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Original article

Exploration of polyacrylamide microplastics and evaluation of their toxicity on multiple parameters of *Oreochromis niloticus*

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ABSTRACT

Due to the increased production of polyacrylamide microplastics in the environment impacting the adverse effects on aquatic organisms have become a global concern. The present study aimed to evaluate the toxicity of different concentrations (0.018, 0.03 0.09 g/1) of polyacrylamide microplastics on *Oreochromis niloticus*. Polyacrylamide microplastics were characterized by Fourier transform infrared and Raman spectroscopy. The FTIR technique revealed the spherical morphology and size range of polyacrylamide (0.1–0.4 mm) with 99 % purity. Raman Spectroscopy results showed peaks around (1100 cm^{-1} and 1650 cm^{-1}) in gills, these peaks confirmed the presence of polyacrylamide microplastics in the gills of *Oreochromis niloticus*. Polyacrylamide microplastics significantly shortened the antioxidant enzymes (Catalase and Reduced glutathione) proceedings and the increase was observed in Malondialdehyde and Lipid peroxide levels in high-dose treated groups. Moreover, total protein contents were expressively increased, while other blood parameters (AST, ALP, ALT) were significantly decreased. Hemoglobin (g/dl), Erythrocyte (106/µl) and TLC ($1x10^9$/l) levels were reduced significantly ($p < 0.05$) in high concentrations. The administration of polyacrylamide microplastics induced different histological changes in the gills, liver and intestine of *O. niloticus*. It was concluded that polyacrylamide microplastics are toxic agents having harmful effects on fish health.

1. Introduction

Globally, plastic pollution is rapidly increasing with 57 % raise in plastic production, while management of waste products is still a challenge for researchers. Due to the increased production of plastic particles and poor management of waste materials, many plastic particles are discharged into water bodies (McDevitt et al., 2017). Pollution caused by plastic particles is one of the major environmental issues for both marine and freshwater habitats, causing harmful effects on aquatic flora and fauna (Eriksen et al., 2014). The large-sized plastic particles turned into microplastics consequently through gradual degradation, fragmentation, UV radiation (Photo - oxidation), biological degradation caused by micro-organisms and mechanical transformation (waves abrasion) in the aquatic and terrestrial ecosystem (Cole et al., 2013). Further degradation of microplastics results in the formation of the nanoplastics which have more toxicological effects and fats as compared to microplastics (Da Costa et al., 2016).

Microplastics are composed of fragmented particles which are formed through microbial decomposition, weathering and photolysis processes and are discarded into the aquatic environments. Their distribution in the aquatic environment depends upon their size, density and shape. Density determined the position of microplastics in the water column. Polymers like polypropylene (PP) and polyethylene (PE) being less dense float in the water column whereas polymers having high density (polystyrene) accumulate into water sediments making them more available to deposit,

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filter and suspension feeders in the water and produce harmful effects on aquatic flora and fauna (Collard et al., 2018, Crawford et al., 2017).

The most frequently identified microplastics in both aquatic and terrestrial environments are polystyrene (PS), polyethylene glycol (PEG), polypropylene (PP), Xylene and polyacrylamide (PAM), less than five millimeters in size (Do Sul and Costa, 2014). Many studies have been conducted to investigate the mechanism of toxicity caused by the accumulation of microplastics based on their uptake efficiency. Results showed that micro and nanoplastics could induce oxidative stress, genotoxicity, histological alterations and reactive oxygen species in various aquatic organisms such as aquatic lungworms, mussels and invertebrates (Avio et al., 2015; Mazurais et al., 2015;). When these particles accumulate in the tissues of an organism, being toxic they affect the physiology of organisms including increased mortality rate, reduced feeding activity, inhibit growth rate as well as fertility and metabolic rate (Rochman et al., 2013).

To assess the health of aquatic organisms and other biological factors of aquatic ecosystems, fish are widely used as biomonitors. From all kinds of aquatic habitats, more than 250 fish species have been reported to contain microplastics in large amounts (Wang et al., 2018). Fish is an essential nutrient in the human diet and is also present in the global aquatic product industry for consumers. Therefore, we need to protect our aquatic environment against to pollution on various environmental and ecological effects (Caglar et al., 2019; Mesut, 2021). The aquatic ecosystems and living organisms suffer from environmental impact by emissions of volatile organic substances, and pollution of water by oil chemicals and many various hazardous agents (Selamoglu, 2021).

Oreochromis niloticus is warm water and fast-growing fish species. Tilapia as a commercially important fish species can survive in adverse environmental conditions. Tilapia can feed on algae and any other plant-based food. This feeding habit diminishes the cast in farming tilapia fish as well as decreases fishing pressure on prey species. Therefore, we prefer tilapia for our research (Christopher et al., 2009).

Currently, there is no standardized method for the detection of microplastics toxicity. However, for the isolation of microplastics various methods were used. These methods ranged from simple dissection including histology to the complete digestion of organic tissues using enzymes or dissolving chemicals (Lusher et al., 2017). Recent studies on microplastics indicated that hepatic stress, cell necrosis, hormonal imbalance and physiological abnormalities are produced in Nile tilapia when exposed to polyethylene glycol (PEG). Hormonal imbalance and oxidative stress in gill tissues in Zebrafish (*Danio rerio*) are produced as a result of bioaccumulation of polystyrene microplastics (Lu et al., 2016).

Today microplastics are usually determined by using Fourier transform infrared (FTIR) and Raman spectroscopy. In these methods, polymers are determined based on the energy absorption of characteristics of functional groups. The current study was designed to explore the toxic effects of microplastics in various organs of fish (*Oreochromis niloticus*) as these are elaborated in the production of reactive oxygen species (ROS) that induce oxidative stress which is proved to be a common mechanism to cause cell damage. Inference of various studies illustrated that even a small amount of microplastics incorporated into the living cells involved in the generation of a large amount of ROS such as Superoxide anions, Hydroxyl radicals and Hydrogen peroxide (Khabbazi et al., 2015).

In the light of the above-mentioned data, still there is a need to assess the toxicity of polyacrylamide microplastics in the aquatic ecosystem by evaluating the blood parameters, oxidative damage and tissue histology of freshwater fish. The current study would be useful in the management of aquatic toxicity and environmental safety.

2. Materials and methods

2.1. Characterization of polyacrylamide microplastics

Microplastics were characterized by Fourier transform-infra red (FTIR) and Raman Spectroscopy using the facility of a Hi-tech laboratory. The powder of microplastics (10 mg) was subjected to FTIR using 10 mg of KBr pellet to prepare the sample disc for analysis. The range for scanning of the spectroscope was 400–4000 cm-1. Lambert-beer's law was applied to correlate the physical properties and chemical composition (Hameed et al., 2015). The concentration of the sample was estimated by using the equation as given below:

The concentration of the sample A = . C. D

Here ε is the molar absorption coefficient, C is for Sample concentration and D is Sample thickness.

2.2. Experimental work

Oreochromis niloticus of average body weight (60–70 g) were purchased from the Government fish hatchery, Satyana road, Faisalabad. Fish were acclimatized in a cemented tank of 102 × 52 × 73 cm (LWH) for fifteen days prior experiment. The physicochemical parameters of un-chlorinated tap water were analyzed. During the acclimatization, the temperature of the tank was maintained at 25 °C. The dissolved oxygen (6.4–7.5 mg/l) and pH (6.8–7.4) were maintained. Ammonia concentration (NH_3), total dissolved solids and total hardness were measured as 0.5–0.7 ppm, 48–52 ppm and 6.4–7.6 ppm respectively. Photoperiod was maintained at 12-hour day and night cycle. The fish were fed with a commercial feed comprising 65 % of protein and 10 % fats. Polyacrylamide microplastics 99 % pure were purchased from the Sigma-Aldrich company.

2.3. Determination of LC_{50} of polyacrylamide

The 96 h LC_{50} value of polyacrylamide was determined for *Oreochromis niloticus* (n = 80; approximately 60–70 g) in the glass aquarium. During the trial, fish were not fed. After 15 days of acclimatization, all fish showing no physical abnormalities, internally and externally active and no parasitic problems were placed in four equal groups. Fish in groups (G1-G3) were exposed to different doses of polyacrylamide for 28 days. Fish in the control group named C. All the fish were physically monitored on daily basis for any behavioral signs.

