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Intestinal Toxicity of Glyphosate in *Clarias gariepinus*: A Biochemical and Histological Assessment

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Abstract: **This study evaluated the biochemical and histopathological effects of glyphosate on the intestine of the African catfish, *Clarias gariepinus*. Fish were exposed to 5 mg/L and 10 mg/L glyphosate for 10 days. Results showed a significant, dose-dependent decrease in intestinal protein and carbohydrate levels, along with sex-specific changes in the pyloric intestine somatic index. Histological examination revealed severe structural damage, including villi degeneration, epithelial disruption, goblet cell alterations, and nuclear pyknosis, with greater severity at higher concentrations. These findings indicate that glyphosate causes marked metabolic and intestinal toxicity in *C. gariepinus*, highlighting its potential ecological risk to freshwater organisms.**

Index Terms: **Intestine, Histopathology, Biochemical alterations, Herbicide toxicity**

I. INTRODUCTION

Freshwater ecosystem is increasingly contaminated by agricultural chemicals like glyphosate due to surface runoff, posing serious threats to aquatic organisms like fish species (Yan *et al.,* 2022). GLY has been widely occurred in aquatic environments at concentrations generally vary from 3 to 700 μg/L, and as high as 1.2 mg/L was found in freshwater & fishponds (Yan *et al.,* 2022; Ding *et al.,* 2021) also be detected in freshwater aquaculture products (Yan *et al.,* 2022) contaminated with domestic waste and combined with surfactants, which enhance its adherence to plant surfaces and cellular uptake (Giommi *et al.,* 2022). These commercial formulations, such as Roundup™, increase glyphosate's bioavailability in the environment, making it more potent and potentially more toxic to aquatic organisms (Braz-Mota *et al.,* 2015). Glyphosate's have high water solubility (12 g/L at 25°C) allows it to persist sin water bodies, where it industrial effluents (Dey *et al.,* 2016). When this contamination reaches beyond certain allowed concentrations it makes water pollution. These pollutants can be categorized into physical, chemical and biological parameters. Pesticides and/or herbicides that pollute water by runoff from agricultural land, direct applications such spray drift and aerial spraying or discharge as manufacturing industry effluents therefore they are potent biological toxins. It now poses a risk to aquatic organisms that are outside its intended targets, including shell and fin fish (Samanta *et al.,* 2016). In agriculture field are glyphosate, DDT, Cloroacetamide, Carboxamide mainly use.

Glyphosate (GLY) is the most frequently used broad-spectrum organo-phosphorus herbicide worldwide (Yan *et al.,* 2022). GLY is most extensively used in agriculture, some is also used pervasively for controlling submerged aquatic weeds and algae in ponds (Yan *et al.,* 2022). Glyphosate is in the toxicity class of III (on I to IV scale, where IV is least dangerous) for oral and inhalation exposure (Samanta *et al.,* 2016). In pure form glyphosate is a colorless, crystalline solid, yet it is most commonly applied as part of a water-soluble formulation

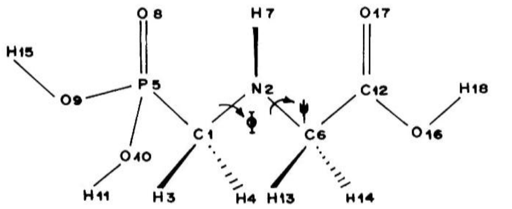


Fig. I: Chemical structure of glyphosate with central conformational angles (P. Kaliannan *et al.,*

2002).
Molecular structure of glyphosate is $C_3H_8NO_5P$. Molecular Weight- 169.07g/mol., H-bond donor count-4, H-bond acceptor count-6, Rotating bond count-4, This is canonicalized compound Glyphosate inhibits shikimate pathway found in plant, fungi and microorganism (karpiesiuk *et al.,* 2023). Several in vitro studies have shown that glyphosate is toxic to animal cells, such as mouse bone marrow cells, granulosa cells and placental cells (Qui *et al.,* 2020;).

