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Oxidative stress, haematology and histology biomarkers of African clariid catfish *clarias gariepinus* (Burchell 1822) to glyphosate-based herbicide GBHG, '*Glycot*'

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Abstract
Fish were exposed to acute doses: 31, 33, 35, 37, 39 and control (0.00) mg glycot L^{-1} of clean water and obtained 96h LC_{50} value of 33.9 mgL^{-1} and a logarithmic probit line y = 20.11x - 25.80, R^2 = 0.972. Sub -acute doses: 3.39, 6.78, and 16.95mgL^{-1} of the same, corresponding to $^1/_{10}$ LC_{50}=3.39 mgL^{-1}, $^1/_5$ LC_{50}=6.78 mgL^{-1}, ½ LC_{50}=16.95 mgL^{-1}' were generated. The 96 h LC_{50} of the herbicide to the fish corresponds to 96h safety dose of $^1/_{100}$ LC_{50} = 0.339 mgL^{-1}. Catalase CAT was significantly ($p<0.05$) inhibited in treatments compared to control, and it ranged from the highest value of 0.74 umol mm^{-1} mg $protein^{-1}$ in control to the lowest value of 0.21 umol mm^{-1} mg $protein^{-1}$ in exposed group to 16.95 mg/L on day 4 but returned to control value on day 8 in all the treatments (figure 2). Superoxide disimutase SOD was significantly ($p<0.05$) inhibited among exposed fish compared to control on days 1-4. A range of 11.60 U mg protein $^{-1}$ in control on day 1 to lowest value of 6.50 U mg protein $^{-1}$ returned to control value on day 8. Similarly GPX was significantly inhibited to a lowest value of 4.25 in fish exposed to 3.5mg/L compared to the highest value of 9.36 in control fish on day 4 but returned to control value with a high value of 9.25 $umol^{-1}$ min $protein^{-1}$. Similarly, Lipid peroxidation LPO ranged from the highest value of 6.8 mMole/TBARS/ mg protein in 16.95mg/L to a lowest value of 6.41 mMole/TBARS/ mg protein in control on day 4, returned to the control value on day 8. The hematological parameters were significantly reduced in the treated values of PCV and ranged from 20.00±0.19 – 23.33±0.55 %) below the value of 28.33±0.61% recorded in the control. Similarly, the red blood cells RBC, and haemoglobin HB recorded inhibited ranges of 8.58±0.21 - 9.10±0.03 x 10^6 mm^3 below their elevated respective controls of 10.06±0.03 x 10^6 mm^3 and 8.73±0.05. However the white blood cells WBC and platelets PL recorded elevated ranges of 12934.00±544.68 - 13700.00±485.72 x 10^3 mm^3 and 15933.33±322.79 above their respective control values of 9466.66 0.09±96.86 x 10^3 mm^3 and 15633.33±181.89. Histology of gills exposed to glyphosate indicated lamellae degeneration, primaryand secondary lamellae degenerations/talengietasis/oedema, liver exposed to glyphosate showing intercellular dilatations, hyperplasia/cirrhosis and thickening of portal tract/fatty degeneration while muscles exposed to the sames showed evidence of splitting of fibre, atrophy and cell degeneration. The present finding indicated that glyphosate based herbicide glycot impaired haematology, antioxidative stress enzymes and vital histology of *C. gariepinus* and could serve as an early warning signs toward ecotoxicological hazards prevetion and remediation in aquatic ecosystems.

Keywords: glyphosate, oxidative stress, haematology, histopathology

1. Introduction
In the bid to increase agricultural production, most countries in the world (Nigeria Inclusive) have undergone serious remarkable process of intensification and modernization as regards to their farming system in order to increase productivity in satisfying the nutritional needs of its citizens. Pesticide usage has increased dramatically worldwide within the past two decades inorder to assure sustainability in agricultural production. With the use of herbicides, the inhibitory effect of weeds on the production of commercial crops has been greatly reduced. Due to their systemic effect, glyphosate-containing herbicides have great success in controlling perennial plants. For this reason, glyphosate is a leading and increasingly utilized chemical around the world (Baylis, 2000) [19]. It is frequently used in agricultural areas, especially in rice, corn and soybean fields, for garden care, forestlands, and to get rid of unwanted plants with large leaves in pastures and green areas (Dallegrave *et al.*, 2003) [35]. On the other hand, these pesticides which play an important role in the agricultural productivity and have been widely applied to protect agricultural crops; can impact and cause severe damage to non-target organisms, both in the terrestrial and aquatic environment (Magar and Shaik, 2013; Grung *et al.*, 2015) [74, 56]. The indiscriminate or excessive use of glyphosate leads it to being washed-off and seeps into nearby water bodies forming free radicals which lead to water pollution (Ansari *et al.*, 2011) [10]. Water pollution is a major problem of this century owing to the addition of various pollutants through many ways (Voltz *et al.*, 2005) [124]. Pollution of water is mainly due to contamination with hazardous chemicals from agricultural runoff and waste water from household & different industries. One of the major chemicals from agricultural runoff are pesticides which play an important role in the agricultural productivity and have been widely applied to protect agricultural crops. These contaminants however change the natural qualities of water thereby causing fish stress. Fish have an important role in the food chain therefore investigation of the effects of pesticides on fish have a relevant significance in evaluation of adverse effects of pesticides to human health (Begum and Vijayaraghavan, 1996) [20]. Fish are among the group of non-target aquatic organisms which represent the

largest and most diverse group of vertebrates and a number of characteristics make them excellent experimental models for toxicological research (Raisuddin and Lee, 2008) [97]. In this sense, fish are often used as indicators of pesticide water contamination due to the fact that they respond to it with physiological and behavioral alterations (Banaee *et al.*, 2011, 2013; Murthy *et al.*, 2013) [16, 83]. One of the materials used as a biological indicator in the studies made for this purpose is blood and various tissues taken for pathological studies. These materials that constitute biological indicators also show the effects of environmental and human based stress factors and ecosystem sensitivity ((Luskova, 1997; Ozkan *et al.*, 2009, Gül *et al.*, 2008; Aksu *et al.*, 2008) [94, 4, 4]. The biomonitoring and risk assessment processes as suggested by Viana *et al*. (2013) [121], should include analyses at different levels of biological organization, from sub-cellular and cellular analysis of tissues and organs, to those of population and community levels. Therefore, Van der Oost *et al*. (2003) [118] also suggested the use of biomarkers as biological indicators from an exposure to a stressor responding in various ways which can be successfully applied.