2.4. Toxicity of polyacrylamide

Fish were fed by polyacrylamide for 28 days to study their toxic effects. Doses of polyacrylamide were selected as 1/50th (0.018 g/l), 1/30th (0.03 g/l) and 1/10th (0.09 g/l) fractions of calculated LC_{50}. *Oreochromis niloticus* (n = 80) of average weight (60–70 g) were divided into four groups with 20 fish in each aquarium of 40 L capacity. Fish were exposed to polyacrylamide @ 0.00, 0.018, 0.03 and 0.09 g/l in C, G1, G2 and G3 respectively for 28 days on daily basis. Fish were fed daily and 90 % water was changed before dosing. Weight and length of fish were measured before and after each exposure period along with general observations including physiological abnormalities such as weakness, unbalancing, lesions, surface running, gasping, breathing and bottom running on the regular basis.

Fish from each treatment group were immediately anesthetized with 75 µl/liter of clove oil in a bucket for 3–5 min. Blood was collected from the caudal vein of fish and stored in EDTA-coated tubes (Yong Kang Medical Devices CO., ltd, China) at room temperature. After collection of blood, total of 80 fishes were dissected to collect sampled organs (gills, liver and intestine) and preserved them in 10 % formalin in normal saline for histological study. The relative weight of each organ was calculated by the formula;

$$\text{Relative weight} = \text{Organ weight/Body weight} \times 100$$

2.5. Determination of antioxidant enzyme activity

2.5.1. Preparation of tissue homogenate

0.25 g of tissue sample (gills) was homogenized with 2.5 ml of 0.1 M Tris-HCl buffer in a bullet blender (American company Alchemy-Pro 900) while keeping the pH 7.4 at 4 °C and crude tissue homogenate was then centrifuged at a speed of 10,000 r.p.m for 20 min in the centrifuge (Sigma 2–16 K) at 4 °C. The supernatant was stored at −20 °C and processed for further oxidative stress biomarkers (Jabeen et al., 2011).

2.5.2. Estimation of lipid peroxide (LPO)

For the estimatation of lipid peroxidase, 0.1 ml of homogenate tissue was treated with 0.9 ml fox reagent (7.6 mg xylenol orange, 88 mg butylated hydroxytoluene, 9.8 mg ammonium iron sulfate and 90 ml methanol was added into 10 ml of 250 mM sulphuric acid). The 2 ml eppendrop tubes consist of this solution were incubated at 37 °C for 30 min. After incubation the color of the solution was established and the sample was taken in the cuvette and the value was read in a spectrophotometer at 560 nm. The lipid peroxidase concentration was verbalized as mM/g of tissues (Liu et al., 2013).

2.5.3. Malondialdehyde (MDA) assay

For MDA test firstly 0.2 ml sample of tissue homogenate supernatant was taken in falcon tube then added 0.2 ml of 8.1 % sodium dodecyle sulphate (SDS), also added 1.5 ml of 20 % acetic acid and 1.5 ml of 8 % (TBA) solution. Whole solution became 3.4 ml, after that 0.6 ml distilled water were mixed in 3.4 ml solution so, the total concentration of the solution was 4 ml. This solution was placed at 95° C in the water bath for 1 h then cool out the solution at room temperature, add 1 ml distilled water then the solution became 5 ml. The second phase of solution preparation was including mixture of *n*-butanol and pyridine (1: 15) at concentration of 5 ml. Both solutions of 5 ml concentration were collected in the same falcon tube for each sample then the total solution of 10 ml was formed. This solution was centrifuged at 4000 r.p.m for 10 min, after that spectrophotometery was done at the wavelength of 532 nm and readings were noted for each sample (Ohkawa et al., 1979; Dastan et al., 2017).

2.5.4. Reduced glutathione (GSH) assay

For the determination of glutathione content tissue homogenate was mixed with 50 % of trichloro acetic acid and exposed to centrifugation at 1000 r.p.m for five minutes. Mixture was made with 500 µl of supernatant, 100 µl of 0.001 M DTNP (5′ 5′-dithiobisnitrobenzoic acid known as Ellman's reagent) and 2.0 ml of 0.2 M Tris-EDTA buffer with pH 8.9. This reaction mixture was the grant to stay at room temperature for 5 min. After that solution was transferred into the cuvette, placed in a spectrophotometer and the values were read in 412 nm. The values were declared as µM/g of tissues (Feng et al., 2013).

2.5.5. Estimation of catalase activity (CAT)

Catalase activity (CAT) was evaluated by following the procedures mentioned (Aebi, 1974). 50 µl of supernatant was taken in 3 ml cuvette then added 1.95 ml phosphate buffer having neutral pH and 1.0 ml of hydrogen peroxide (H_2O_2) was mixed and checked the absorbance twice at 240 nm for 30 s and again after 15 s. Reading was expressed in units per mL.

2.6. Biochemical parameters

For serum analysis blood was collected in Heparin-coated anticoagulant tubes. Collected blood in the tubes with a coagulation activator was centrifuged at 3800 × g for 8 min at 4 °C for hemolysis-free serum collection. The biochemistry of the hemolysis-free serum was analyzed using standard laboratory procedures. The biochemical parameters in serum ALT (alanine aminotransferase), AST (asparate transaminase), ALP (alkaline phosphate), UREA (urea concentration), CREA (creatinine blood test), TP (total protein), ALB (albumin) and GLOB (serum globulin) were measured (Selamoglu et al., 2012).

2.7. Hematological parameters

The collection of blood samples was stored in EDTA-coated tubes containing calcium @1.5 mg/ml of blood for complete blood analysis. The different variables were estimated including hemoglobin (HGB), platelets (PLT), lymphocytes (LYM), mean cell volume (MCV), red blood cells (RBC), white blood cells (WBC), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) and hematocrit (HCT) by using a hematology auto-mated analyzer (Selamoglu et al., 2012; Bain et al., 2016).

2.8. Histopathology

Tissues (gills, liver and intestine) were fixed in sera containing 60 m1 absolute alcohol, 30 m1 Formaldehyde and 10 m1 glacial acetic acid. Fixed tissues were then dehydrated gradually by 80 %, 90 % and 100 % ethanol for 2–4 h at room temperature. After dehydration, fixed tissues were shifted to cedar wood oil until they became clear at room temperature. Dehydrated tissues were embedded gradually in Benzol for 20 min at room temperature. Then tissues were embedded in a mixture of benzol and paraplast (1:1) for 20 min at 60 °C. After that, tissues were again embedded in paraplast and placed in the incubator for 12 h at 60 °C. Targeted tissues were then shifted in molten paraffin wax to a box. Bubbles were withdrawn and wax was permitted to harden. The box blocks for each targeted tissue were formed and kept in the plastic casters. Parretti wax blocks were removed and mounted on wooden blocks for sectioning. The microtome was used to cut 3–4 µm sections of each tissue. The ribbons with tissues were stretched and carefully transferred to Fisher slides which were warmed and sustained for 24 h. After staining the slides were mounted with Canada balsam. The cover slips were fixed over slides and were kept in an incubator for 12 h. Then, 3 µm thick sections were studied under the light microscope (Talas et al., 2014; Noureen et al., 2018).

2.9. Statistical analysis

In this research work, collected data presented as mean ± S.E. The collected data was equally distributed in each group. SPSS Software was used for statistical analysis. ANOVA was applied to perform statistical analysis. Tukey's test was applied to compare the means of different parameters of Oxidative stress, Hematology and Serum analysis of control and all treated groups.

3. Results

3.1. Physical observations

Prior to the experiment, the 96 h LC_{50} value (0.9 g/l) of polyacrylamide microplastics was determined for *Oreochromis niloticus*. Probit analysis was used to determine the LC_{50} using Minitab version 17. In the present study, *Oreochromis niloticus* was exposed to different concentrations (0.018, 0.03 and 0.09 g/l) of polyacrylamide for 28 days. After the exposure of polyacrylamide no mortality was observed in all treated groups (G1-G3). Results showed various physiological abnormalities such as an increase in swimming rate, swallowing in eyes, dark lesions on different parts of body, weight loss, mucus secrets from gills and fins, and gasping in all treated groups was increased in a dose-dependent manner.

