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Histological Alterations in Some Organs of African Catfish (*Clarias gariepinus*) Exposed to Sub-lethal Concentrations of Glyphosate [N-(phosphonomethyl) glycine]

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ABSTRACT

This study used a static bioassay to investigate the histological effects of glyphosate on the gill, liver and muscle of African catfish (*Clarias gariepinus*) fingerlings. This was done with a view of further characterising the effect of glyphosate on *C. gariepinus* fingerlings and other aquatic life forms. Six-week old *C. gariepinus* fingerlings with an average weight of 10.02 ± 0.2 g were stocked into three exposure sets (control, 2.75 ppm (25% of the 96 h LC50 value) and 5.50 ppm (50% of the 96 h LC50 value)) in triplicate at 30 fish per tank for 70 days. The 96 h LC50 value was 11.00 mg/L. Histological examination of the *C. gariepinus* exposed to various sublethal concentrations of glyphosate showed that major histological changes in their organs were concentration dependent such as gill arch vacuolation, excessive mucosal secretions, lifting of epithelial, and epithelium thickening, hyperplasia and telangiectasis in the gills, discolouration, change in form and consistent alterations involving hyperplasia, narrowing of the central nerve, necrosis, pkynosis, blood congestion and vacuolation of the liver, mild hyperplasia and inflammatory responses in the muscle of the fish. The severity of histological alteration was more pronounced in fish organs exposed to 5.50 ppm of glyphosate concentration. This study concluded that the toxicant (glyphosate) is highly toxic to *C. gariepinus* particularly at a concentration of 5.50 ppm, therefore its use near farm lands or adjacent water bodies should be discouraged.

Keywords: Alterations, *Clarias gariepinus*, glyphosate, histology, organs

INTRODUCTION

The increasing intensification of agriculture practices with the use of agrochemical products, coupled with accidental spillage, careless handling and surface runoffs from point of application into adjacent waterbodies has led to increasingly contamination of waterbodies worldwide (Cavas and Konen, 2007; Ramirez-Duarte *et al*., 2008; Sabae *et al*., 2014; Thanomsit *et al*., 2016; Bawa *et al*., 2017; Samanta *et al*., 2018).

Glyphosate, N-(phosphonomethyl) glycine commonly called Roundup® became the most widely used herbicide due to its efficacy, cost effectiveness and non-persistence in the environment (Samanta *et al*., 2018). Glyphosate which has more than 24 days of half-life in vegetation, has been reported to have a half-life ranging between 9 and 91 days in aquatic environment (Giesy *et al*., 2000). Although, glyphosate is readily degradable in soil and water by microbes (Rueppel *et al*., 1977; Samanta *et al*., 2018) but because of its strong adsorptive nature, glyphosate has tendency of contaminating surface water rather than moving to the groundwater (Reuter, 2011).

The toxicity of glyphosate based herbicide in aquatic environment has been documented on aquatic organisms with lasting effects (Tomlin, 2006; Hued *et al*., 2012; Shitmae *et al*., 2013). Among the non-target aquatic organisms, fish has been the most reported organism that is highly sensitive to toxicity of various aquatic pollutants especially herbicides during their early life stages (Jiraungkorskul *et al*., 2003; Adedeji *et al*., 2008; Ladipo *et al*., 2011; Ayanda and Egbamuno, 2012; Bawa *et al*., 2017). However, other studies had shown the effects of glyphosate formulations on fish survival, growth

and reproduction of fish in the laboratory (Olurin *et al*., 2006; Ayoola, 2008b; Okayi *et al*., 2010; Ayanda and Egbamuno, 2012). According to WHO, toxicity of glyphosate to organism varies depending on test organism and test condition.

Due to the toxicity nature of these chemicals, there is a need to understand in depth the damage this chemical can cause at cellular and subcellular level. Therefore, histological damage of the gills, liver and muscle are highly considered sensitive for detecting such potential adverse effects of pollutant damage due to over time bioaccumulation, because of direct exposure, respiration, biotransformation and xenobiotic metabolism respectively (Gernhofer *et al*., 2001; Mohamed, 2009; Bawa *et al*., 2017).

African catfish (*Clarias gariepinus*) was considered as test fish for toxicity study because of their hardness, fast growth rate, ability to adapt in extreme environmental conditions and good fish of high economic value in Africa (Adewumi and Olaleye, 2005). Also, being a bottom dweller where the name "mudfish" was derived, they can accumulate the pollutant chemicals in the aquatic environment overtime. Therefore, this study aimed to determining the sub-lethal toxicity of glyphosate, N-(phosphonomethyl) glycine and its effect on the histology of muscle, gills and liver of African catfish (*C. gariepinus*) under a laboratory environment.

MATERIALS AND METHODS

Range Finding and Acute Toxicity Test

The concentrations of the glyphosate used for the range finding test followed the methods described by Reish and Oshida (1986) and (Obuotor, 2004). From 11.1 ml of Round-up®, 4 g/L of glyphosate was prepared with proper dilution to obtain five different logarithmic concentrations (4, 0.4, 0.04, 0.004, and 0.0004 g/L) of glyphosate in a well labelled tank A – E respectively. Control tank, which was without glyphosate, was labelled as F. The test fish were exposed into the different glyphosate concentrations at 10 fingerlings per treatment in triplicate. Fish mortality was observed after 1 h, 3, 6, 12, 48, 72 and 96 h of exposure (Reich and Oshida, 1986). Toxicity range value was then estimated from the probit analysis and Spearman–Karber method of estimating mortality results (Carter *et al*., 2000).

Acute Toxicity Testing

Eight graded concentrations (1.00, 4.00, 8.00, 12.00, 16.00, 20.00, 24.00 and 400.00 ppm) of commercial glyphosate formulation (Roundup®) which was used for 96 h acute toxicity bioassay in a static exposure system (Obuotor, 2004) were prepared from the result obtained from the range finding tests. Ten randomly selected fingerlings were exposed to each test concentration in triplicates. Mortality was monitored and recorded in each of the exposure tank after 1 h, 3, 6, 12, 24, 48, 72 and 96 h. Data collected were analysed using Trimmed Spearman Karber method to determine LC50 value at 96 h.