Oxidative stress however is a cellular response to stressors like glyphosate, caused by an imbalance between the production of a particular biological system to quickly detoxify the intermediates generated or repair the resulting damage (Di Giulio and Meyer, 2008; Repetto and Repetto, 2009) [41].

Nevertheless, the establishment of normal haematological values in fish species could be used as an important diagnostic tool for monitoring fish health under culture conditions. Haematological parameters are closely related to the response of the fish species to the environment where fish lives and could exert some influence on the haematological characteristics (Gabriel *et al.*, 2001) [53]. It was noted that the haematological characteristics of a number of culturable fish species have been studied with the aim of establishing normal ranges and any deviation from it may indicate a disturbance in the physiological process. Hematological parameters like Red Blood Cell (RBC) count, Hemoglobin (Hb/dl), Packed Cell Volume (PCV), Mean Corpuscular volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular hemoglobin concentration (MCHC) and total white blood cell count (WBC) have been used as biomarkers for physiological and pathological changes in fisheries. These parameters remain veritable tools in determining the sub-lethal concentration of pollutants such as glyphosate in fish as it reveals the alterations in the blood composition and immune mechanisms (Svoboda 2001; Witeska, 2003), Hematology has been widely used for the detection of physiological alterations following different stress conditions. Hence, hematological techniques are the most common methods to determine the sub lethal effects of pollutants (Kumar *et al.*, 2011) [34]. In this same vein, according to Van Dyk and Pieterse (2008) [119], histology is also an important diagnostic and sensitive tool to detect the direct effects of chemical compounds on target organs and can therefore, be used to assess the health of fish populations in contaminated aquatic systems. The advantage of histology as a biomarker lies in its intermediate location with regard to the level of biological organization. Furthermore, the histological changes appear as a medium-term response to sub-lethal stressors, and histology provides a rapid method to detect the effects of irritants, especially chronic ones, in various tissues and organs (Devi and Mishra, 2013; Binukumari and Vasanthi, 2014) [39, 222]. Thus, the histopathological changes due to various pesticides have been extensively studied in different fish species (Ullah and Zorriehzahra, 2015) [116]. Histological abnormalities in fish exposed to pollutants include impaired growth and reproduction, which reduces the fish population and possible extinction of fish at continuous exposure. The aim of this study was to investigate and determine the toxicological impact, lethal toxicity and other alterations of glyphosate herbicide on post juveniles of *Clarias gariepinus* exposed to sub-lethal doses in the laboratory in order to provide toxicological data on glyphosate use on common tropical fish. The prospect of encouraging polyculture especially the culture of Clarias gariepinus in a rice farm will continue to be mere wishes if we continue to shy away from studies like this. I believe that this project can give us a sub-acute toxicity results that can inform a farmer on the recommended level of concentration of glyphosate that over a specific period of time, will not lead to an acute toxicity.

Glyphosate is an active ingredient of preparation Roundup which has herbicides effect (Andrea *et al*., 2009). Glyphosate is a herbicide commonly used by crop farmers to control weeds in Nigeria. Its uses around aquatic habitat pose a serious threat on aquatic organisms particularly fish. There is therefore need to determine the tolerance limit of fish to glyphosate. The knowledge of the study will help the fish toxicologists, scientific researchers, aquaculturist and farmers in general know more about toxicology and the use of herbicides. It will help in discovering the importance of monitoring herbicides level in water bodies for their impact assessment on fish production. It will also help to know the exact concentration level of glyphosate that will kill 50% (LC_{50}) of *Clarias gariepinus* post juveniles. The concluded experiment will help also to show the various changes induced by glyphosate in the cellular, hematological, histological and physiological parameters, DNA integrity; which will help in fish health evaluation. *C. gariepinus* respires bimodally and it is hardy and acceptable principally as a consequence of its air breathing ability, feeds on a wide array of natural prey under adverse condition. It has a remarkable fast growth rate and ability to withstand adverse environmental conditions especially low oxygen content, highly resistant to diseases and is highly fecund and easily spawned under captive conditions. It is the commonest catfish due to its high adaptation to pond conditions even in captivity; these includes acceptance of artificial feed, tolerance of crowded conditions, high quality of its flesh (Dede and Igbigbi, 1997) [36]. The objective of this project was to determine the LC_{50} survival and mortality rate of the *Clarias gariepinus* post juveniles after being exposed to varying concentration levels of glyphosate for duration of 96hrs.The specific objectives were to determine; The histopathological changes with emphasis in the muscular tissues at varying concentration level; Observe the sub-acute level of glyphosate toxicity on hematology of *Clarias gariepinus* post juveniles by estimating the Packed Cell Volume (PCV), Red Blood Cell (RBC) count, Hemoglobin (Hb/dl), Platelets and total white blood cell count (WBC) and the oxidative stress caused by glyphosate on the post juveniles of *Clarias gariepinus*.

2. Materials and Methods

A total number of three hundred and sixty (360) Post Juveniles of African Catfish, *Clarias gariepinus* (Burchell, 1822) of about 12–20weeks old with a mean weight of 300g±0.96g and 22cm±0.02cm of length were procured from ISRAELEX global Ltd farms, located in Amachara Mpu, Aninri L.G.A of Enugu State, Nigeria and were used for this study. This is due to the more sensitive nature of juveniles than adult for toxicity test (Solbe, 1995; Odiete, 1999) [108, 90]. The fish were transported in well aerated fish transit tanks to Heldin Fisheries Laboratory Emene, Enugu state-Nigeria. The fish were acclimatized under laboratory conditions for 2 weeks (14days) in a plastic aquaria tank of 10,000 gallon capacity, containing dechlorinated and aerated tap water. During this period of acclimatization, pond water was changed daily to clear the faecal matter from the water. Feeding was twice daily at 3% of their body weight with Copens feed (4mm) at 08.00hrs & 17.00hrs. They were not fed 24hrs before the commencement of the experimental study to empty their stomach and avoid pollution of the water with faecal matters. Moreover, termination of feeding was necessary because feeding increases the rate of respiration and excretory products, which may however influence the toxicity of test solution. Glysate® (a commercial formulation of glyphosate 41%SL) with batch no ASRHY1706 – a product of Nantong Jinagshan agrochemical & chemicals, Jinagshu- China and supplied by Lionseal Industrial limited –Lagos, Nigeria.