3.2. FTIR spectroscopy

The fourier-transform infrared (FTIR) technique was used to identify the functional groups bound with polyacrylamide. FTIR spectra showed bands at 3378, 3257, 1663, 1619 and 1026 cm^{-1}. Few absorption bands of the biomolecules (microplastics) used as reduction and stabilization (capping) agents, could also be observed in the spectrum. FTIR spectroscopy also confirmed the spherical morphology and size range of polyacrylamide (0.1–0.4 mm) and proved it impurity-free. Traces of absorption bands of the biomolecules (microplastics) used as reduction and stabilization (capping) agents are also visible in the spectra (Fig. 1).

3.3. Raman spectroscopy

All the samples treated with polyacrylamide showed vibrational modes/humps at various points in the scan but no sharp peak was observed. In polyacrylamide treated slides there were peaks at 1100 cm-^{1} and 1650 cm^{-1} in gills, these peak confirmed the presence of polyacrylamide microplastics in gills. Vibrational modes and peaks of minimum intensity at 1100 cm^{-1} was also observed in the liver sample indicating that few polyacrylamide microplastics have been reached there. In intestine few vibrational modes were the indication of the traces of polyacrylamide but minimum as compared to gills and liver (Fig. 2).

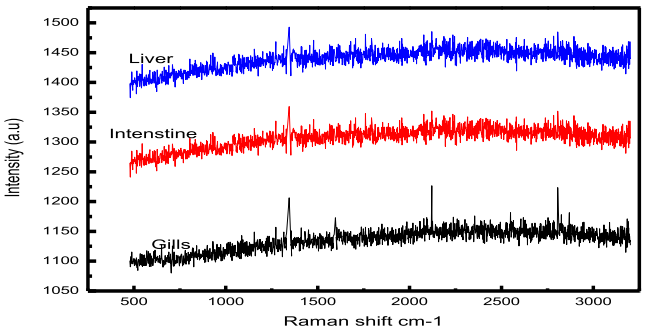

Fig. 2. Characterization of polyacrylamide microplastics by Raman spectroscopy.

3.4. Toxicity phase

3.4.1. Body weight change

Microplastics can potentially cause physiological changes in aquatic flora and fauna. The presence and toxicity of plastic debris are rapidly increasing in aquatic ecosystem. Table 1 showed the toxic effects of polyacrylamide on the body weight of *Oreochromis niloticus* treated with different doses. After the exposure of polyacrylamide, the body weight was significantly decreased (58.24 ± 0.24) in high dose treated group G3. Gills absolute (1.85 ± 0.07) and relative weight (3.32 ± 0.13) was also decreased. Liver and intestine weight was decreased significantly in all treated groups. Fig. 3 (A & B) showed significant results ($p < 0.05$) of polyacrylamide on absolute and relative weight organ (gills, liver and intestine) in different exposed groups (low = G1, medium = G2 and high = G3 dose treated) after 28th day of exposure.

3.4.2. Oxidative stress enzymes

The toxicity of microplastics inhibited the production of Glutathione levels (GSH). Microplastics have negative effects on the production of glutathione activity but enhance the production of ROX resulting in cellular abnormalities. Table 2 showed significant ($p < 0.05$) decrease in catalase (423.2 ± 204.4) and glutathione (896 ± 31.69) activities in all treated groups of polyacrylamide in a dose-dependent manner as compared to control group. Table 2 also showed significant ($p < 0.05$) increase in malondialdehyde (1670 ± 23.63) and lipid peroxidase (1957 ± 42.16) activities in all treated groups of polyacrylamide in a dose-dependent manner as compared to control group (Fig. 4).

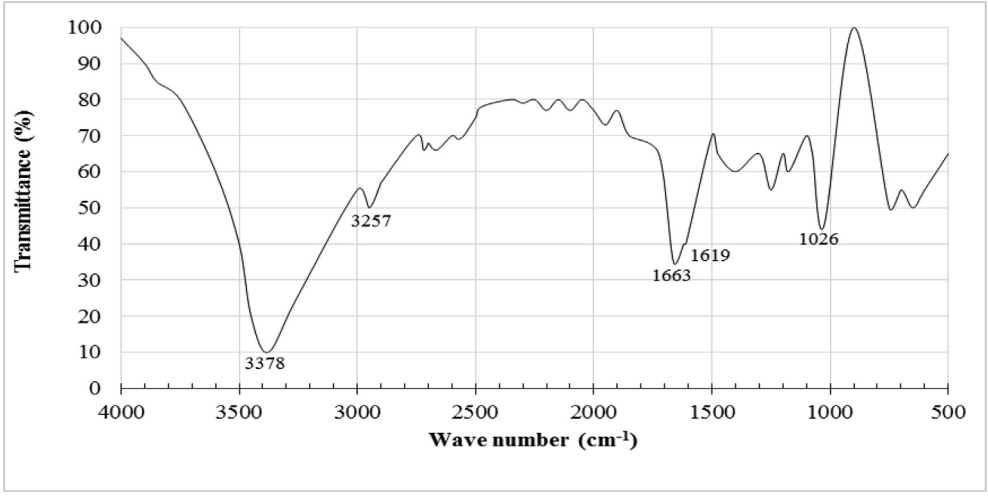

Fig. 1. Characterization of polyacrylamide microplastics by FTIR spectroscopy.

Table 1
Effect of different concentrations (control, 0.018, 0.03, 0.09 g/l) of polyacrylamide microplastics on body weight, body length, organs weight (gills, liver and intestine).

Polyacrylamide treated groups	Control Group	G1 (0.018 g/l)	G2 (0.03 g/l)	G3 (0.09 g/l)
Weight (g)	60.54 ± 0.23^{a}	59.32 ± 0.31^{b}	58.81 ± 0.28^{c}	58.24 ± 0.24^{d}
Length (cm)	16.38 ± 0.41^{a}	16.43 ± 0.29^{a}	16.43 ± 0.23^{a}	16.42 ± 0.23^{a}
Gill (Absolute)	2.11 ± 0.08^{a}	2.01 ± 0.1^{a}	1.93 ± 0.08^{ab}	1.85 ± 0.07^{b}
Gill (Relative)	3.46 ± 0.15^{a}	3.41 ± 0.12^{a}	3.37 ± 0.13^{ab}	3.32 ± 0.13^{b}
Liver (Absolute)	0.85 ± 0.02^{a}	0.82 ± 0.02^{b}	0.79 ± 0.03^{bc}	0.76 ± 0.01^{c}
Liver (Relative)	1.39 ± 0.018^{a}	1.28 ± 0.015^{ab}	1.24 ± 0.015^{ab}	1.24 ± 0.024^{bc}
Intestine (Absolute)	1.66 ± 0.007^{a}	1.63 ± 0.005^{a}	1.63 ± 0.009^{b}	1.59 ± 0.014^{c}
Intestine (Relative)	2.73 ± 0.015^{a}	2.68 ± 0.008^{a}	2.64 ± 0.014^{b}	2.65 ± 0.017^{c}

C: control group, G1: low dose (0.018 g/l), G2: medium dose (0.03 g/l), G3: high dose (0.09 g/l).