Chronic Toxicity Test

Six weeks old African catfish (*C. gariepinus*) fingerlings with an average weight of 10.02 ± 0.2 g were obtained from Prime Aquaculture Ltd., Ikorodu, Lagos. The fishes were brought to the Fish Culture Laboratory, Department of Zoology, Obafemi Awolowo University, Ile-Ife, acclimatized for 14 days in an aerated in a glass tank of 150 L. The stocked fingerlings were fed with 2 mm Coppen's® feed (45% crude protein) at 4% their body weight twice a day. After acclimatization, the fish fingerlings randomly selected were carefully introduced into three exposure sets (control, 2.75 ppm (25% of the 96 h LC50 value) and 5.50 ppm (50% of the 96 h LC50 value)) in triplicate at 30 fish per tank of 40 litres capacity for 70 days. During these periods, the experimental test fingerlings were fed with Coppen's ® feed at 4% body weight. The test solution in each tank was renewed every 72 h with freshly prepared solutions. During the experiment, the water pH, temperature and dissolved oxygen (D.O.) measured *in situ* using a portable pH meter (Model with resolution 0.01 pH and accuracy of ± 0.05 pH), mercury in glass thermometer and Milkawauke D.O. meter respectively had average values of 25.7 ± 0.1 ℃, 6.79 ± 0.06 and 6.22 ± 0.17 mg/L also respectively.

Sample Collection

At the end of experiment after 70 days, five fish each were collected from the treatments. The collected fish were euthanized with 150 mg/l

tricane methanesulphonate (MSS-22, sigma) then the muscle, gill and liver of the fish were dissected out, and the collected tissues were fixed in 10% formalin.

Histological Examinations

Persevered gills, muscles and liver samples (preserved in 10% formalin for about 24 h at 4 ^{o}C) which were dehydrated in sequential order of alcohol 70, 80, 90 and 100% respectively for 30 minutes were then cleared in xylene, infiltrated with paraffin at 56 ^{o}C, and embedded in paraffin wax. Each of the selected tissues were then sectioned at 6-7 µm by means of a rotatory microtome (Model 157 B and H), dehydrated and stained with heamatoxylin and counterstained with eosin. The sections were then examined under a microscope (model 4551 cutting Acces) which has photographic attachment used for photomicrograph. The slides photomicrographs were then used to describe and compare the appearance, histological structure, physiological condition, and arrangement of the tissues from in the exposed tanks and the control according to Luna (1968); Olurin *et al*. (2006) and (Ogundiran *et al*., 2009).

Statistical Analysis

Toxicity range value was estimated from the Probit analysis and Spearman – Karber method of estimating mortality results (Carter *et al*., 2012).

RESULTS

Toxicity Indices

The lethal concentrations were calculated using both the probit method and the Spearman-Karber method (Table 1) giving an ppm and 20.00 ppm for the Probit and Spearman-Karber method respectively. The LC90 of the acute toxicity test was 58.00 ppm while the 95% confidence lower and upper limits were 30.00 ppm and 340.00 ppm respectively. The No Observable Effect Concentration was 2.00 ppm with 95% lower and upper confidence limits of 0.00 ppm and LC50 values of 11.00 ppm and 10.00 ppm, respectively with a corresponding 95% lower confidence limit of 7.00 ppm and 10.00 ppm. The 95% upper confidence limit was recorded as 17.00 4.00 ppm µg/l respectively. The inhibition concentration (IC25) was seen to be 1.00 ppm. The 95% lower confidence limit was 0.00 ppm while the 95% upper confidence limit was calculated as 3.00 ppm. The observed behavior of the exposed fish at different concentration include: imbalance and incoherent coordination, erratic swimming, gulping of air and restlessness.

Table 1. Toxicity indices for acute toxicity test

Index	Concentration (ppm)	95% LCL (ppm)	95% UCL (ppm)
LC50	11.00	7.00	17.00
LC90	58.00	30.00	340.00
NOEC	2.00	0.00	4.00
IC25	1.00	0.00	3.00

LC50- Lethal concentration that killed 50% of the test fish
LC90- Lethal concentration that killed 90% of the test fish
NOEC- No observable effect concentration
IC25- Inhibition concentration with 25% reduction in growth
LCL- Lower confidence limit
UCL- Upper confidence limit

Histological Changes in the Gills

The control fish gills appeared normal in form and consistency during gross examination with a bright red colour. Slide photomicrograph examinations revealed normal structure of the gill arch showing blood spaces in the lamellae. Primary lamellae appeared fine and distinct with no signs of epithelial lifting, no signs of inflammation, cellular hypertrophy and hyperplasia. The base of primary lamellae showed cells of similar size which were sparsely distributed evenly. Normal blood space congestion was observed in one of the lamellae as show in Figure 1.

There were observable changes in the gill tissues of fish following treatments with glyphosate during the experiment showing histological alterations. Gross examination of gill tissues of fish in Treatment A (2.75 ppm) showed a fairly firm consistency and bright reddish colour comparable to that of the control (Figure 2). Histological examination showed gill arch vacuolation, thickening of the primary lamella, which became blunt and not finely distinct with signs of mucosal secretions, slight epithelial lifting were seen hyperplasia (Figure 2 (b)). Severe diapedesis and blood congestion were also visible in the lamella (Figure 2 (a), (b)). Lamella base were observed to have a thickened denser cellular network compared to the control group suggesting increase in cells and mucous productions with an area of inflammation (Figure 2 (c), (d)).

Gill tissues in Treatment B (5.50 ppm) showed a severe alteration in the gill histology. Gross examination of the tissue showed colour changes from light red to brown colour. Microscopic examination of the tissue revealed severe damage of the gills due to glyphosate exposure. Severe cellular erosion was seen in the gill tissues, lamella rupture was conspicuously obvious, epithelial erosion and cellular hypertrophy were also observed in lamella base (Figure 3 (a), (b), (c), (d)).