Fishes are particularly sensitive to contamination Hence, these pollutants such as insecticides may significantly damage certain physiology and biochemical processes. The different kinds of insecticides can cause serious impairment to physiological and health status of fishes (Sabra and Mehana 2015). The intestine of *Clarias gariepinus* is crucial in toxicological and histological studies due to its sensitivity to pollutants. It absorbs toxicants, helping study bioaccumulation and the effects of pollutants like herbicide (Van Dyk *et al.,* (2009). Histopathological biomarkers are used extensively for documenting and quantifying exposure level, as well as expressing the effects of environmental stressors. A number of studies on histopathological biomarkers in the evaluation of fish health exposed to contaminants (Samanta *et al.,* 2016). The major advantages of using histopathological biomarkers in environmental monitoring are that they allow the investigation of specific target organ toxicity including stomach, intestine etc. (Samanta *et al.,* 2016).

II. MATERIALS AND METHODS

A. Collection and maintenance of fish

The freshwater African catfish, *Clarias gariepinus*, was selected for the present study because of its availability from local market and its convenient size. The body weight of fish ranged between 150-300 gm and their length varied between 25-35 cm. The fish were maintained in glass aquarium containing 30 lit of tap water, under normal conditions of light and temperature. The fishes fed with minced goat liver every alternate day and water was changed at the interval of one day. The fish were acclimatized for 10 days by keeping 6 fishes (3 male and 3 female) in each aquarium prior to their use in the experiment. The toxicant used in this experiment, glyphosate (RoundUp), was purchased from a nearby agricultural market and diluted in accordance with the dose determined using LC50 values that had previously been collected from a number of research papers (Samanta *et al.,* 2018 and Topal *et al.,* 2015).

B. Experimental setup

The three aquaria were taken filled with 30 lit tap waters. In each aquarium 3 male and 3 female fishes were kept. The fishes in the aquarium were grouped into control and experimental group and labelled them accordingly. The experimental fishes were treated by adding 10mg /liter (High dose) and 5mg/liter (Low dose) of glyphosate in the aquarium water for 10 days.

C. Histological study of Intestine

After completion of duration of experiment, the fishes were anesthetized with 2-phenoxyethanol to dissect out the intestine. After dissection, intestine was cut into small pieces and fixed in alcoholic Bouin's fluid for 24 hours. Intestine fixed in Bouin's fluid was transferred to 70% alcohol for 24 hours. Dehydration was carried out in 90% alcohol, absolute alcohol, cleared in xylene and embedded in paraffin wax. Blocks were made and cut at 7- micron thickness with the help of rotary microtome at 35ºC room temperature. The sections of intestine were spread on slides and then the slides were stained by using HE double staining method for histological observations56

The sections of intestine were deparaffinized in xylene and were passed through descending grades of alcohol. The sections were stained in hematoxylin and were kept in running water for some time. 1% HCl were used to remove the excess stain if tissues get over stained and then stained with eosin for 2-3 min. They were then differentiated in 90% alcohol and dehydrated in absolute alcohol. Then it was cleared in xylene and mounted in DPX. The slides were then observed under microscope at various magnifications (100X and 400X).

D. Study of biochemical techniques

Preparation of the tissue extract for biochemical estimation

After completion of duration of exposure, the surviving fishes from both control and experimental group were anesthetized and intestine dissected out. Dissected intestines were washed in PBS saline solution (PBS;1X, pH 7.4). After washing intestine shocked in tissues paper and 100mg tissue were homogenized in 1ml PBS saline solution (100mg/ml) by using mortar and pestle. Homogenized tissue then centrifuged at 3000 RPM for 15min twice to get extract. The obtained extracts were kept at -20º till further use for the biochemical estimation of carbohydrate and protein. Prior to use, the extracts were allowed to thaw at room temperature and after thawing the extracts were used for the biochemical estimation of various biomolecules. Following methods were used for the biochemical estimation of various biomolecules.