2.1 Water Quality Parameter

Pipe borne water was dechlorinated by allowing it to stand for 72hr in an overhead tank during which it was aerated. And the physio-chemical properties of water such as Dissolved oxygen, Temperature, pH) were analysed by direct reading using dissolved oxygen meter, thermometer and pH meter (AOAC, 2005; APHA, AWWA, WPCF, 2005) [12, 13]. Dissolved oxygen was determined by dipping the electrode head of the dissolved oxygen meter into the experimental containers and allowed to read, observation was taken when the meter stabilized and the average reading was taken. Temperature was determined using dry bulb Mercury-in-glass thermometer. The thermometer was dipped in the experimental aquaria bowls and reading was taken at least 2 minutes when Mercury is stabilized and the average was taken (AOAC 2005) [12]. This was determined by dipping the electrode of the pH meter into the experimental bowls for few minutes. Reading was taken by observing the indicator and recording the sharp point where the reading pointer stabilizes. This was done in each experimental bowl and average was taken.

2.2 Range Finding Test (Pilot Study)

For each test concentration of the herbicide, two (2) fish were exposed in 25 liters capacity aquaria tanks with 10 litres of water, 24hrs before commencement of acute toxicity test. The concentrations of toxicant were 2.5mgl^{-1}, 3.0mgl^{-1}, 3.5mgl^{-1} and 4.0mgl^{-1}. The fish were not fed during the 96hr experimental period. Mortality was recorded within these period and suitable concentrations were obtained for the definitive test.

2.3 Acute Toxicity Test

The 96 h LC_{50} value of glyphosate was determined by randomly exposing a set of ten (10) post juvenile fish specimen in six (6) treatment groups. The five selected concentrations of glyphosate herbicide (31, 33, 35, 37, and 39 mgl^{-1}) were run in triplicates. Another triplicate set of ten (10) post juveniles were simultaneously maintained in water without the herbicide and considered as control. However, the Mortality and Survival rate were monitored and recorded at 24, 48, 72 and 96 hours intervals respectively. The median lethal concentration (LC_{50}) value was determined from the bioassay results using the probit analysis methods Lichfield and Wilcoxon, 1949; Finney (1971) [51].

2.4 Sub-Acute Toxicity Test

In order to investigate the hematology, histology and micro nucleus effects caused by glyphosate in the blood cells and tissues, the sub-lethal test was carried out after the acute definitive test. Fish were exposed to four (4) sub lethal concentrations gotten through serial dilution of the 96h LC_{50} glyphosate dose for 192 hours (8 days). The resultant concentrations of the herbicide were ($^1/_{10}$ LC_{50}=3.39mgl^{-1}, $^1/_5$ LC_{50}= 6.68mgl^{-1}, ½ LC_{50}=16.95mgl^{-1}). A set of 10 fish were maintained in herbicide-free (0.00mgl $^{-1}$) water; this served as the control and treatments were all replicated thrice. The test solution was changed and re-treated every 48hours to counter-balance the decreasing pesticide concentration because of its hydrolysis in water. Samples for the oxidative test, haematology and histology examinations were taken at 24hrs (day 1), 96hrs (day 4) and 192hrs (day 8).

2.5 Oxidative stress enzyme activities

Catalase (CAT) activity was assayed according to the method of Takahala *et al.* (1960) as described by Clairborne (1985). This method was based on the disappearance of H_2O_2 at 240 nm. The reaction mixture consisted of 1 ml of 0.05M H_2O_2 in 0.1M sodium phosphate buffer (pH 7.4), at 25˚C, 1.9 ml of distilled water and 0.1 ml of liver homogenate. Change in absorbance was measured with spectrophotometer at 240nm (Nanomoles). However, Catalase activity was calculated in terms of micromole H_2O_2 consumed per minute per milligram of protein. Superoxide dismutase (SOD) activity) blood was determined by the technique of Misra and Fridovich (1972) [78]. The assay was based on the ability of the enzyme SOD to inhibit the autoxidation of epinephrine-adrenochrome and its transition at an alkaline pH. 0.5ml of tissue homogenate was diluted with 0.5ml of distilled water, to which 0.25ml of ice-cold ethanol and 0.15ml of ice-cold chloroform was added. The mixture was properly mixed using a Cyclo mixer for 5 minutes and centrifuged at 2500 rpm. To 0.5ml of supernatant, 1.5ml of carbonate buffer (0.05M, pH 10.2) and 0.5ml of EDTA solution (0.49M) were added. The reaction was initiated by the addition of 0.4ml of epinephrine (3mM) and the change in optical density/minute was assessed spectrophotometrically at 480nm against reagent blank. SOD activity was expressed as the amount of enzyme units/mg protein. Change in optical density per minute at 50% inhibition of epinephrine to adrenochrome transition by the enzyme was taken as the enzyme unit. Glutathione Perioxidase (GPx) activity was assayed as described by Paglia and Valentine (1967) with modifications according to Lawrence and Burke (1978). The reaction mixture contained 50mM potassium phosphate buffer (pH 8.3), 1mM EDTA, 1mM sodium azide, 0.2mM nicotinamide adenine

dinucleotide phosphate (NADPH), and 1 U/mL glutathione reductase. The reaction was initiated with the addition of 1.5mM cumene hydroperoxide. The enzyme activity was estimated from the rate of oxidation of NADPH. The reagents were mixed and the absorbance measured at 340 nm. Enzyme activity was expressed in mmol/minute/milligram protein

2.6 Haematological analysis
Blood was collected from fish through the caudal vein by means of heparinized plastic syringe after the administration of clove oil in order to reduce stress. It was then stored in ethylenediaminetetracetic acid (EDTA) tubes. The blood samples were analysed, using automated blood analyzer (Pentra XL 80, Pentra 60C+, BIORAD D-10HPLC, Automated Coagulometer, Japan). The following parameters were measured: red blood cell count (RBC), haemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and total white blood cell count (WBC).

2.7 Stastical analysis
Data are expressed as mean ± standard deviation and were analysed using the statistical package SPSS 20.0 computer program (SPSS Inc. Chicago, Illinois, USA). Differences in the test concentra-tions and control were subjected to one-way analysis of variance (ANOVA), followed by Turkey's multiple range tests to determine significant mean differences. The Pearson correlations between the test biomarkers and blood parameters as well as the principal component analysis to assess the variability associated with each biomarker exposed to different concentrations of GBHB were determined using XLSTAT® 2017. The statistical significance was determined at 95% level of probability.