Histological Changes in the Liver

The liver tissues of fish in the control group showed no conspicuous alteration in the cells as shown in Figure 4. The cells appear normal with a large spherical central vein. Hepactic sinusoides appear firm and free of fluid congestions with high vascularisation and haemorrhage around the spheres of the central nerve. Hepatocytes appear firm with a darkly stained nucleus and nucleolus. Glycogen vacoules were visible as spherical bright spaces. Amongst the control group liver tissues, there were no signs of necrosis, cellular degeneration, pyknosis, hypertrophy, hyperplasis and necrosis. However, the sinoides were highly vascularised with blood concentrated around the central vein. Liver tissues of fish exposed to glyphosate showed alterations in tissue form and structure. Gross examination revealed brownish-yellowish colourations of the liver lobes. Histological examination of fish in Treatment A (2.75 ppm) revealed higher vascularisation of the sinusoids supplying the central bile duct with evidence of hypatocytes hyperplasia and telangiectasis (Plate 5 (a), (b)). Mild pyknosis were observed suggesting cell deaths and degeneration although this was minimal in the Treatment B (5.50 ppm). Blood congestion and vasodilation was seen in the liver tissues along the sinusoides which is as a result of increased liver function. The form and consistency of the bile duct was also observed to be distorted and narrowing (Figure 6 (a), (b)).

Histological Changes in the Muscles

The fish muscles showed no serious distortion. Both white muscle and red muscle of the fish were observed to be show normal histological structures with a revealing striated muscle cells (Figure 7a). The cells possessed a darkly stained nucleus. No severe histological distortions were observed in the tissues of all treatments unless an area of suspected inflammation.

Treatment A (2.75 ppm) had its muscle tissues just like the control group with only signs of mild cellular hyperplasia, nevertheless, the structure appeared just like the Control (Figure 7b). However, treatment with the highest glyphosate concentration (5.50 ppm) showed signs of mild inflammation and cellular distortions with likely hyperplasia (Figure 7 (c)).

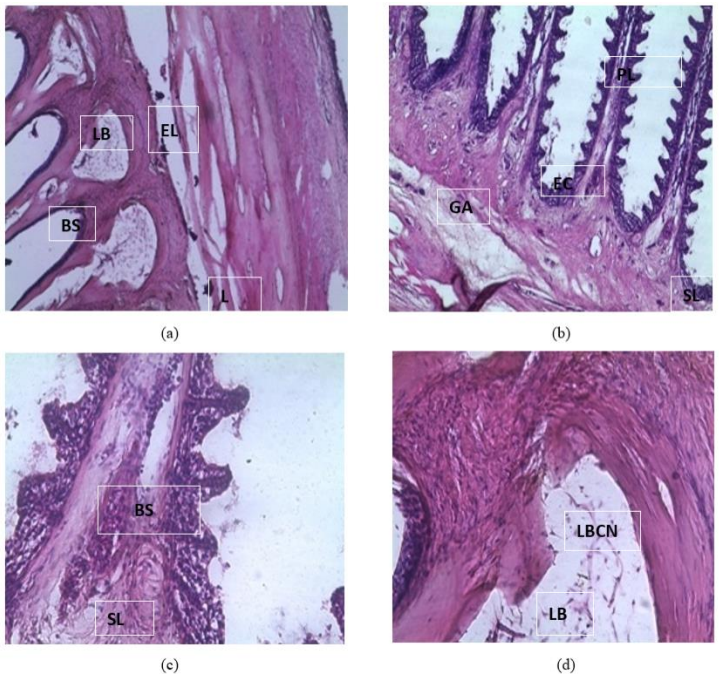


Figure 1. Histological photomicrographs of the gill tissues of *C. gariepinus* under control condition showing normal (a) LB-lamella base, EL-epithelial lifting, L-lamella, BS-blood spaces, (X100) (b) PL-primary lamella, GA-gill arch, EC-erythrocyte cell, SL-secondary lamella, (X100) (c) BS-blood spaces, SL-secondary lamella, (X400) and (d) LBCN-lamella base cell network, LB-lamella base, (X400)

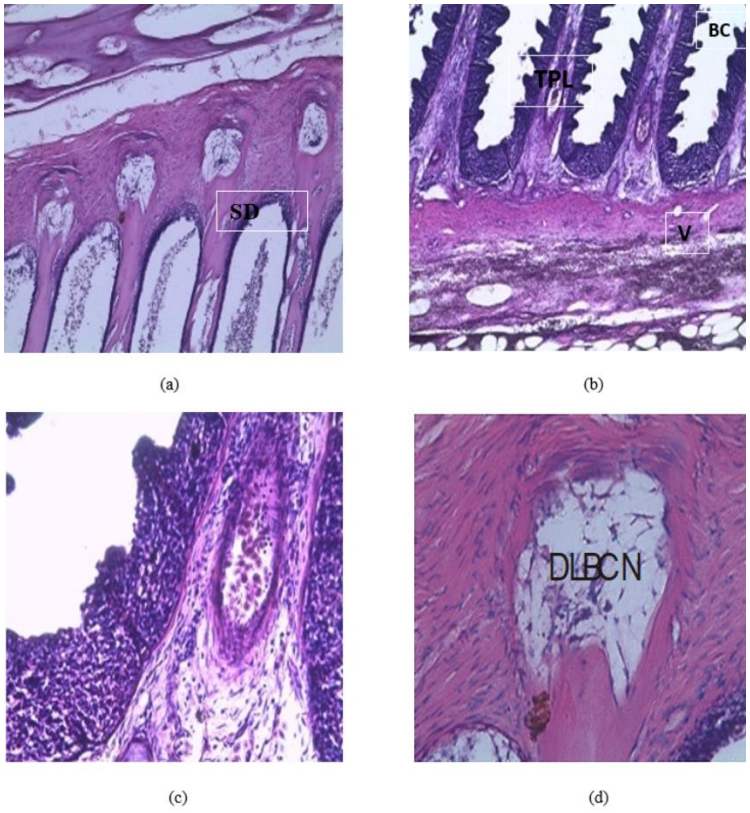


Figure 2. Histological photomicrographs of the gill tissues of *C. gariepinus* exposed to 2.75 ppm concentration of glyphosate showing (a) SD- severe diapedesis, (X100) (b) BC- blood congestion, V-vacoulation, (X100) (c) TPL-thickened primary lamella, BC-blood congestion, CHP-cellular hyperplasia, (X400) and (d) DLBCN-dense lamella base cell network (X400)