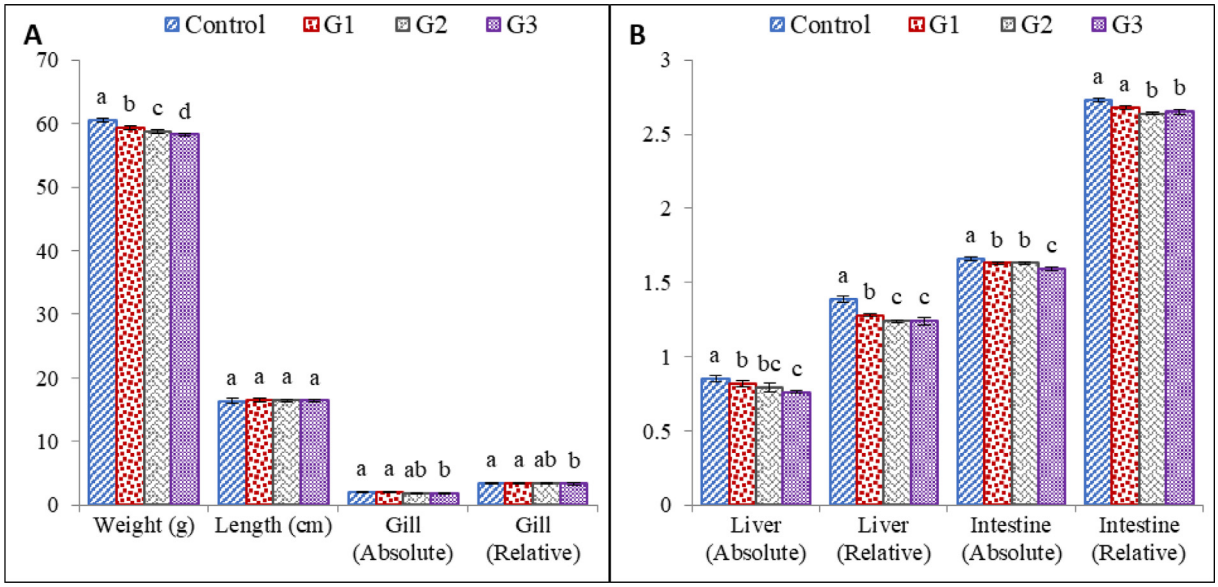

Fig. 3. Effect of different concentrations (control, 0.018, 0.03, 0.09 g/l) of polyacrylamide microplastics on; A) body weight, body length, organ gills; B) liver absolute, liver relative, intestine absolute and intestine relative).

Table 2
Effect of different concentrations (control, 0.018, 0.03, 0.09 g/l) of polyacrylamide microplastics on oxidative stress enzymes.

Anti-oxidant enzymes (Parameters)	Control Group	G1 (0.018 g/l)	G2 (0.03 g/l)	G3 (0.09 g/l)
CAT (Unit/ml)	2017 ± 1489.4^{a}	608.1 ± 1149.7^{b}	538.7 ± 331.6^{b}	423.2 ± 204.4^{b}
MDA (N moles/g)	414 ± 11.82^{b}	1158 ± 22.68^{d}	1660 ± 18.87^{b}	1670 ± 23.63^{b}
GSH (µmole/g)	1256.9 ± 38.09^{a}	1186.9 ± 35.69^{a}	1081.1 ± 40.99^{b}	896 ± 31.69^{c}
LPO (mM/100 g)	1312 ± 56.68^{d}	1613 ± 38.65^{b}	1903 ± 40.48^{a}	1957 ± 42.16^{a}

C: Control group, G1: low dose (0.018 g/l), G2: medium dose (0.03 g/l), G3: high dose (0.09 g/l), CAT: catalase, MDA: malondialdehyde, GSH: glutathione, LPO: Lipid peroxidase.

3.4.3. Effects of polyacrylamide microplastics on biochemical parameters

After the administration of polyacrylamide microplastics, aspartate transaminase (AST), alanine transaminase (ALT), total protein, urea and creatinine concentration was significantly ($p < 0.05$) increased in high dose (0.09 g/l) of polyacrylamide microplastics treated groups as compared to non-treated group (51.7 ± 0.67, 9.69 ± 0.43, 1.37 ± 0.09, 15.35 ± 1.00, 46.4 ± 0.09 respectively) Fig. 5 A & B. While, albumin (ALB) and globulin (GLOB) concentration was decreased (5.99 ± 0.08, 2.96 ± 0.07 respectively) in polyacrylamide treated groups see Table 3; Fig. 5 (A & B). Microplastics based hepatotoxicity caused an increase in the activities of hepatic damage markers such as aspartate transaminase (AST), alanine aminotransferase (ALT). The increase in ALT is possibly caused due to any of the causes like the obstructive jaundice, hepatic cirrhosis, hepatitis, or liver tumor. Increase in the level of AST might be related to non-hepatic cause such as heart damage. Polyacrylamide exposure may exert more critical effects on the liver function in young or juvenile organisms as compared to adult ones. After the exposure of polyacrylamide, the treated groups experienced a significant ($p < 0.05$) escalation in the value of erythrocyte sedimentation rate (ESR) in high dose treated group G3.

3.4.4. Effects of polyacrylamide microplastics on hematological parameters

In the present study, sub-lethal effects of microplastics on various Hematological parameters (erythrocytes, total leucocytes count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, MCH concentration, leucocytes, neutrophils, lymphocytes and monocytes) of *Oreochromis niloticus* after 28 days of exposure was analyzed. Hematological parameters such as hemoglobin (g/dl), total leucocyte count TLC ($1x10^{9}$/l), ery-

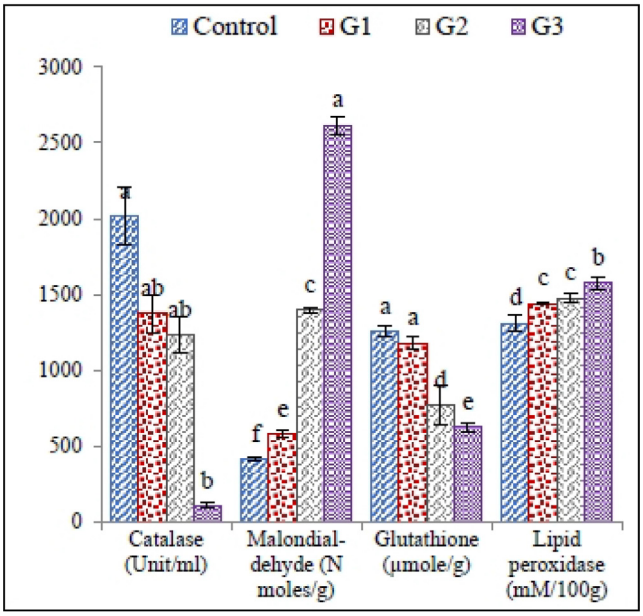

Fig. 4. Effect of different concentrations (control, 0.018, 0.03, 0.09 g/l) of polyacrylamide microplastics on oxidative stress enzymes.

throcyte (10^6/µl), hematocrit, platelets, neutrophils (%) and Eosinophils (%) showed significant ($p < 0.05$) decrease (6.29 ± 0.19, 119. 48 ± 0.62, 1.04 ± 0.02, 19.77 ± 0.43, 168.59 ± 4.65, 2.00 ± 1.13, 0.29 ± 0.47 respectively) after exposure of polyacrylamide as compared to control group Fig. 6 (A, B & C). However, other blood parameters; (MCV) and (MCH) showed a significant ($p < 0.05$) increase after exposure to 0.18, 0.03 and 0.09 g/l of polyacrylamide for 28 days (Table 4).

3.4.5. Histopathology

Histological results showed marked histological alterations in the gills of *O. niloticus* treated with polyacrylamide. Current results showed dose-dependent histological alterations such as clubbed tips of the secondary lamellae (Ct), fusion of secondary lamellae (Fsl), vacuolization (Vc), oedema (Oe), aneurism, hyperplasia (Hp), degeneration of epithelial cells (DEC), erythrocyte cells (Er), fusion filament (Ff), necrosis (Nc), secondary epithelial lamellae (Sel), smooth lamellar filament (Slf), lifting of gill epithelium (Lge), congestion (Con), curvature (Cu) and tip degeneration (Td) was observed in high dose treated group (Fig. 7). Current results showed marked histological alterations in the liver including increased kupfer cells (IKC), rupture of hepatocyte membrane (RH), congested blood vessels (CBV), increased pycnotic nuclei (IPN), degenerated nuclei (DN), hypertrophied hepatic cells (HHC), dilated sinusoids (DS) and hypertrophy and hyperplasia of bile duct cells (HHB) (Fig. 8). Results presented (Fig. 9) also showed histological alterations in intestine including ruptured intestinal epithelial cells (RE), lumen blocked (BL), abundant calciform cells (AC), thin wall of villi (Tn), vacoulation (V), mild lumen blocked (ML), villi with necrotic endocytes (VN), swelling of goblet cells (SG).