2.8 Histological Examinations
Fish were anesthetized on ice; excised and fresh specimens of Body tissue samples were collected from the fish muscles across all the treatments from both control and herbicides-exposed fish for histological studies. The specimens were rapidly fixed in 10% formalin (normal saline) for at least 24hr. After 24hrs, the tissues were processed for histological examinations using standard histological techniques as described by Culling (1983) [33]. The fixed specimens were processed by passing through graded series of alcohol; (70%, 95% and absolute ethanol). They were further passed through xylene and infiltrated with paraffin. From the prepared paraffin blocks, sections of the muscular tissues were cut at 5µm using a Microtome followed by heamatoxylin and eosin staining. Permanent sections were read under light microscope and the images recorded. The readings of the histology specimens were blind to the treatment groups.

3. Results
3.1 Water Quality Parameters
The physio chemical parameters of the test water and cumulative lethality in various treatment levels is presented in table 1. The results showed that there were no significant differences between the different treatments and the control. The values of the physiochemical analysis of test water ranged from pH reading of 8.40 ± 0.1 – 9.8 ± 0.60; temperature reading vales of 25 ± 0.5 – 25.81 ± 0.05 and dissolved oxygen values of 5.0 ± 0.00 – 5.5 ± 0.01 respectively. The water quality did not cause mortality of experimental fish because it fell within the standard range of water quality for aquaculture.

Table 1: Physiochemical analysis of test water and cumulative mortality of *Clarias gariepinus* exposed to different concentrations of GBHG for 96 hours

Concentration (mgL^{-1})	pH	Temperature ^0C	Dissolved oxygen
Control	8.40 ± 0.1	25 ± 0.5	5.0 ± 0.00
31	9.30 ± 0.3	24.88 ± 0.4	5.4 ± 0.04
33	9.40 ± 0.1	25.68 ± 0.5	5.2 ± 0.00
35	9.65 ± 0.85	25.81 ± 0.1	5.0 ± 0.01
37	9.75 ± 0.15	25.68 ± 0.05	5.3 ± 0.03
39	9.80 ± 0.60	25.81 ± 0.05	5.5 ± 0.01
Aquaculture Standard	6.7 – 8.5	26 - 28	5.0 – 10

Conc. (mg/L)	Log Conc. (mg/L)	Fish exposed	Cumulative mortality				% Survival	% Mortality	Probit mortality
			24	48	72	96			
Control 0.0	0	10	-	-	-	-	100	0	-
31	1.50	10	-	1	1	1	70	30	4.48
33	1.52	10	-	2	1	1	60	40	4.75
35	1.54	10	1	1	1	2	50	50	5.00
37	1.56	10	1	1	2	3	30	70	5.52
39	1.59	10	1	2	2	3	0	90	6.28

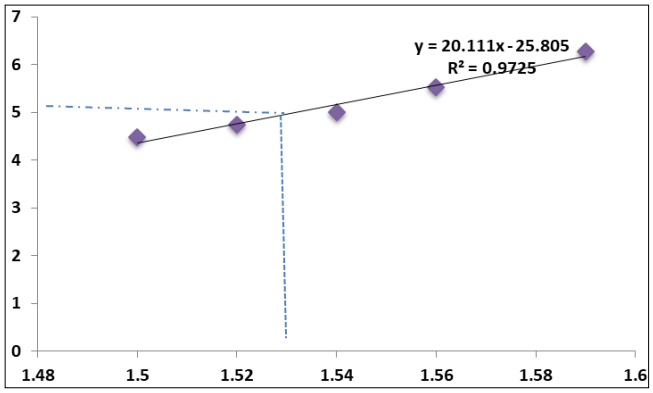
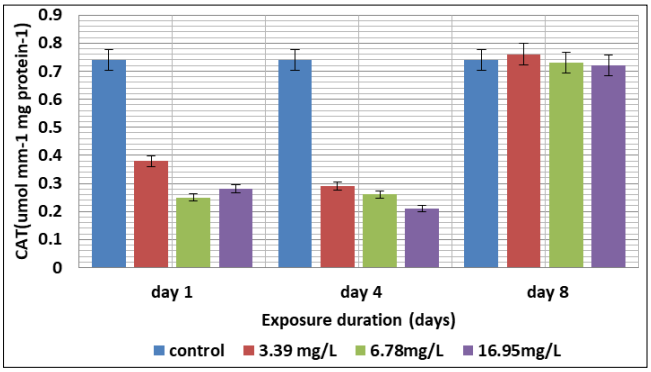
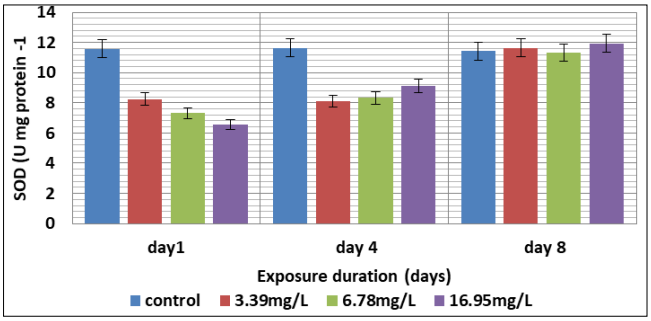
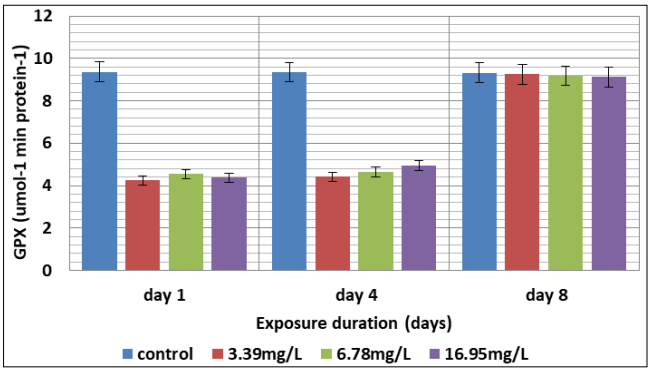