E. Estimation of Protein by Lowry's method

The total protein concentration in intestine was estimated by Lowry *et al.,* method (1951). The standard protein solution was prepared by dissolving 5 mg of standard protein bovine serum albumin in 1 ml of distilled water. The blank and unknown tube contained 4 ml of distilled water and 4 ml of tissue extract respectively. To each test tube, 5.5ml of reagent (50ml of 2% sodium carbonate in 0.1N NaOH + 1ml of 0.5% copper sulphate solution in 1 % sodium potassium tartarate solution) was added and kept undisturbed for 10-15 minutes. To each test tube, 0.5 ml of folio-ciocaltteau reagent with equal amount of water was added with vigorous shaking and was kept for 30 minutes. The colour intensity with blue color was observed at 650 nm on the spectrophotometer, (Visiscan Spectrophotometer (Type: 167). The standard graph with four known tubes of bovine serum albumin, and finally, the actual amount of protein was determined from the extracted samples.

F. Estimation of Carbohydrates by Dubois method

The total carbohydrate concentration in intestine was estimated by Dubois *et al.,* method (1956). The standard carbohydrate solution was prepared by dissolving 1 mg of sugar in 6 ml of distilled water. The solution was assisted by adding distilled water (0.001mg/ml). The blank and unknown tubes contained 1 ml of distilled water and 1 ml of tissue extract (0.2 ml extract + 0.8 ml distilled water) respectively.

Now, 1 ml of 5% phenol reagent and 5 ml of concentrated sulphuric acid was added since the heat required for color development was provided by exothermic reaction of sulphuric acid and water, it was desirable to add the acid from a fast-flowing pipette (5-10 seconds employing time directly on to the surface of the water layer) to all the tubes and kept undisturbed for 30 minutes. The colour intensity with yellowish brown colour was observed at 490 nm on the spectrophotometer, (Visiscan Spectrophotometer (Type: 167). The standard graph was drowned for five known tubes of sugar, and finally the actual amount of carbohydrate was determined from the extracted samples.

G. Statistical Analysis

The observational data for all parameters were given as individual values and as mean ± standard error (SE) and differences were significant at $p<0.05$. Statistical analysis was done using

III. RESULTS

A. Effect of glyphosate on pyloric intestine somatic index of fish

In the present study sex specific alterations in the pyloric somatic index in the glyphosate exposed fish. In male pyloric somatic index decreases significantly whereas in female pyloric somatic index increases as compared to control (Fig.II). The somatic index of male fishes from control group showed 1.50±0.02 whereas the somatic index of male fish exposed to 5mg/Lit and 10mg/Lit glyphosate showed 1.22±0.02 and 1.14±0.01 mg/100mg respectively (Table-I). Similarly, the pyloric somatic index of female fishes from control group showed 0.92±0.01 whereas the somatic index of female fish exposed to 5mg/Lit and 10mg/Lit glyphosate showed 0.99±0.02 and 1.52±0.03 respectively (Table -I).

Table. I Showing the effect of glyphosate on pyloric intestine somatic index of African catfish, *Clarias gariepinus* (Values are expressed in Mean ±SE) N = 06 for each group p<0.05*, p<0.01**, p<0.001***)

Groups / Sex	Control	5mg/L Malathion	10mg/L Malathion
Male	1.50±0.02	1.22±0.02***	1.14±0.01***
Female	0.92±0.01	0.99±0.02***	1.52±0.03***