4. Discussion

Water pollution caused by different pollutants is considered one of the major environmental issues in different regions of the world (Browne et al., 2013). However, in freshwater ecosystems, the toxic effects of various toxicants, such as the microplatics, on aquatic organisms remain a huge challenge. To understand the knowledge about the toxicity of microplastics in freshwater ecosystem, the current study showed that these particles, even in small concentrations induce toxicity by producing harmful effects on aquatic flora and fauna (Araujo et al., 2018). Toxicity of microplastics based on uptake efficiency. Results showed that micro and nanoplastics could induce oxidative stress, genotoxicity, histological alterations and reactive oxygen species in various aquatic organisms such as aquatic lungworms, mussels and invertebrates (Avio et al., 2015).

Microplastics have many hazardous impacts on the physiology of humans and other animals. Microplastics serve as a carrier for a variety of elements and different toxicants i.e. additives for various industrial production processes and cause permanent contamination by adsorption processes in water bodies (Li et al., 2018). Microplastics produce toxicity in aquatic organisms. Microplastics

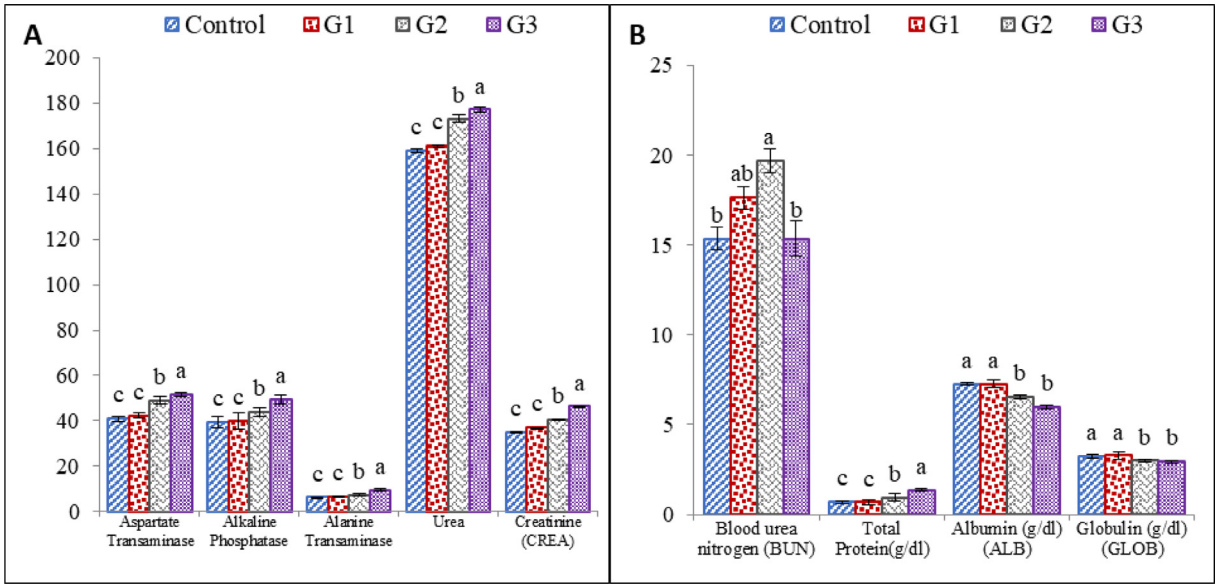

Fig. 5. Effect of different concentrations (control, 0.018, 0.03, 0.09 g/l) of polyacrylamide microplastics on biochemical parameters; A) aspartate transaminase, alkaline phosphate, alkanine transaminase, urea, creatinine; B) blood urea nitrogen, total protein, albumin, globulin.

Table 3
Effect of different concentrations (Control, 0.018, 0.03, 0.09 g/l) of polyacrylamide microplastics on biochemical parameters.

Serum Analysis	Control Group	G1 (0.018 g/l)	G2 (0.03 g/l)	G3 (0.09 g/l)
Aspartate Transaminase	40.9 ± 0.97^{c}	42.4 ± 0.98^{c}	49.0 ± 1.59^{b}	51.7 ± 0.67^{a}
Alkaline Phosphatase	39.5 ± 2.55^{c}	39.8 ± 3.55^{c}	44.0 ± 2.07^{b}	49.5 ± 1.79^{a}
Alanine Transaminase	6.40 ± 0.19^{c}	6.65 ± 0.18^{c}	7.45 ± 0.32^{b}	9.69 ± 0.43^{a}
Urea	159 ± 0.79^{c}	161 ± 0.57^{c}	173 ± 1.62^{b}	177 ± 0.98^{a}
Creatinine (CREA)	35.2 ± 0.08^{c}	37.1 ± 0.09^{c}	40.6 ± 0.16^{b}	46.4 ± 0.09^{a}
Blood urea nitrogen (BUN)	15.36 ± 0.62 ^{d}	17.62 ± 0.65 ^{bc}	19.69 ± 0.66 ^{ab}	15.35^{A} ± 1.00 ^{a}
Total Protein(g/dl)	0.69 ± 0.058^{a}	0.68 ± 0.15^{a}	0.93 ± 0.21^{b}	1.37 ± 0.09^{c}
Albumin (g/dl)	7.27 ± 0.09^{a}	7.28 ± 0.21^{a}	6.55 ± 0.09^{b}	5.99 ± 0.08^{b}
Globulin (g/dl)	3.25 ± 0.08 ^{a}	3.28 ± 0.18^{a}	3.00 ± 0.06^{b}	2.96 ± 0.07^{c}

C: control group, G1: low dose (0.018 g/l), G2: medium dose (0.03 g/l), G3: high dose (0.09 g/l), (g/l) gram per liter, (g/dl), gram per deciliter.