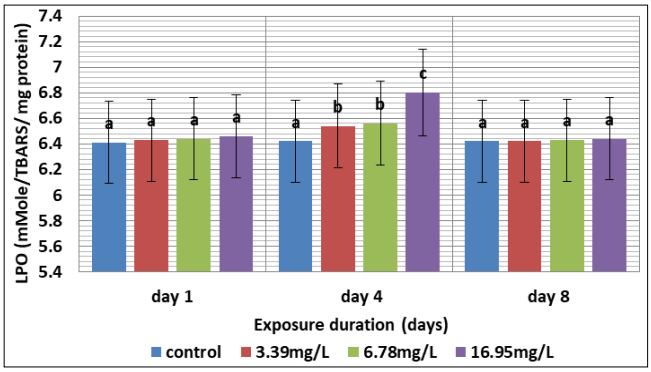
Fig 1: Logarithimic probit line to determine 96 h LC_{50}

Acute toxicity
Fish were exposed to acute doses: 31, 33, 35, 37, 39 and control (0.00) mg glycot L^{-1} of clean water and obtained 96h LC_{50} value of 33.9 mgL^{-1} and a logarithmic probit line y = 20.11x - 25.80, R^2 = 0.972. Sub -acute doses: 3.39, 6.78, and 16.95mgL^{-1} of the same, corresponding to $^1/_{10}$ LC_{50}=3.39 mgL^{-1}, $^1/_5$ LC_{50}=6.78 mgL^{-1}, ½ LC_{50}=16.95 mgL^{-1}' were generated. The 96 h LC_{50} of the herbicide to the fish corresponds to 96h safety dose of $^1/_{100}$ LC_{50} = 0.339 mgL^{-1}.









Different alphabetic superscripts differ significantly ($p<0.05$) between concentrations in each parameter.

Fig 2: Mean of CAT, SOD, GPX and LPO C. gariepinus to GBHG for 8 days

Antioxidative enzymes and lipid peroxidation

Catalase CAT was significantly ($p<0.05$) inhibited in treatments compared to control, and it ranged from the highest value of 0.74 umol mm^{-1} mg $protein^{-1}$ in control to the lowest value of 0.21 umol mm^{-1} mg $protein^{-1}$ in exposed group to 16.95 mg/L on day 4 but returned to control value on day 8 in all the treatments (figure 2). Superoxide disimutase SOD was significantly ($p<0.05$) inhibited among exposed fish compared to control on days 1-4. A range of 11.60 U mg protein $^{-1}$ in control on day 1 to lowest value of 6.50 U mg protein $^{-1}$ returned to control value on day 8 (fig.2). Similarly GPX (fig.2) was significantly inhibited to a lowest value of 4.25 in fish exposed to 3.5mg/L compared to the highest value of 9.36 in control fish on day 4 but returned to control value with a high value of 9.25 $umol^{-1}$ $min\ protein^{-1}$. Similarly, Lipid peroxidation LPO (fig.2) ranged from the highest value of 6.8 mMole/TBARS/ mg protein in 16.95mg/L to a lowest value of 6.41 mMole/TBARS/ mg protein in control on day 4, returned to the control value on day 8.

Table 2: Hematological parameter of *Clarias gariepinus* exposed to different concentrations of GBHG for 8 days.

Parameters	Concentrations (mg/L)	Exposure days		
		1	4	8
PCV	Control	28.00±0.38 ^{a}	27.33±0.40 ^{a}	28.33±0.61 ^{a}
	3.39	23.00±0.33 ^{b}	23.00±0.19 ^{b}	23.33±0.55 ^{b}
	6.78	21.33±0.29 ^{b}	20.33±0,29 ^{b}	21.33±0.11 ^{b}
	16.95	21.33±0.58 ^{b}	21.00±0.66 ^{b}	20.00±0.19 ^{b}
RBC	Control	10.05±0.03 ^{a1}	10.06±0.03 ^{a1}	10.05±0.03 ^{a1}
	3.39	8.60±0.06 ^{b1}	8.61±0.06 ^{b1}	8.61±0.06 ^{b1}
	6.78	9.09±0.12 ^{a2}	9.10±0.12 ^{a2}	9.10±0.12 ^{a2}
	16.95	8.58±0.21 ^{b1}	8.58±0.21 ^{b1}	8.59±0.21 ^{b1}
WBC	Control	9433.33±125.21 ^{a1}	9400.00±153.96 ^{a1}	9466.66±96.86 ^{a1}
	3.39	13033.33±544.44 ^{a2}	12934.00±544.66 ^{a2}	13133.66±544.72 ^{a2}
	6.78	13333.33±357.66 ^{a2}	13233.33±363.28 ^{a2}	13400.00±360.55 ^{a2}
	16.95	13633.33±481.25 ^{b1}	13566.66±477.00 ^{a2}	13700.00±485.72 ^{b1}
Hb/dl	Control	8.73±0.05 ^{a1}	8.63±0.07 ^{a1}	8.73±0.05 ^{a1}
	3.39	7.43±0.09 ^{b1}	7.56±0.09 ^{b1}	7.50±0.12 ^{b1}
	6.78	7.70±0.12 ^{a2}	7.73±0.13 ^{a2}	7.70±0.11 ^{a2}
	16.95	7.86±0.11 ^{a2}	7.73±0.12 ^{a2}	0.96±0.10 ^{a2}
Platelets	Control	15600.00±203.67 ^{a}	15600.00±220.94 ^{a}	15633.33±181.89 ^{a}
	3.39	13900.00±472.58 ^{a}	13866.66±449.41 ^{a}	13933.33±496.40 ^{a}
	6.78	15866.66±327.91 ^{a}	15533.33±298.96 ^{a}	15933.33±322.79 ^{a}
	16.95	15633.33±401.07 ^{a}	15600.00±397.67 ^{a}	15600.00±384.41 ^{a}

Mean Values with different alphabetic superscripts (a1, a2, b1 and b2) differ significantly ($p<0.05$) between concentrations in each parameter.

Haematology

The hematological parameters were significantly reduced in the treated values of PCV and ranged from 20.00±0.19 – 23.33±0.55 %) below the value of 28.33±0.61% recorded in the control (table 2). Similarly, the red blood cells RBC, and haemoglobin HB recorded inhibited ranges of 8.58±0.21 - 9.10±0.03 x 10^6 mm^3 below their elevated respective controls of 10.06±0.03 x 10^6 mm^3 and 8.73±0.05. However the white blood cells WBC and platelets PL recorded elevated ranges of 12934.00±544.68 - 13700.00±485.72 x 10^3 mm^3 and 15933.33±322.79 above their respective control values of 9466.66 0.09±96.86 x 10^3 mm^3 and 15633.33±181.89.