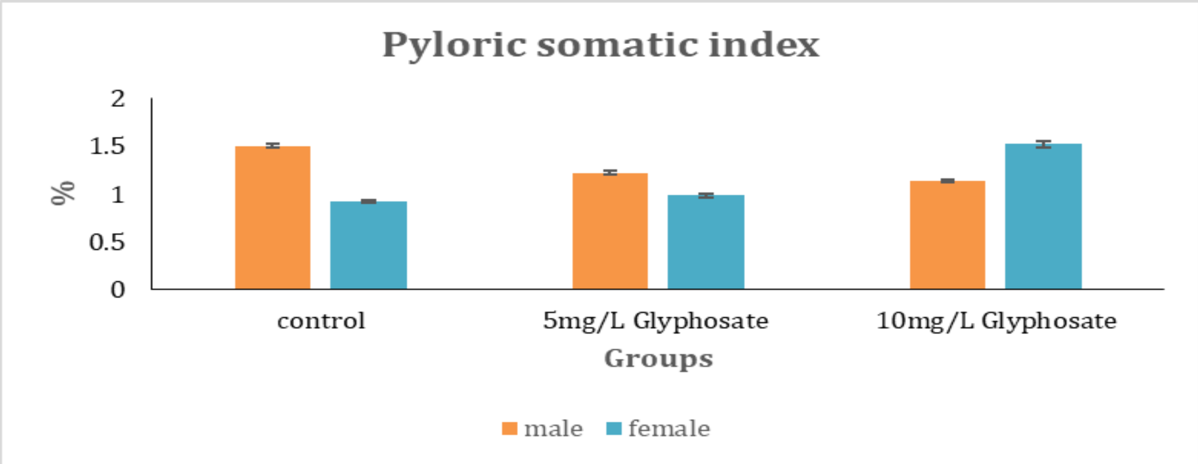


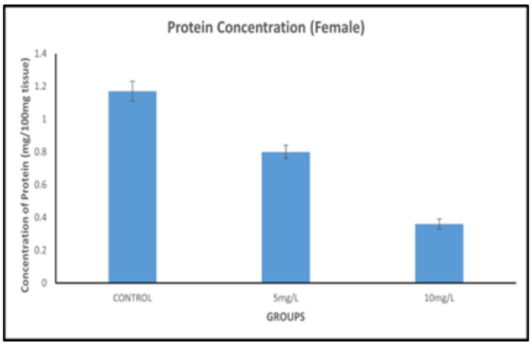
Fig II. Showing effect of the glyphosate on the pyloric somatic index of male and female African catfish *Clarias gariepinus*

B. Effect of the Glyphosate on the protein concentration in Intestine

The concentration of protein in Intestine of fishes from experimental group exposed to glyphosate showed the marked decreased in the protein concentration as compared to control group (Fig. III and IV). The protein concentration of male fishes from control group showed 0.72±0.05 mg/100mg, Similarly, the protein concentration of female fishes from control group showed 1.17±0.06 mg/100mg, whereas the protein concentration of female fish exposed to 5mg/L and 10mg/L Glyphosate showed 0.80±0.04 mg/100mg and 0.36±0.03 mg/100mg respectively (Table.4.2), whereas the protein concentration of male fish exposed to 5mg/L and 10mg/L Glyphosate showed 0.40±0.03 mg/100mg and 0.25±0.02 mg/100mg respectively (Table.II).

Table II. Showing effect of the Glyphosate on the protein concentrations in glyphosate of African catfish, *C. gariepinus*

Groups / **Sex**	Control	5mg/L Glyphosate	10mg/L Glyphosate
Male	0.72±0.05	0.40±0.03***	0.25±0.02***
Female	1.17±0.06	0.80±0.04***	0.36±0.03***



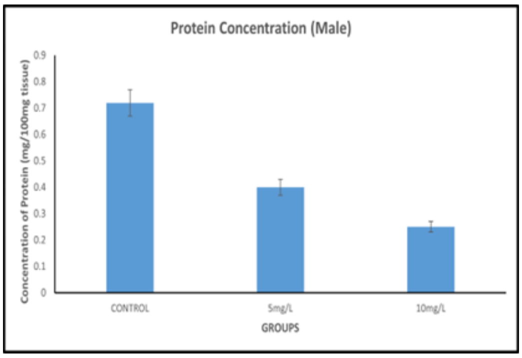


Fig III and IV- Showing effect of the glyphosate on the protein concentrations in intestine of male and female African catfish Clarias *gariepinus.*