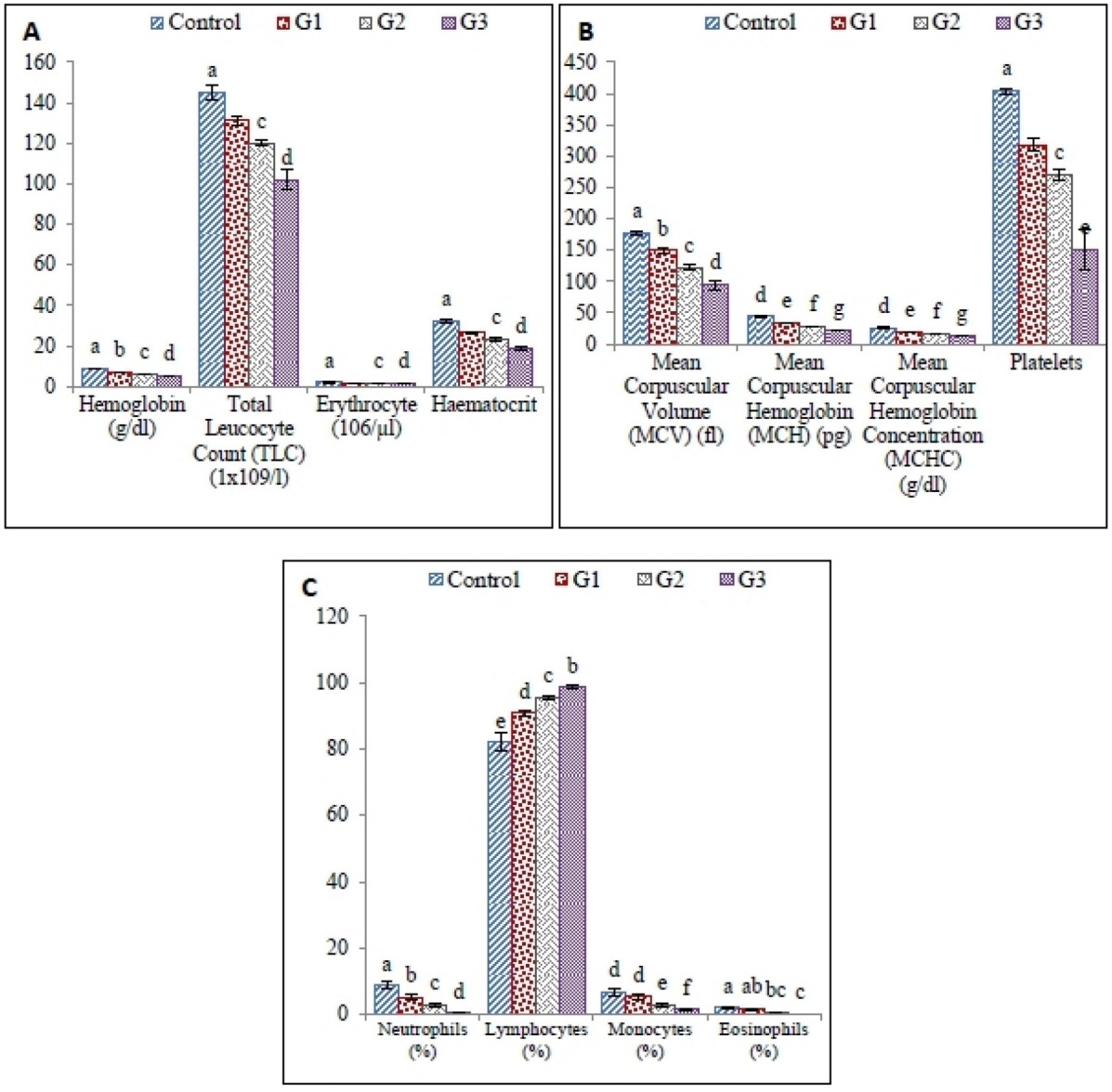

Fig. 6. Effect of different concentrations (control, 0.018, 0.03, 0.09 g/l) of polyacrylamide microplastics on hematological parameters; A) Hemoglobin, total leucocyte count, erythrocyte, haematocnt count; B) Corpuscular volume, corpuscular hemoglobin, corpuscular hemoglobin concentration, platelets; C) Neutrophils, lymphocytes, monocytes, eosinophils.

combined with other pollutants in aquatic flora and fauna (Schwaferts et al., 2019). They combined with pharmaceutical and many other toxic products, transporting these products to aquatic organisms resulting in unexpected environmental hazards (Revel et al., 2018). When microplastics exposed to fish skin and taken up by fish, they caused serious health problems. The mortality rate was also enhanced by the toxicity of microplastics (Raza et al., 2018).

Table 4
Effect of different concentrations (Control, 0.018, 0.03, 0.09 g/l) of polyacrylamide microplastics on hematological parameters.

Hematology Parameters	Control Group	G1 (0.018 g/l)	G2 (0.03 g/l)	G3 (0.09 g/l)
Hemoglobin (g/dl)	8.4 ± 0.19^{a}	7.75 ± 0.05^{b}	7.10 ± 0.13^{c}	6.29 ± 0.19^{d}
TLC ($1x10^{9}$/l)	144.1 ± 3.62^{a}	135.27 ± 1.03^{b}	126.79 ± 0.74^{d}	119.48 ± 0.62^{c}
Erythrocyte (10^{6}/µl)	1.92 ± 0.10^{a}	1.50 ± 0.06^{b}	1.27 ± 0.01^{d}	1.04 ± 0.02^{c}
Haematocrit (%)	32.07 ± 1.20^{a}	26.46 ± 0.51^{b}	22.57 ± 0.68^{c}	19.77 ± 0.43^{d}
MCV (fl)	176.48 ± 1.93^{c}	183.19 ± 0.43^{b}	187.38 ± 0.72^{b}	193.39 ± 0.60^{a}
MCH (pg)	44.08 ± 1.50^{d}	51.73 ± 0.65^{c}	56.44 ± 0.79^{b}	62.27 ± 0.76^{a}
MCHC (g/dl)	26.30 ± 1.35^{d}	31.93 ± 0.69^{c}	38.52 ± 0.76^{b}	44.13 ± 0.70^{a}
Platelets	404.00 ± 4.26^{a}	258.28 ± 3.63^{c}	206.38 ± 2.93^{d}	168.59 ± 4.65^{c}
Neutrophils (%)	8.58 ± 1.06^{a}	5.08 ± 0.71^{b}	2.89 ± 0.86^{c}	2.00 ± 1.13^{c}
Lymphocytes (%)	82.01 ± 2.77^{d}	89.08 ± 0.72^{d}	97.28 ± 1.55^{bc}	115.29 ± 3.02^{a}
Monocytes (%)	6.48 ± 0.95^{d}	9.08 ± 0.72^{c}	12.00 ± 1.03^{b}	15.29 ± 1.47^{a}
Eosinophils (%)	2.00 ± 0.80^{a}	0.89 ± 0.72^{bc}	0.59 ± 0.69^{c}	0.29 ± 0.47^{c}

TLC: Total leucocyte count, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean cell hemoglobin concentration, (g/dl), gram per deciliter, (pg) picograms, (fl) femtoliters.

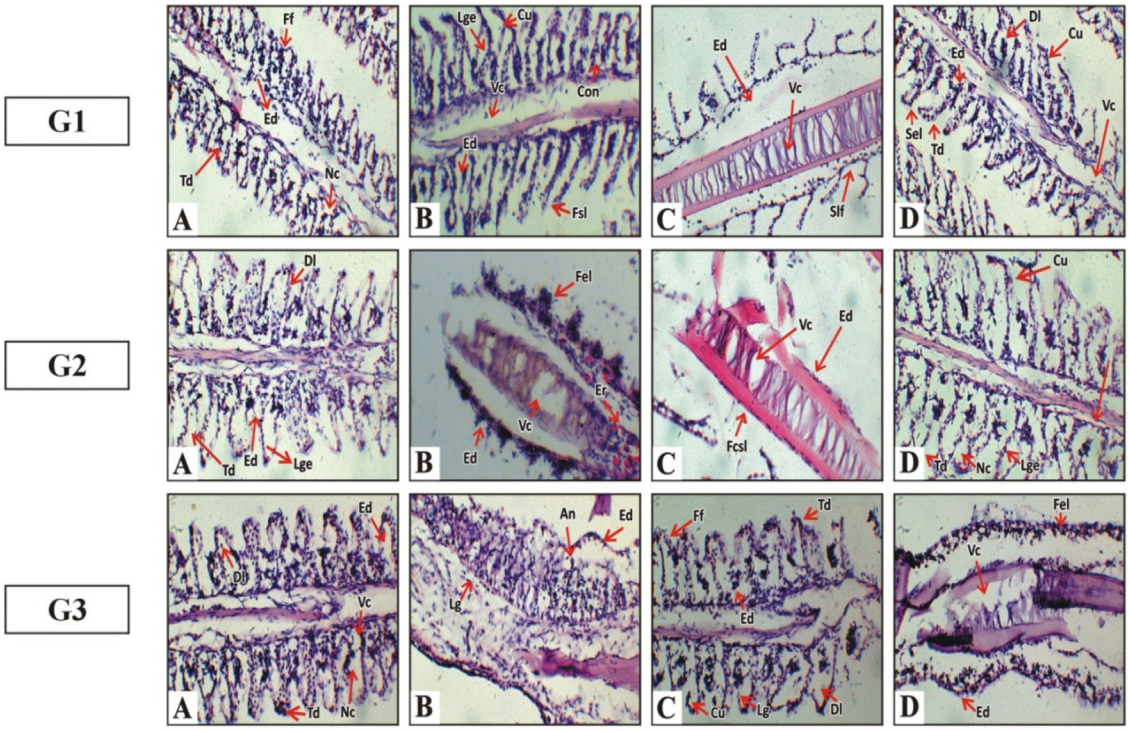

Fig. 7. Histological changes in the gills of *Oreochromis niloticus* in all treated groups of polyacrylamide microplastics. G1: low dose, G2: medium dose, G3: high dose; Fusion filament (Ff), Oedema (Ed), Tip degeneration (Td), Fusion of secondary lamellae (Fsl), curvature (Cu),vacuolization (Vc), Congestion (Con), lifting of gill epithelium (Lge), Necrosis (Nc), Dilation (Dl), Secondary epithelial lamellae (Sel), aneurism (An), Erythrocyte cells (Er), Secondary lamellae (SL), smooth lamellar filaments (Slf),

Zhang et al., 2019 studied the toxic effects of polystyrene on *Oreochromis niloticus* after seven days of exposure. Results showed that distribution and accumulation of polystyrene microplastics in the tissues of *Oreochromis niloticus* increased the accumulation of reactive oxygen species in tissues. Biochemical results showed that the level of cytochrome P450 was significantly increased after the exposure of polystyrene after seven days as compared to control group. Moreover, Superoxide dismutase (SOD) activity was significantly increased while Malondialdehyde (MDA) activity decreased significantly in treated groups.