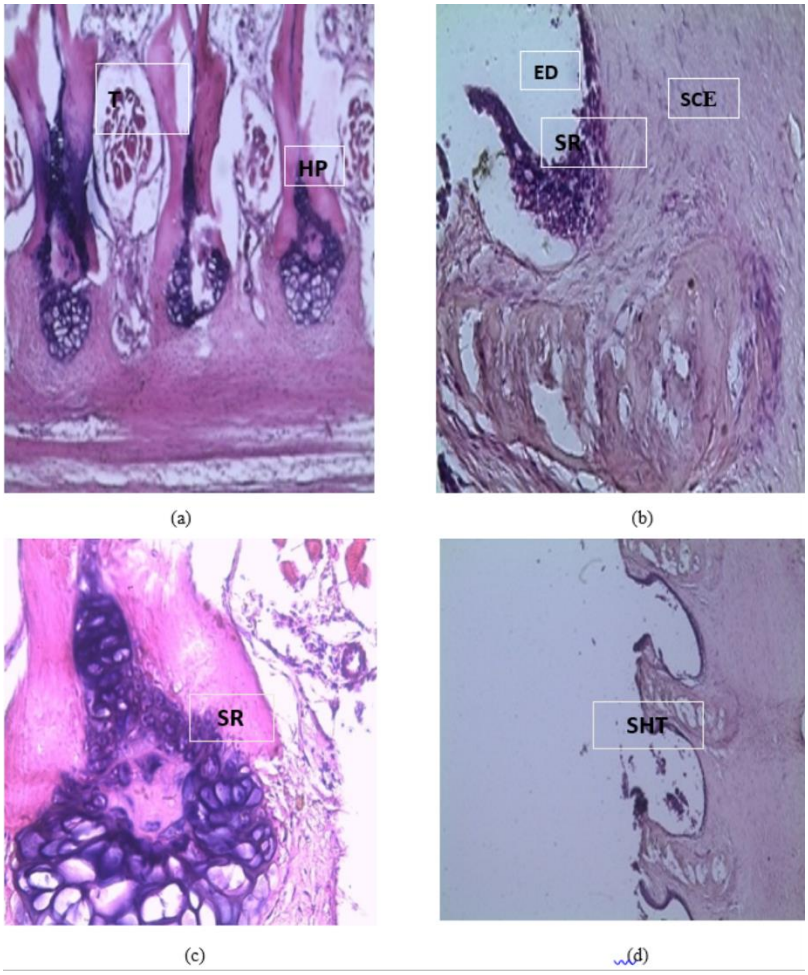


Figure 3. Histological photomicrographs of the gill tissues of *C. gariepinus* exposed to 5.50 ppm concentration of glyphosate showing (a) T-telangiectasis, (X100) (b) ED-epithelial degeneration, SR-severe rupture, SCE-severe cellular erosion, (X100) (c) SR-severe rupture, (X400) and (d) SHT-severe hypertrophy, (X400)