C. Effect of Glyphosate on carbohydrate concentration in Intestine

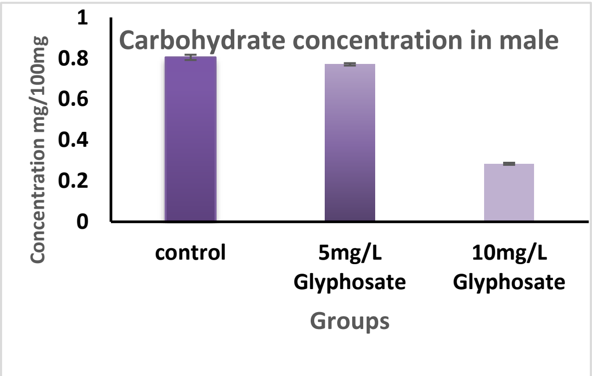
The concentration of carbohydrate in Intestine of fishes from experimental group exposed to glyphosate showed the marked decreased in the carbohydrate concentration as compared to control group (Fig. V and VI). The carbohydrate concentration of male fishes from control group

showed 0.72±0.01 mg/100mg,
whereas the carbohydrate mg/100mg whereas the carbohydrate
concentration of female fish exposed to 5mg/L and 10mg/L Glyphosate showed 0.66±0.01 mg/100mg and 0.32±0.00 mg/100mg respectively (Table.III).

Similarly, the carbohydrate concentration of female fishes from control group showed 1.16±0.00 mg/100mg and 0.28±0.00 mg/100mg respectively (Table.III). concentration of male fish exposed to 5mg/L an10mg/L Glyphosate showed 0.72±0.01

Table III. Showing effect of the Glyphosate on the carbohydrate concentrations in liver of African catfish, *C. gariepinus*

Groups / Sex	Control	5mg/L Glyphosate	10mg/L Glyphosate
Male	0.72±0.01	0.77±0.00**	0.28±0.00***
Female	1.16±0.00	0.66±0.01***	0.32±0.00***



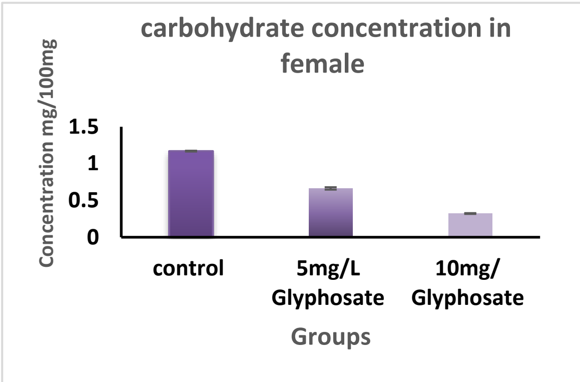


Fig. V and VI Showing effect of the glyphosate on the Carbohydrate concentrations in intestine of male and female African catfish *Clarias gariepinus*

D. Effect of glyphosate on histological structure of Intestine

The intestine of control group of fish *Clarias gariepinus* showed normal structure with four layers viz., serosa, submucosa, muscularis and mucosa. The normal structure of intestine consists of intestinal gland, lamina propria, mucus secreting goblet cells, Intestinal gland (Fig. VII). Histological examination of intestine of fish treated with 5mg/Lit glyphosate for 10 days showed toxic lesion and such lesions were increased with increase in the concentration of glyphosate. Hyperplasia of goblet cells, curving of intestinal villi, degeneration of serosa layer, disintegration in the tips of intestinal villi, cracked clay appearance, pyknosis, vacuolar hydropic degeneration of cells and dilation of lamina propria were found.

(Fig. VIII). Histological examination of intestine of fish treated with 10mg/Lit glyphosate for 10 days showed pyknosis, narrowing of muscularis layer and fusion of villi, reduction of lamina propria and distortion of enterocytes, degeneration of intestinal gland, loss of architecture and blunting of villi, reduction in goblet cells, pyknosis and degeneration of cuboidal epithelial layer (Fig. IX).