Increased production of reactive oxygen species occurs as a result of exposure to polyacrylamide. Reactive oxygen species increased the production of different antioxidant enzymes which induced various cellular abnormalities and enhanced the production of TBARS (Ates et al., 2008). Increased concentration of Malondialdehyde is an indicator of toxicity caused by free radicals. MDA is considered as an important tool for evaluating the health of aquatic organisms (Fuat et al., 2012). Therefore, activities of Malondialdehyde (MDA) and Lipid peroxidase (LPO) increased as a result of a higher generation of reactive oxygen species because toxicity of microplastics enhanced the production of ROX (Reactive Oxygen Species) (Selamoglu et al., 2011; Besseling et al., 2014).

In the present study, sub-lethal effects of polyacrylamide on oxidative stress biomarkers including CAT, MDA, LPO and GSH were observed in the gills of *O. niloticus* after 28 days of exposure. Results showed significant increase in the activities of Malondialdehyde (MDA) and lipid peroxide (LPO) in gills of *O. niloticus* while decreased activity of catalase was observed in high dose groups. Polyacrylamide induced hormonal imbalance, endocrinal perturbation, behavioral impairments, hepatic stress including CYPIA expression, genomic toxicity, cellular necrosis, changes in metabolic rate as well as reduced enzymatic activity (Oliveira et al., 2013).

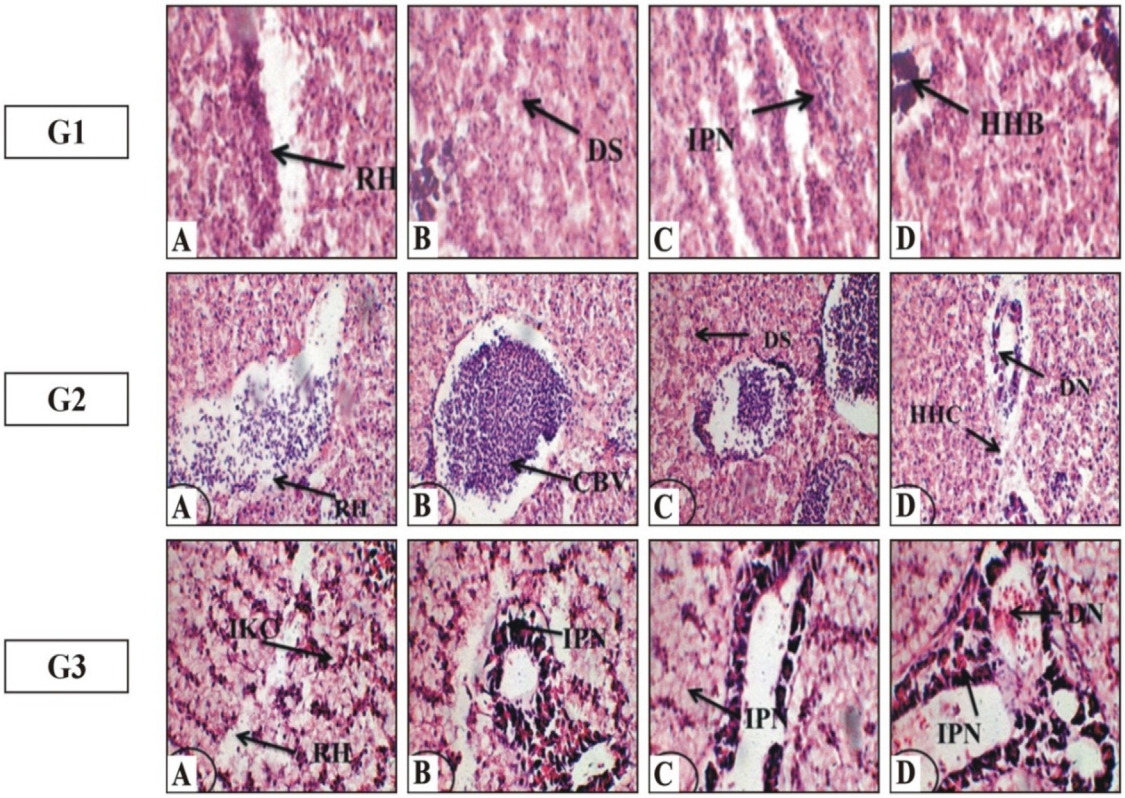

Fig. 8. Histological changes in the liver of *Oreochromis niloticus* in all treated groups of polyacrylamide microplastics. G1: low dose, G2: medium dose, G3: high dose; Rupture of hepatocyte membrane (RH), dilated sinusoids (DS), increased pycnotic nuclei (IPN), hypertrophy and hyperplasia of bile duct cells (HHB), congested blood vessels (CBV), degenerated nuclei (DN), hypertrophied hepatic cells (HHC), increased kupfer cells (IKC),

Haghi and Banaee (2017) studied the exposure of sub-lethal concentrations of paraquat (0.25 and 0.45 mg/l) and microplastics (1.5 and 2.5 mg/l) separately and in combination in *Cyprinus carpio* for two weeks. Results of blood biochemical analysis indicated that the level of glucose and the activity of aspartate transaminase (AST), alkaline phosphatase (ALP), and creatine phosphokinase (CPK) increased when exposed to 0.45 mg/l paraquat. Moreover, activity of ALP and CPK was significantly increased in fish exposed to 2.5 mg/l microplastics. Results showed significant reduction in total protein, globulin, cholesterol, and triglyceride levels and glutamyl transferase activity. The activity of alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) increased significantly ($p < 0.05$) as compared to control group. While Albumin and creatinine level significantly increased in the fish treated with a mixture of paraquat and 2.5 mg/l microplastics. Results showed that the enhancement of microplastics dose in the water increased the toxicity of paraquat at significant level in fish. Results showed that variations in the biochemical parameters of the blood were produced when fish exposed to paraquat and microplastics.

In our study biochemical parameters such as creatinine, uric acid, AST, ALT and ALP significantly increased after the exposure of polyacrylamide in treated groups as compared to control group. Similar results were observed in common goby (*Pomato schistusmicrops*) after the administration of microplastics (dos Santos Norberto, 2014). Alanine aminotransferase can be related to the activity of enzymes. The following findings were matched with the work done by (Abdel-Khalek et al., 2016) who studied the clinical effects on liver enzymes after the exposure of graphene oxide for 27 days.

The hematological changes may caused through hematopoietic mechanism which involves the inhibition of heme biosynthesis. Microplastics can block the activities of three important enzymes which are responsible for the synthesis of heme, i.e., delta amino-levulinic acid synthase, delta amino-levulinic acid dehydratase (ALAD) and ferro chelatase. Ferro chelatase is involved in the deposition of iron into porphyrin ring but microplastics can disturb its pathway. Consequently, inhibition of heme synthesis reduces the amount of circulating hemoglobin in the blood that assertively hinders cytochrome *P* 450-dependent phase-1 metabolism (Alvares et al., 1976). Inhibition of heme biosynthesis can cause morphological alterations in blood cells in the form of erythrocyte nucleus inversion, thrombocyte and erythrocyte aggregation, infiltration of blast cells and lymphocyte degeneration (Suljevic et al., 2020a).