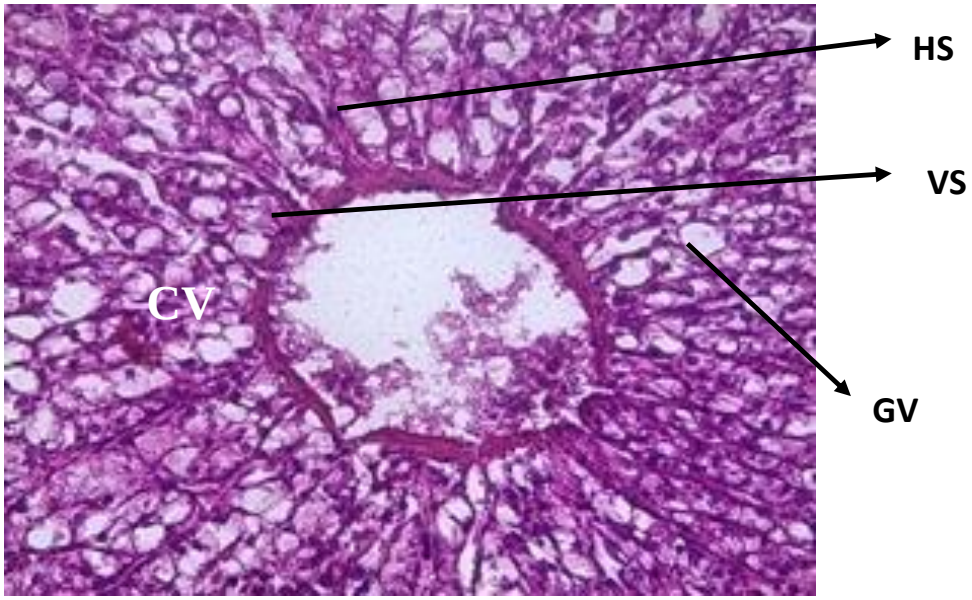
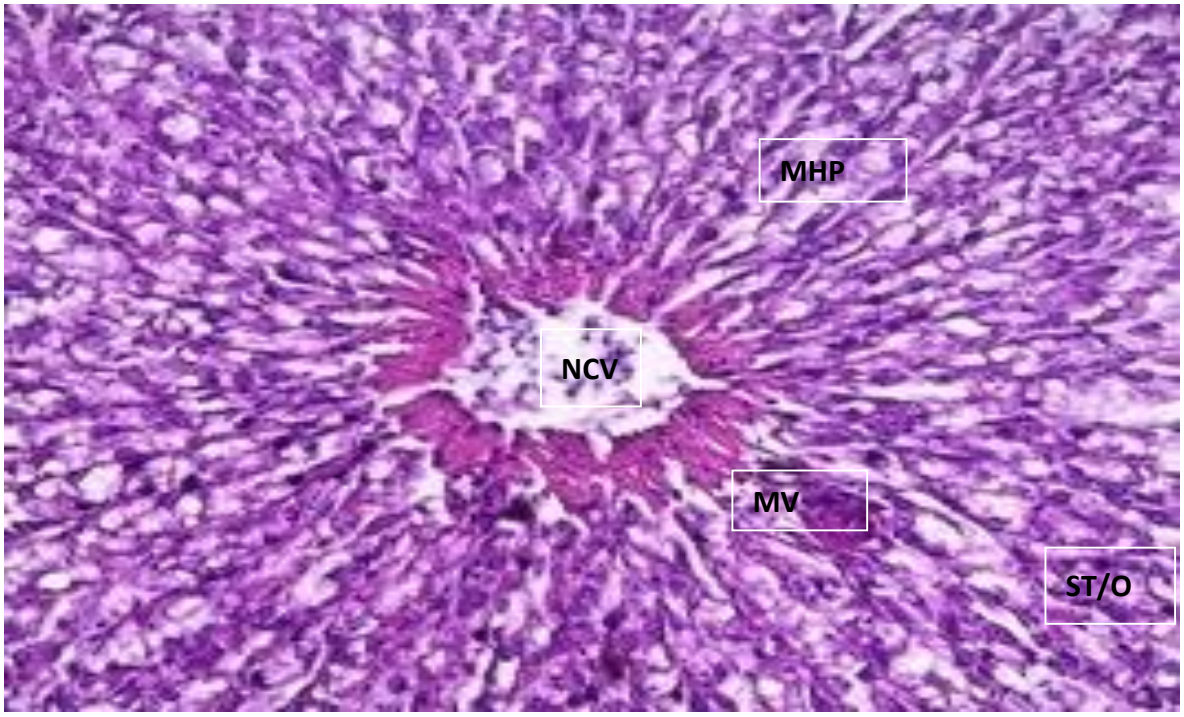
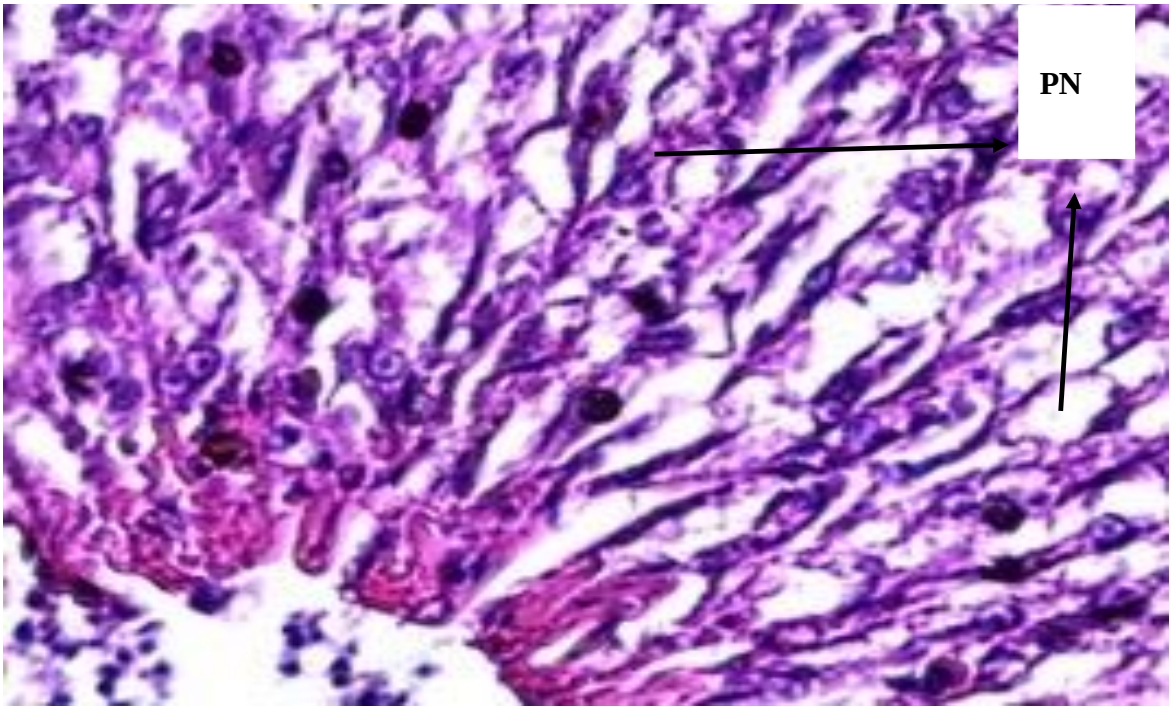


Figure 4. Histological photomicrographs of the liver section of *C. gariepinus* under control treatment showing HS-hepactic sinusoides; VS-vascularised sinusoides; CV-central vein; GV-glycogen vacuole (X400)

