics and consequently great research effort is asserted to understand the transport, exposure, and ecological effects of microplastics in these systems (Horton *et al*., 2017).

In conclusion,

Microplastic ingestion has histological and histochemical adverse effects on the different organs of Nile tilapia fish, including gills, liver, and kidneys. Furthermore, microplastics that evade filtration by the liver and kidneys can collect in these organs and possibly cause severe health issues over time, even if MPs may not have immediate effects, they could cause suspended effects. There is a need for programs for recycling plastic products to reduce the discharge of plastic garbage to water and incorporate disposable plastic packaging manufacturers into the extended producer responsibility system and increase the recycling rate.

Ethical Approval:

The experiment was carried out according to the national regulations on animal welfare.

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