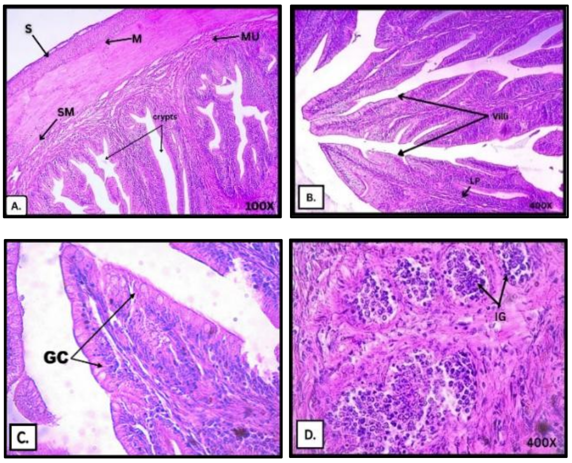


Fig. VII Microphotograph showing a histological structure of intestine of control group of African catfish, *Clarias gariepinus*

A) Intact serosa layer, muscularis, submucosal layer and crypts of Lieberkühn. HE. (100X). B) Villi and lamina propria of Intestine. HE. (400 X). C) Intestinal gland. HE. (400 X). D) Goblet cells. HE. (400 X). (S- Serosa. M- Mucosa. SM- Submucosa. MU- Muscularis. V- Villi, LP- Lamina propria, IG- Intestinal gland. GC- Goblet cells)

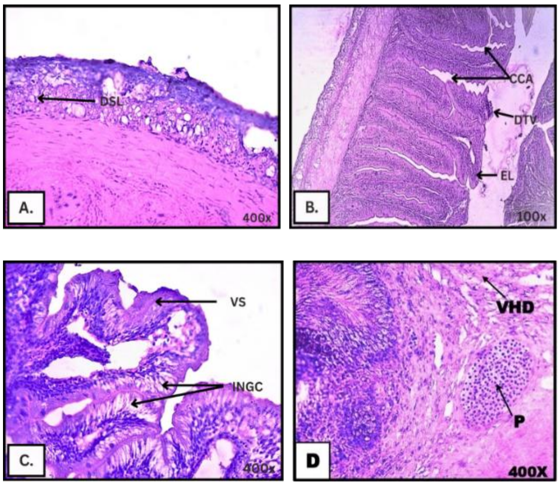


Fig.VIII Microphotograph showing a histological structure in the intestine of African catfish, *Clarias gariepinus* exposed to 5mg/L glyphosate.
A. Degeneration of serosa layer. HE. (400X), B. Disintegration in the tips of intestinal villi, Epithelium lifting and cracked clay appearance HE. (100 X). C. Villi shortening, hyperplasia of goblet cells. HE. (400 X). (P- Pyknosis, VS- Villi shortening, EL - Epithelium lifting , CCA-Cracked Clay Appearance, DTV- Disintegration in the tips of intestinal villi, DSL - Degeneration of Serosa layer, INGC-increase no. of goblet cell, VHD - Vacuolar Hydropic Degeneration of cells)

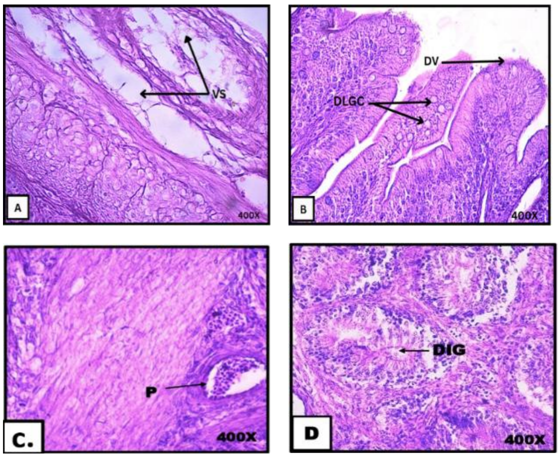


Fig. IX Microphotograph showing a histological structure in the intestine of African catfish, *Clarias gariepinus* exposed to 10mg/L glyphosate. A. Loss of architecture and blunting of villi HE. (100 X). B. Narrowing of muscularis layer and fusion of villi. HE. (100 X). C. Degeneration of intestinal gland HE. (400 X). D. Pyknosis. HE. (400 X). (P- Pyknosis, DV- Distortion of Villi, VS-vacuoles in serosal layer, LA- Loss of Architecture, DLGC- Decrease no. of goblet cell, DIG - Destruction of Intestinal Gland, DCE- Degeneration of Cuboidal Epithelial layer.