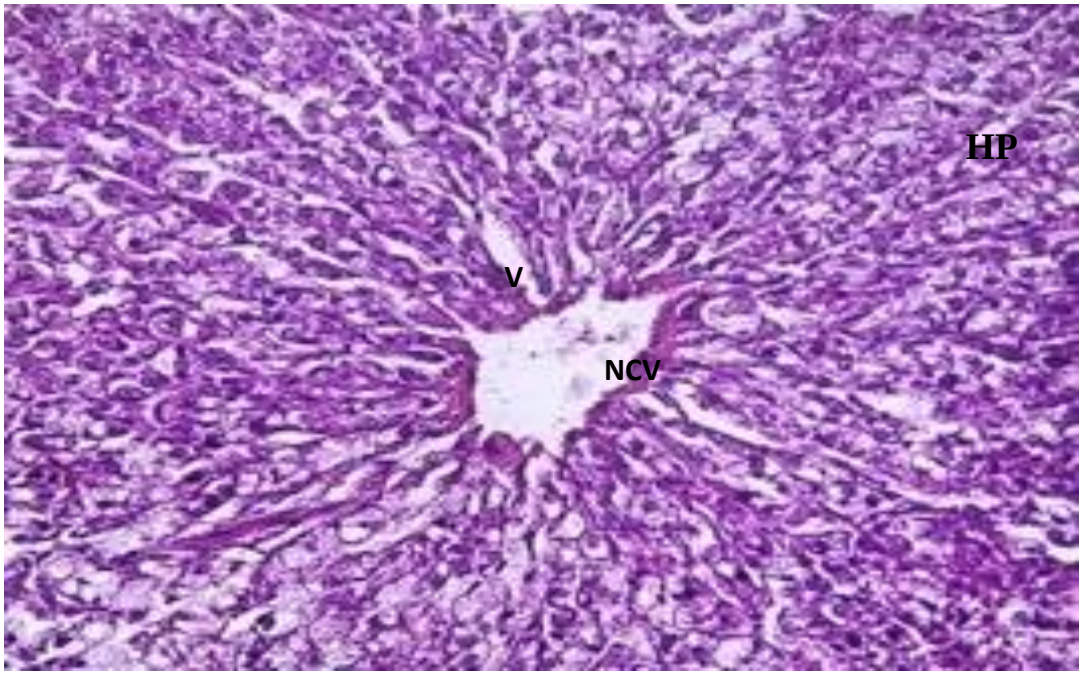


(a)

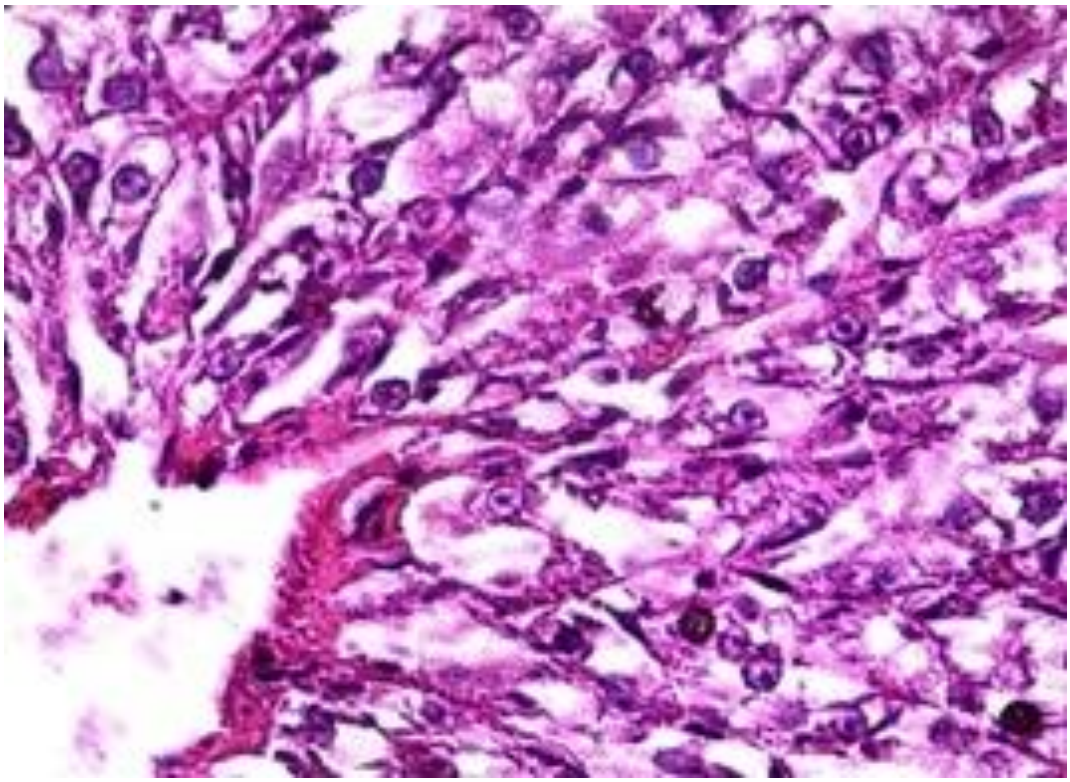


(b)

Figure 5. Histological photomicrographs of the liver section of *C. gariepinus* exposed to 2.75 ppm concentration of glyphosate showing (a) MHP-mild hyperplasia; NCV-narrowing of the central nerve; ST/O-sinusoidal telangiectasis/oedema, (X400) and (b) PN-pyknotic nuclei, MV- mild vacuolisation (X1000)

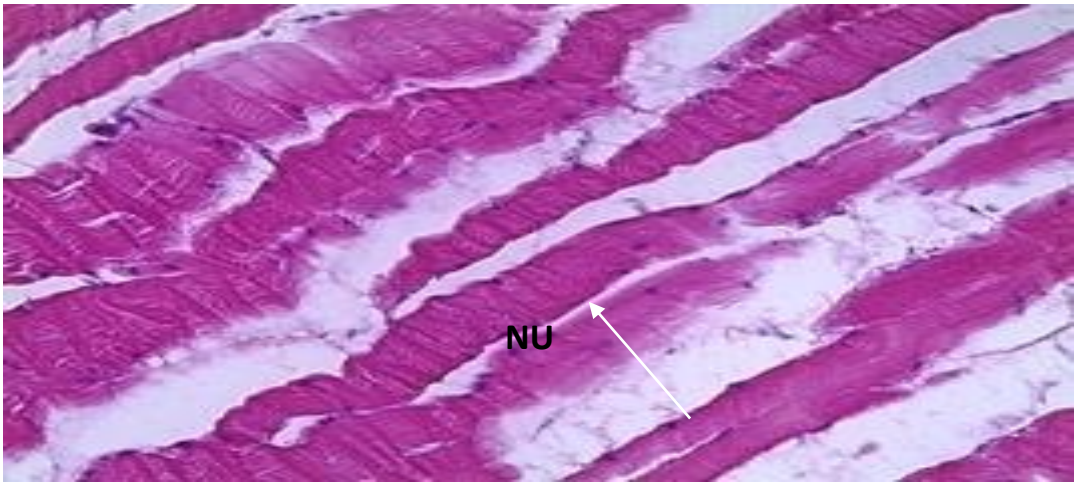


(a)