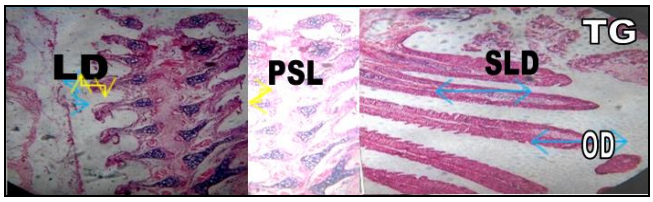


Plate 1: Photomicrograph of gills exposed to glyphosate showing lamellae degeneration, primary and secondary lamellae degenerations/talengietasis/oedema

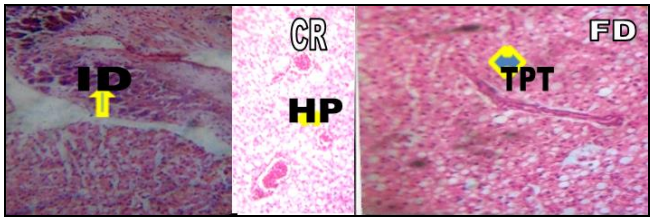


Plate 2: Photomicrograph of liver exposed to glyphosate showing intercellular dilatations, hyperplasia/cirrhosis and thickening of portal tract/fatty degeneration

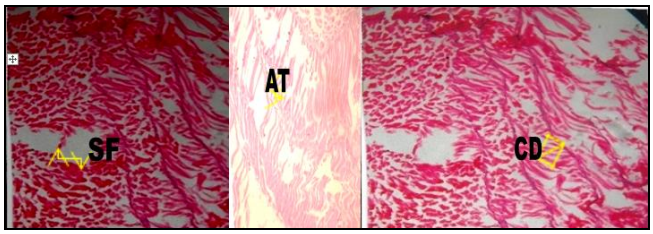


Plate 3: Photomicrograph of muscles exposed to glyphosate showing splitting of fibre, atrophy and cell degeneration

Discussion

Herbicide Toxicology

A herbicide is a pesticide, described by The United States Environmental Protection Agency (USEPA) as an agent used to prevent, destroy, repel or mitigate any pest ranging from insects, animals and weeds to microorganisms such as fungi, molds, bacteria and viruses. A herbicide, then, is any compound capable of killing or severely injuring plants and may be used for the elimination of plant growth or the killing off of plant parts (Amdur *et al*., 1991) [8]. All around the world, herbicides are widely used in agricultural activities for the control of water plants, which may impede the flow of water during the summer, when sudden heavy rain can cause flooding (Annune *et al.*, 1994) [11] and they are followed by some other pesticides such as insecticides and fungicides (He *et al.*, 2012; USEPA, 2011) [61]. While the direct effect of herbicides addition is the loss of macrophytes, non-target organisms such as fish may also be affected through loss of habitat and food supply (Ervnest, 2004) [45]. Several toxicological tests can be conducted on each herbicide. From these tests, a variety of hazard indicator values were derived. All herbicides exert their actions in one or more of the following ways - Plant-growth regulators, Photosynthetic inhibitors, Bleaching Agents (Pigment Inhibitors), Lipid Synthesis Inhibitors, Cell Wall Synthesis Inhibitors, Cell Division Inhibitors, Amino acid Inhibitors, EPSP Inhibitors, Energy-Production Inhibitors, Cell Membrane Destructors, General Cell Toxicants and Herbicides with Unknown Mode of Action (DiTomaso, 2010) [37].

Glyphosate toxicity

Glyphosate, the active ingredient which is the 48% acid equivalent of the 180 isopropylamine salt (IPA salt) of glyphosate (N-phosphonomethyl glycine) organophosphorous compound, is used as a non-selective broad spectrum, post emergent, systemic herbicide and crop desiccant and for control of a great variety of annual, biennial and perennial grasses, sedges, broad-leaved weeds and woody shrubs, used in fruits orchards, vineyards, conifer plantations and many plantation crops. It is perhaps the most important herbicide ever developed.

Glyphosate, a herbicide which inhibits the synthesis of aromatic amino acids such as tryptophan, tyrosine and phenylalanine in plants (Santos *et al.*, 2007) [99] may leak into water bodies after agricultural use or when directly applied to water systems to control macrophyte plants (Soso *et al.*, 2007) [110]. Since glyphosate has a high solubility in water, both soils and aquatic systems are continually being contaminated. So, it may lead to developmental, morphological, physiological and biochemical modifications on non-target organisms (Tate *et al.*, 1997) [113]. Substantial reports on the first and second generation groups of the original glyphosate Roundup and several other group of glyphosate based herbicides have been reported (Monsanto, 1995; Glusczak *et al*., 2009; Modesto and Matinez, 2010; Modesto and Martinez, 2010) [81, 59, 103, 79], but the toxicity effects of GBHG is almost unavailable in literature except maybe a lowerered value of 24.6 mgL^{-1} 96h LC_{50} reported by Ani *et al*. (2017) on the same species of fish juveniles compared to ours of 33.39 mgL^{-1}. The present value also proved to be higher than respective values of 1.05mgL^{-1} and 13.6 mgL^{-1} by Ayoola (2008) [14] and Langiano and Martinez (2008) [64] when they exposed *Oreochromis niloticus and Prochilodus lineatus* to glyphosate and glyphosate-based herbicides respectively. However, the reported value of 108mgL^{-1} of glyphosate to tadpole's juveniles by Clements *et al*. (1997) [30] is somewhat higher than the present, as well as respective higher values of 620 mgL^{-1} and 975 mgL^{-1} 96h LC_{50} reported by Shiogiri *et al*. (2012) [107] when *Cyprinus carpio* and *Palloceroscaudi maculatus* were exposed to glyphosate and glyphosate commercial formulation (Rodeo) herbicides probably due to species and formulation variations. The present study indicated that GBHG formulation was less toxic compared with other reported cases which could be an indication of toxicity improvement on the mixture of POEA surfactants and other unspecified inert adjuvants and preservative substances. It has been shown that many manufacturers regard as a trade secret and seldom disclose (Cox and Surgan, 2006; Mesnage *et al.*, 2013) [31, 77], and have been implicated to be responsible for the high level of toxicity reported on glyphosate based

commercial formulations and roundup in general. Mesnage *et al*. 2019 [76] noted that POEA talloamine present in GBH was more toxic compared to the original glyphosate in Roundup.