Hemolysis or blood clotting can be a cause of toxic effects of metallic exposure. Exposure of fish to sub-lethal concentrations of these particles resulted in changed hematological parameters, for example a significant increase in WBCs (white blood cells), a significant decline in RBCs (red blood cells), Hb (hemoglobin) and platelets count (Orun et al, 2008; Selamoglu et al., 2012). Furthermore, these particles significantly raised the levels of alanine aminotransferase (ALT), aspartate transaminase (AST), urea and creatinine. Likewise, toxic effects can be visualized as aggregation of platelets and changes in the plasma coagulation time (Kaya et al., 2016). Microplastics are extremely toxic and alter the normal hematological and serological parameters (Khabbazi et al., 2015). In the present study the hematological parameters such as Hemoglobin (g/dl), TLC ($1x10^9$/l), Erythrocyte (10^6/µl), Haematocrit, Platelets, Neutrophils (%) and Eosinophils (%) showed significant ($p < 0.05$) decrease after exposure to 0.018, 0.03 and 0.09 g/l of polyacrylamide for 28 days. However, other blood parameters;

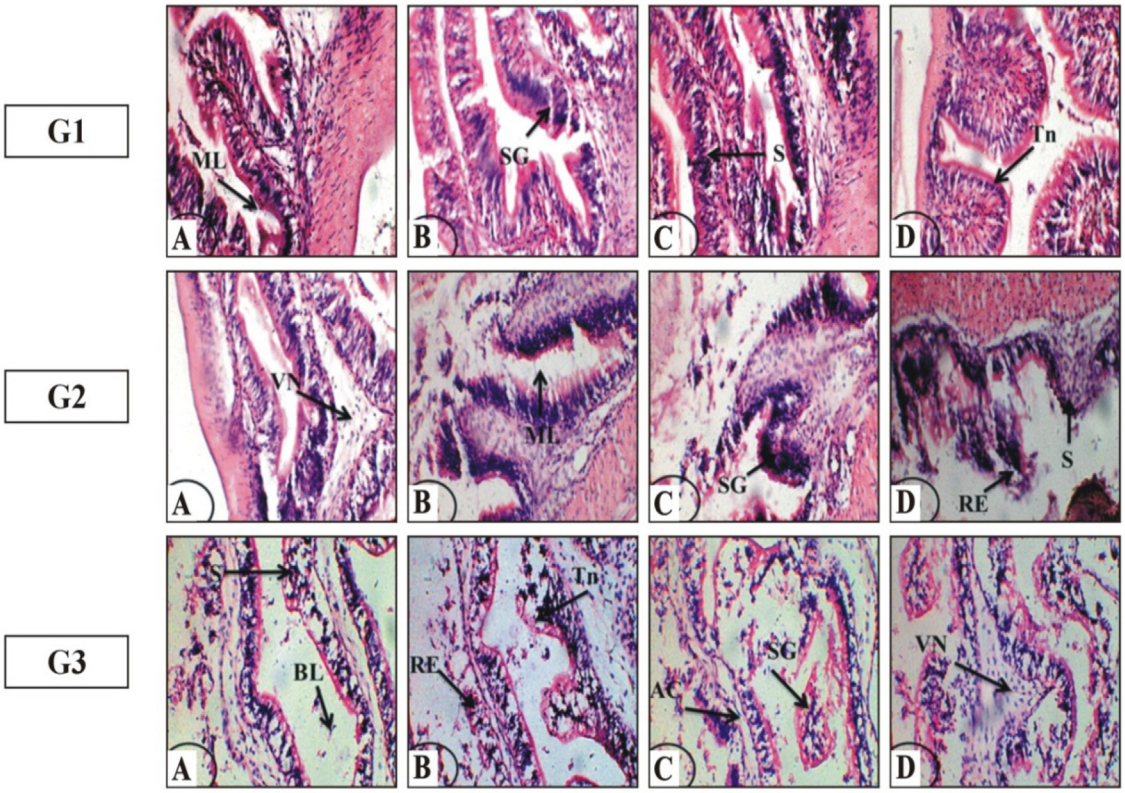

Fig. 9. Histological changes in the intestine of *Oreochromis niloticus* in all treated groups of polyacrylamide microplastics. G1: low dose, G2: medium dose, G3: high dose; Mild lumen blocked (ML), swelling of goblet cells (SG), swelled mucosa (S), Thin wall of villi (Tn), villi with necrotic endocytes (VN), ruptured intestinal epithelial cells (RE), lumen blocked (BL), abundant calciform cells (AC), villi with necrotic endocytes (VN).

(MCV) and (MCH) showed a significant ($p < 0.05$) increase in treated groups.

Histopathological study gives useful data concerning tissue changes before external manifestation. Histopathological changes in animal tissues are reliable and direct indicator of environmental hazards. Therefore, histopathological evaluation remains as an important part of assessment of the adverse effects of xenobiotics on organisms (Nataraj et al., 2017). Romano et al. (2017), studied the exposure of fragments of polyvinyl chloride (PVC) in silver barbs fry in raising concentrations of 0.3, 0.6, and 1.0 mg/l for 96 h, significant increase was observed in the activity of trypsin and chemotrypsin in fish after the exposure of 0.6 and 1.0 mg/l polyvinyl chloride as compared to 0 or 0.3 mg/l polyvinyl chloride. Polyvinyl chloride was also evaluated in both proximal and distal portion of intestine in the fish encountered to 0.6 and 1.0 mg/l polyvinyl chloride respectively, and these pieces of microplastics were related with restricted thickness of the mucosal epithelium. There was no damage observed in other organs and gills.

Histological parameters are important for supporting the biochemical assays. Moreover, histological changes in the tissues of organisms provide evidences for the detection of toxicity caused by various toxicants. Histopathological studies are also useful for the evaluation of potential effects caused by the accumulation of heavy metals in fish (Talas et al., 2014). Histological analysis of gills revealed significant alterations such as clubbed tips of the secondary lamellae (Ct), fusion of secondary lamellae (Fsl), vacuolization (Vc), oedema (Oe), aneurism, Hyperplasia (Hp), degeneration of epithelial cells (DEC), Erythrocyte cells (Er), Fusion filament (Ff), Necrosis (Nc), Secondary epithelial lamellae (Sel), Smooth lamellar filament (Slf), lifting of gill epithelium (Lge), Congestion (Con), Curvature (Cu) and Tip degeneration (Td) in high dose treated groups as compared to low and medium dose after 28 days of exposure to polyacrylamide. Marked histological alterations were observed in the liver including increased kupfer cells (IKC), rupture of hepatocyte membrane (RH), congested blood vessels (CBV), increased pycnotic nuclei (IPN), degenerated nuclei (DN), hypertrophied hepatic cells (HHC), dilated sinusoids (DS) and hypertrophy and hyperplasia of bile duct cells (HHB). The results also showed histological alterations in intestine including ruptured intestinal epithelial cells (RE), lumen blocked (BL), abundant calciform cells (AC), thin wall of villi (Tn), vacoulation (V), mild lumen blocked (ML), villi with necrotic endocytes (VN), swelling of goblet cells (SG) and swelled mucosa (S).

5. Conclusion

Our findings indicated that high concentrations of polyacrylamide microplastics induced dose-dependent effects on *Oreochromis niloticus*. In the current study, the presence of polyacrylamide particles has been confirmed in the gills of *Oreochromis niloticus* by FTIR and Raman spectroscopy. Exposure to polyacrylamide caused alterations in fish health parameters by decreasing antioxidant enzymes (CAT and GHS). Hemoglobin (g/dl) and total protein levels were significantly decreased after exposure to polyacrylamide. It was observed that high concentrations of polyacrylamide caused significant increase in total protein contents, while other blood parameters (AST, ALP, ALT) were significantly decreased in high dose treated group. In addition to it, histopathological changes were observed in gills, liver and intestine of *Oreochromis niloticus*. Results showed that higher concentrations of polyacrylamide microplastics caused more damage in fish tissues as compared to low doses.

CRediT authorship contribution statement

Bilal Rasool: Conception and Design. **Tahira Younis:** Acquisition of data, Analysis and Interpretation of data, Manuscript writing. **Tehreem Raza:** Conception and design & Interpretation of data. **Zeeshan Javed:** Interpretation of data. **Muhammad Asrar:** Acquisition & Interpretation of data. **Faiza Jabeen:** Analysis & Interpretation of data. **Maleeha Manzoor:** Resources, Acquisition of data, Writing - review & editing. All authors have read and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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