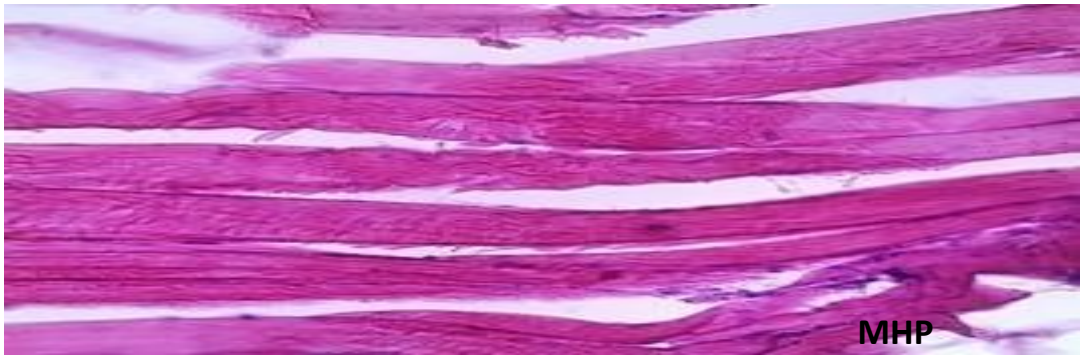


(b)

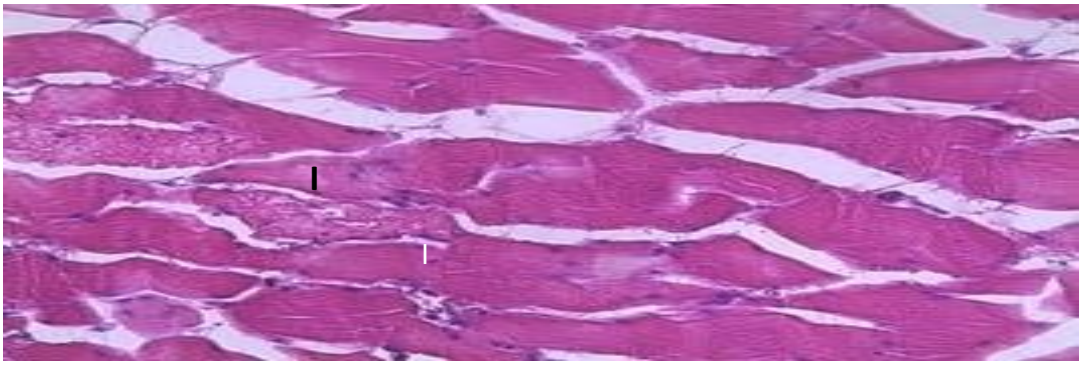
Figure 6. Histological photomicrographs of the liver section of *C. gariepinus* exposed to 5.50 ppm concentration of glyphosate showing (a) HP-hyperplasia; NCV-narrowing of central vein, V-vacuolation, (X400) and (b) VD-vasodilatation; N-necrosis; BC-blood congestion (X1000)



(a)



(b)



(c)

Figure 7. Histological photomicrographs of the muscle section of *C. gariepinus* under (a) control treatment, Treatment A (2.75 ppm) showing normal muscle cell structure revealing the striated muscle cells with nucleus (X400) (b) Treatment A (2.75v ppm) showing similar muscle cells with fish in the control with signs of mild cellular hyperplasia (X400) and (c) Treatment B (5.5 ppm) showing signs of mild inflammation and cellular distortions (X400)

DISCUSSION

The Probit and Spearman-Karber LC50 values were 11.00 ppm and 10 ppm respectively. This value was above the 96 h LC50 value of glyphosate herbicide of 0.04 ppm reported by Ayoola (2008b) and above the 1.8 ppm reported by Okayi (2010) in a toxicity test of glyphosate to juvenile *C. gariepinus* and with a corresponding 95% lower confidence limit of 7.00 ppm and 10.00 ppm. The 95% upper confidence limit was recorded as 17.00 ppm and 20.00 ppm for the Probit and Spearman-Karber method respectively.

The result of LC50 value of the acute toxicity test in this study revealed that *C. gariepinus* showed a higher sensitivity to glyphosate when compared to the Tilapia fish,

Oreochromis niloticus which recorded an LC50 value of 1.05 mg/l in a 96 h acute toxicity test (Ayoola, 2008a) but was however lower in comparison with *Cachama blanca* (*Piaractus brachypomus*) which can tolerate glyphosate more at LC50 value of 97.47 mg/L in acute toxicity test. This observation was in consonance with earlier reports on the increasing toxicity with increased concentrations of xenobiotics (Ayoola, 2008b; Ogundiran *et al*., 2009; Hadi and Alwan, 2012; Selvanathan, 2013). Bioaccumulation rate, organism responses, and reaction of different compound of the pesticide composition determines the toxicity level of any pesticide (Neibor and Richardson, 1980). Folmar *et al*. (1979) also reported acute toxicity of glyphosate in fish as a consequence of the POEMA surfactant.

Normal fish behavior observed in the control group with incoherent coordination and balance, erratic swimming, gasping for air and restlessness observed in the exposed treatments was also reported by Bawa *et al*. (2017) and Samanta *et al*. (2018) on histological alteration of *Cyprinus carpio* and *Oreochromis niloticus*, respectively exposed to glyphosate. This behavior could be attributed to respiratory impairment, nerve and or brain damage, probably because toxicant glyphosate that is present water affects the gills which are the first point of contact.