Heamatological Effects

Hematological alterations, specifically an increase in hematocrit, were detected in *P. lineatus* exposed for 96 h to Roundup® at 5 mg/L (Modesto and Martinez 2010) [79]. Increases in both erythrocyte and leukocyte numbers suggest that defense mechanisms in response to contaminant exposure were activated in the fish (Cazenave *et al*. 2005) [25]. This result is different from an earlier study by Glusczak *et al*. (2006) [59] where a decrease in hematocrit was observed in *L. obtusidens* after exposure to Roundup®. However, it can be said that exposure to glyphosate-based herbicides can alter the hematology of fish though there are variation of results in the direction of these blood and immune responses. Haematological concerns of glyphosate based herbicides and indeed other pollutants have been used as a health status biomarker indicator of stressed animals (Modesto and Martinez, 2010) [80]. The present indication of inhibited PCV, RBC and HB by subacute doses of GBHG and elevated WBC and PL compared to the control corroborated with report of Glusczak *et al*. (2006) [58] on the same active principle glyphosate but of different species and formulations on *Leporinus obtusidens*, suggest to the fact that there was a lowering in the production level from the haemopoietic areas in the exposed group because of haemodilution (Modesto and Martinez 2010) [80] or it may have been hampered by some of unspecified inert substances in the formulation and rendered inefficient to produce sufficient parameters below the control. In order to respond to the forgoing, the fish elevated its WBC and PL to counter the effect of the herbicide and restore normalcy of the blood demand of the fish. However, it disagreed with the reported elevations on PCV, RBC and HB and lowered WBC and PL in fish exposed to a glyphosate based herbicide at short duration (Svodova, 1994) [111]. On the contrary, increased total number of leukocytes and platelets could be a defense response in the presence of surfactants and other inert portion of herbicide into the blood stream or as the organism's adaptive response ploy for towards effective immune defense (Barreto-Medeiros *et al*., 2005; cazenave *et al*., 2005; Dong *et al*., 2017; Li *et al*., 2017) [25, 38, 61].

Oxidative Stress

Oxidative stress, a pathological process relating to over-production of reactive oxygen species (ROS) in tissues is one important general toxicity mechanism for many xenobiotics. Under normal conditions, ROS and other pro-28 oxidants are continually detoxified and removed in cells by antioxidant systems (Li *et al.,* 2009) [61]. The most important antioxidant enzymes are superoxide dismutase (SOD), Catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase (GST). The non-enzymatic defense system includes Vitamins E, C, and A, glutathione and carotenes (Filho, 1996; Li *et al.,* 2010a) [50, 61]. These systems can prevent the formation of ROS, which can react with susceptible biological macromolecules and produce lipid peroxidation, resulting in oxidative stress (Zhang *et al.,* 2008; Li *et al*., 2010b) [128, 61]. Many antioxidant responses including oxidative stress biomarkers and antioxidant enzyme activities, are used in environmental risk assessment (Song *et al.,* 2006) [109].

Anti-oxidative stress enzyme activity effect of GBHG

Antoxidants are substances that significantly delay or prevent the oxidation of oxidisable substrate (Franco and Martenez-Pinilla, 2017) [52]. The antioxidants produced by the body of animals act enzymatically to decrease the excess of free radicals through enzymatic components such as SOD,CAT and GPX (Halliwel *et al*., 1995) [60]. In our finding, inhibition of all three antioxidants CAT, SOD and GPX below their respective controls occurred on day 1 but there was further inhibition of CAT on day 4 followed by an elevation to control level on day 8 compared with elevation in both SOD and GPX on day 4, followed by further elevation to control level on day 8. Voet and Voet (1990) [123] noted that antioxidants work in tandem to dismutate oxygen radicals in which SOD converts superoxide anion to hydrogen peroxide which is broken down to oxygen and water by catalase. The SOD– CAT system has been noted to be the first line of defense against oxygen toxicity, due to the inhibitory effects on the formation of oxygen radicals (Pandey *et al*., 2003) [96], and these enzymes were frequently used as biomarkers, that indicated the production of reactive oxygen species (ROS) (Monteiro *et al*., 2006) [82]. The reduction in SOD activity after day 1 of exposure to the herbicide may be related to the production of oxidants. An excess of hydrogen peroxide may have reduced SOD activity, while the superoxide anion may be responsible for further decrease in CAT activity on day 4 (Bagnyukova *et al*., 2006; Scandalios, 2005) [17, 100]. Thus, it may be reasonable to assume that hydrogen peroxide was responsible for the reduction observed in SOD activity while the reduction of CAT activity was due probably to accumulated superoxide anions not sufficiently neutralized by SOD. The activities of enzymes involved in animal's antioxidant system have been known to be a complex pathway of interactions among enzymes, because the activity and substrate product of one enzyme may influence the other. In the present work, the inhibition of CAT and SOD limited the antioxidant defenses of the fish during the first 4 days of exposure to the herbicide. But was restored on day 8 when the activities of the antioxidants approached the control level. Although GPX has been noted to function principally in the removal of organic peroxides, Maran *et al*. (2009) [75] reported its involvement in the metabolism of hydrogen peroxide. The significant increased activity of GPX in the fish after day 4 to the herbicide indicated that the antioxidant pathway was stimulated, probably due to the increased production of peroxides. Thus, the activation of GPX may be an indication of adaptive response to compensate the inhibition of CAT at the period of exposure.

Reactive oxygen species left un-neutralized reacted with membrane lipids which produced lipid peroxidation, considered as one of the main consequences of oxidative stress (Ahmad *et al*., 2000; Ansari *et al*., 2011; Nwani *et al*., 2013) [1, 10, 87]. In this work, the occurrence of lipid peroxidation was indicated by a transient increase in LPO in fish to 17.5mgL^{-1} to the herbicide on day 4. However, LPO levels returned to control levels after day 4. Thus, it can be inferred that the antioxidant defense before day 8 of exposure was insufficient due to significant decreases in SOD, CAT and GPX activities which led to increased lipid peroxidation as a function of the presence of GBHG.

However these defenses returned to basal levels on day 8 and then were enough to combat the ROS, which prevented incidence of oxidative damage. Lushchak *et al*. (2009) [17] using a similar method to quantify lipid peroxidation found that the herbicide Roundup original also did not affect lipid peroxidation in the liver of the goldfish after 96 h of exposure.