IV. DISCUSSION

Glyphosate, a widely used herbicide, has been recognized for its potential harmful effects on non-target aquatic organisms, including fish (Nwani et al., 2010; Ayoola, 2008). The findings of this study demonstrated that glyphosate exposure caused both histopathological and biochemical changes in the intestine, indicating significant toxic stress. Proteins play a vital role in the architecture and physiology of the cell. Catabolism of proteins and amino acids triggers in energy production in fish. They serve as both structural components and functional molecules (e.g., enzymes, receptors) (David et al. 2004).

The results of the biochemical analysis of protein and carbohydrates in the intestine of fish exposed to glyphosate showed marked decreased in in their content as compared to control. Similar findings were also reported by Samanta *et al.,* (2014), Sancho et al., (1998) and David et al. (2004). A reduction in protein content may indicate protein catabolism for energy under stress, reduced protein synthesis due to impaired ribosomal activity, or tissue degeneration (Ayoola, 2008). Muley *et al., (*2007) reported tissue specific increased in free amino acids and the decreased in total protein indicating that there is significant protein hydrolytic activity because of elevated protease activity in the intestine. Samanta et al., (2019) reported during glyphosate exposure, protease activity showed significant elevation in stomach, intestine and liver of fish due to higher metabolic pathways to meet high energy demand to compensate herbicidal stress. The decline in protein content could be associated with glyphosate induced alteration in the protease activity to fulfil the demand of energy in response stress caused by glyphosate. According to Samanta *et al.,* (2014), exposure to a sublethal dosage of a combination of glyphosate caused a decrease in total carbohydrate levels was observed in the intestinal tissues of glyphosate-treated fish compared to controls. This reduction may be attributed to increased glycolytic activity in response to stress, leading to rapid depletion of carbohydrate reserves for energy. Glyphosate-induced oxidative stress can also impair carbohydrate metabolism enzymes, resulting in inefficient glucose utilization and further depletion of energy stores.

In the present study, histological examination of intestine of fish exposed to glyphosate displayed various degenerative changes and severity of changes increases with increases with increase in concentration of glyphosate. Histological structure of intestine of fish exposed to low dose of glyphosate showed increase in no. of goblet cells, degeneration of serosa layer, disintegration in the tips of intestinal villi, cracked clay appearance, villi shortening, crypt damage, mild epithelial lifting, disorganization of intestinal mucosa whereas fish treated with a higher dose of glyphosate revealed loss of architecture, epithelial disruption, Severe villi erosion and nuclear pyknosis, distorted villus architecture, loss of the intestinal lining's epithelial cells, and a decrease in the size and number of goblet cells.

Similar results were also reported by Erhunmwunse *et al.,* (2014); Larsen *et al.,* (2012); Fathi *et al.,* (2020); Ayoola, (2008) have reported significant histopathological alterations in the intestine exposed to various toxicants. The findings also have resemblance to those of De Maria Serra *et al.,* (2021), who reported that severe dysplasia in small and large intestine, inflammatory infiltration, congestion and epithelial disruption. Samanta *et al.,* (2016) reported exposure to glyphosate resulted in vacuolization of enterocytes, detachment of epithelial cells, severe villi erosion and nuclear pyknosis. The distorted villus architecture, marked inflammatory infiltration, and severe epithelial necrosis are consistent with findings from other studies about the impact of various toxicants on fish intestines (Fathi *et al*., 2020). Such alterations suggest impaired nutrient absorption and weakened intestinal integrity, which can adversely affect the overall health and growth of the fish (Fanta et al., 2003).

Gul et al., (2004) suggested that glyphosate exposure leads to oxidative damage within intestinal tissues as they reported the elevation of malondialdehyde (MDA) levels, along with decreased activities of enzymes like superoxide dismutase (SOD) and catalase (CAT) in the intestinal tissues. Nwani et al., (2010); Ayoola, (2008) and Lushchak, (2011) also reported glyphosate-induced oxidative stress and tissue damage in various fish species.

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