The histological changes in fish are important aspect in substance toxicology to understand the extent to which changes in the structural organisation are occurring in the organs due to environmental pollution (Myers *et al*., 1998; Ramirez-Duarte *et al*., 2008; Samanta *et al*., 2018). Various researchers had studied and reported environmental pollution and its effects on aquatic animals of economic importance. Study on effect of glyphosate on fish histology is gaining popularity due to its significance in toxicology (Olurin *et al*., 2006; Ayoola, 2008b; Ayoola and Egbamuno, 2012). Selvanathan *et al*. (2013) studied histopathology changes in *C. batrachus* exposed to mercury and cadmium while Hadi and Alwan (2012) assessed the changes in the histology of *Tilapia zillii*'s gills, liver and kidney. Histological changes in the kidney, liver, and gill of carps exposed to sublethal toxicity of glyphosate concentration was also researched by Neskovic *et al*. (1996). Similarly, Ayoola (2008b) carried out a study of glyphosate on histology of *C. gariepinus* and *T. zillii* in a short toxicity test. In this study, the histological changes in the muscles, gills, and liver of *C. gariepinus* juveniles were examined after a 70 days of exposure to sublethal concentrations of glyphosate.

Fish gills are the principal organ of gaseous exchange between the fish and the water environment and a direct opening between the internal environment and the external environment (Sullivan and Somero, 1993). Histological alterations in several fish gills as a result of direct and indirect exposure to pesticides and various contaminants have been documented by many authors (Mallat, 1985; Richmonds and Dutta, 1989; Ayoola, 2008a; Ayoola, 2008b; Ogundiran *et al.*, 2009; Hadi and Alwan, 2012, Selvanathan *et al*., 2013). Gills which have interface area that is very large between internal and external environment of fish, and playing important role in exchange of gases, ion osmo-regulation, acid-base balance and excretion of nitrogenous wastes, are the first contact organ of several pollutants that are partially sensitive to adverse environmental conditions (Heath, 1987; Ogundiran *et al*., 2009). Primarily, pesticides and other contaminants enters the fish through the gills while the liver function mainly in detoxification (Dutta *et al*., 1994).

In this study, major histological changes were observed in the gills of fish exposed to sublethal glyphosate concentrations. These changes include gill arch vacoulation, excessive mucosal secretions, epithelial lifting, and thickening of the epithelium, hyperplasia, diapedesis, blood congestion, inflammation, lamellar ruptures, cellular hypertrophy, epithelial erosion and telangiectasis most of which have been documented upon fish exposure to various toxicants in water such as heavy metals, pesticides, and detergents (Nowak, 1992; Ortiz *et al*., 2003; Ayoola, 2008a; Ayoola, 2008b; Hadi and Alwan, 2012). These histological conditions observed in this study were as a result of the toxicity of the herbicide to the gill tissues and this toxic effect may have resulted into osmoregulatory stress and respiratory inefficiency due to reduced surface area, blood congestion in the gill capillaries, cellular distortion and erosion of cells. The damage in terms of rupture of gill

epithelium, cellular hypertrophy and inflammation leads to hypoxia and fish inability to respire. Epithelial lifting and erosion and telangiectasis were direct responses to the action of glyphosate. The severity of this condition in the gills of fish exposed to highest glyphosate concentration indicates its concentration dependence.

Liver is an important organ that plays vital roles such as synthesis of several components of blood plasma, release of glucose to the blood, glycogen storage and detoxification (Ortiz *et al*., 2003). Morphological and histological alterations in liver of fish related to toxic chemical exposures have been extensively studies, showing that these substances cause severe damage to the liver cells (Dutta, 1994; Ortiz *et al*., 2003; Hadi and Alwan, 2012; Ayoola, 2008b). The evaluation of histological alteration in liver of fish is a highly sensitive and precise way to determining xenobiotic compounds effects in field and experimental studies (Figueiredo-Fernandes *et al*., 1979). The alterations in the histology of the fish liver in this study were concentration dependent. Degeneration observed in the structure of the exposed fish indicates the excessive activities of the liver as a detoxifying organ trying to get rid of the toxicant from the body of the animal (Monir *et al*., 2015; Bawa *et al*., 2017). Congestion of sinusoids, leisons in the liver, lipidic vacuoles, focal necrosis, infiltration of leucocytes and pykonic nuclei are reported pathological effects of various herbicides (Couch, 1975; Neskovic *et al*., 1996; Jiraungkoorsul *et al*., 2003; Monir *et al*., 2015; Bawa *et al*., 2017). The pykonic nuclei observed in the liver of the fish exposed to 2.75 ppm glyphosate concentration is an indication of undergoing necrosis or apoptosis. This could be due to alterations in the protein metabolism and synthesis due to failures in the transcription process caused by glyphosate (Papadimitou *et al*., 2000; Jiraungkoorsul *et al*., 2003; Marc *et al*., 2005) The observed vasolidation and necrosis in fish exposed to higher glyphosate concentration (5.50 ppm) confirmed the complete stage of pykonic nuclei leading to blood vessel widening resulting from underlining disease which may be due to glyphosate exposure. The observed necrosis of liver cells could also be due to the inability of the fish to grow new cell (Camargo and Martinez, 2007; Deivasigamani, 2015). Narrowing of the central nerve and vasodilatation in the liver sinusoids could be seen to be as a direct effect of the herbicide to the liver.

The fish muscle tissues in this study showed no apparent histological changes unless for mild hyperplasia and inflammatory responses observed in groups with glyphosate treatment. Inflammation is a common response of animal tissues to toxin as a result of cellular degeneration and haemorrhage (Ladipo *et al*., 2011). However, these were not conspicuous in the examined tissue sample. The skeletal muscle cells consisting of both the white and brown muscles showed normal striation and nucleation of the tissues with distinct adipose tissues suggesting a minimal effect of glyphosate on the muscles of the fish.

CONCLUSION

The pronounced harmful histological changes induced by glyphosate on the organs studied reveals that exposure of fish to chemical environmental stressors can lead to tissue distortion and death of the fish. The toxicity of glyphosate on juvenile fish increases with increasing levels of toxins due to accumulation of the toxic substance and its associated effects. This study revealed that glyphosate herbicide is a potent toxin particularly at a concentration of 5.50 ppm to fingerlings of African catfish (*C. gariepinus*) and results in deadly histological changes in vital organs especially in gills and liver.

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