Histopathological Effects

Histopathology is the microscopic examination of tissue in order to study the manifestations of disease. Specifically, in clinical medicine, histopathology refers to the examination of a biopsy or surgical specimen by a pathologist, after the specimen has been processed and histological sections have been placed onto glass slides. In contrast, cytopathology examines free cells or tissue fragments. Toxic chemicals cause tissue damage and histopathological degradations as the fish show haematological responses to toxicants. Generally, such degradation of histological origin occurs in the vital organs of animals (Velkova-Jordanoska and Kostoski, 2005) [120]. Histopathological changes of gills such as hyperplasia and hypertrophy, epithelial lifting, Aneurysm and increase in mucous secretion have been reported after the exposure of fish to a variety of noxious agents in the water, such as pesticides, phenol and heavy metals (Nowak, 1992) [84]. Cengiz *et al*. (2001) [26] reported alterations in the liver of mosquito fish (*Gambusia affinis*) after exposure to sub-lethal concentrations of endosulfan: hypertrophy, sinusoid enlargement, haemorrhage, pyknosis, vacuolization of cell cytoplasm, infiltration of mononuclear lymphocytes and congestion, and these alterations were time and dose dependent. Similarly Edson *et al*. (2001) [43] reported that after acute (24hr) exposure of juveniles of *Prochilodus lineatus* to organophosphate pesticide, Dipterex 500 (R) (Trichlorfon), the hepatocytes became tumified with vacuolation. Additional alterations were cytoplasmic granulation, nuclear lateralization and the nuclei had various diameters and densities and condensed chromatin in the central region with pyknosis.

Nevertheless, Olurin *et al*. (2006) [92] exposed *Clarias gariepinus* fingerlings to sub lethal concentrations of the herbicide, glyphosate over a 42 day period. After the exposure period, the gills showed marked alterations in the epithelia in response to glyphosate treatment. There was fusion in adjacent secondary lamellae resulting in hyperplasia, with profound oedematous changes, characterised by epithelial detachment. In the liver, the enlargement of the hepatocytes was related to the concentration and duration of exposure to glyphosate. There were also large vacuoles in the hepatocytes, with pyknotic nuclei, and cytolysis that increased with concentration. Focal necrosis was also observed in the hepatocytes.

Histopathology of *Clarias gariepinus* exposed to glyphosate based herbicide

The histopathology of vital organs such as those of gills, liver and muscles (Simonato *et al*., 2008; Gabriel *et a.* 2007) [103, 54, 53] are good biomarkers to evaluate effects of xenobiotics to fish. Gills in particular are very sensitive organ that respond quickly to water pollution caused by xenobiotics (Akaishi *et al*., 2004) [3]. The histopathology result in the present investigation suggests that the mode of action of glyphosate on the exposed group of fish might have been mediated through damages done to vital organs such as gills, liver and muscles with the greatest damage shown to be on the gills showing degeneration in both the primary and secondary lamellae and extensive rupture of the same

By the 8th day, among group of fish exposed to the highest concentration. This may suggest that respiratory impairment across the gill epitheiia were hampered by degeneration effect of the herbicide which may have also caused death of fish among group exposed to the acute concentration as noted by Babatunde *et al.* (2000) [15] when they exposed fingerlings of *Oreochromis niloticus* to gramoxone. Gill lamellae digenarations in various folds reported in this investigation is in agreement with authors (Onusimka and Ufodike 2000; Fafioye *et al.*,2004; Gabriel *et al.*,2007 [93, 54, 47, 53]; Emetine *et al.,* 2012., Cariello *et al.,* 2013 [28] who noted that these changes were early responses and attempts by exposed fish to limit effects of toxicants. Gill oedema, telangiectasis, lamellae erosion and rupture of primary lamellae observed at higher concentration and period in this investigation is agreement with reports of Simonato *et al.* (2008) [103] on juveniles of *Proehidadus lineatus* exposed to crude oil water soluble fractions; Martinez *et al.* (2004) [64] made similar observation on juveniles of *Prochilodus lineatus* exposed to lead and similar to reported effect of kerosene exposure to *Clarias gariepinus* (Gabriel *et al,,* 2007) [54] and heavy metal exposed to *Clarias gariepinus* (Alaa and Ahmed, 2010) [5]. Permanent dilatation of venules and arterioles may have resulted to talengietasia and thus afforded increased flooding that occasioned irreversible changes in xenobiotic expositions.

The liver is the main organ of biotransformation and excretion of xenobiotics and their presence rapidly presents structural, biochemical and molecular alterations (Bnrnett *et al.,* 1999) Histopathological examination of liver indicated liver in this investigation observed rapid proliferation of hepatocytes (hyperplasia) and increased size of the same (hypertrophy), possibly as responses of liver hepatocyte to reduce the effects of toxicity. Dilatations of hepatocytes and shifts in intercellular spaces are in agreement with Simonato *et al.* (2008) [103] and Ricardo *et al.* (2010). Reported fatty degeneration suggests that liver damages were responsible for metabolic disorders, commonly associated with dietary deficiency in response to xenobiotic (Myers *et al.,* 1987; Akaishi iv *al*.. 2004) [27, 3]. Other damages reported at higher concentrations include vacuolation of fat globules; atrophy or disuse of limited areas of liver tissue and shrinkage of hepatocytes (cirrhosis) were reported in petroleum exposed *Clarias gariepimis* (Gabriel *et al.,* 2013) [53] and in the larvae of pejerrey fish *Odontesthes argentinensis* exposed to chronic toxicity of petroleum water soluble fraction (Emeline *et al*., 2012) [44].

Muscle disruptions reported in the present investigation include hyperplasia and hypertrophy at low concentration, during the early stage of the experiment probably as adaptive response to counter the effects of toxicant. Atrophy was due to the disuse of limited areas of tissue owing to insufficient nutrient and cell loss may have led to necrosis and splitting of muscle fibers observed at higher concentration. Period and dose dependent respective un-programmed cell death in muscle tissues caused by glyphosate effects may have resulted to fatty degeneration, suggestive that metabolic disorders occurred as commonly associated with dietary deficiency in response to xenobiotics (Myers, 1987) [27]. These observations corroborates with findings made on fish larvae exposed to other polluting

substances on other species (Akindi *et al.*, 2000; Nasir and Hantough 2010).

Conclusion

The present finding indicated that glyphosate based herbicide glycot impaired haematology, antioxidantive stress enzymes and vital histology of *C. gariepinus* and could serve as an early warning signs toward the avoidance of its ecotoxicological hazards in aquatic ecosystems.

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