HISTOPATHOLOGICAL EFFECTS OF GLYPHOSATE AND ITS TOXICITY TO THE EARTHWORM NSUKKADRILUS MBAE.

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ABSTRACT

This study was carried out to investigate the toxicity of Roundup, aglyphosate-containing herbicide to the earthworm *Nsukkadrilus mbae* and the associated histological changes. One hundred and sixty five earthworms were randomly divided into five groups and exposed to different concentrations of glyphosate (0.0, 13.0, 52.0, 117.0 and 207 mg/Kg soil) for 96 h. The mortality was recorded every 24h and after 96 h, sections of the earthwormfrom each treatment group were prepared for microscopic examination. There was no mortality in the control group and the survivors in the treatment groups were weak. The percentage mortality was least after 24 h and highest after 96 h and the mortality was significantly different (P = 0.05) in the groups except after 24h. The survival rate was generally concentration and duration dependent. The 96 h LC_{50} value was 10.4 mg/Kg soil. The observed histopathological changes included rupture of the cuticullar wall, tissue necrosis and prominent vacuolations. The circular and longitudinal muscle layers had altered structural integrity. These histopathological endpoints could be useful biomonitors of glyphosate toxicities in ecotoxicological studies involving earthworms.

Key words: ***Glyphosate, Nsukkadrilus mbae, contaminated soil, necrosis, biomarker.***

1 INTRODUCTION

Earthworms are importantsoil fauna whose activities directly affect the soil fertility and textural characteristicsas they feed and bring about the aeration of the soil. They are also critically important in the soil food chain [1]in both aquatic and terrestrial ecosystems. In modern times, agricultural intensification and production have tended to depend heavily on the use of agrochemicals either to control weeds or pests in farms. Due to their peculiar ecology in the soil, earthworms areanimal at risk [2] as they are constantlyexposed directly or indirectly to the applied pesticides.Thus, earthworms are good faunal candidates for ecotoxicological research as test organisms for the assessment of environmental impacts of pesticides and other chemical pollutants.

Glyphosate and other glyphosate-containing herbicides such as Roundup are among the few broad spectrum herbicides that are used in the world to boost food production and in horticulture [3-6]. In the United States (US) alone,Donaldson et. al. [7] and Kiely et al [8] noted that in 1987, between 2,600 – 3,600 metric tons of glyphosate-based herbicides were used and that by 2001, the usage had astronomically increased to between 39,000 to 41,000 metric tons. It is our expectation that by 2013 the use of glyphosate formulations in US alone would have increased by 13.5-folds. We further reckon that there is a corresponding worldwide increase in the use of these herbicides.

Roundup contains polyoxyethylene alkyl amine (POEA) as the surfactant [CAS No 61791-26-2] [9]that is more toxic than the pure glyphosate [10-13].This is also true of other surfactants found in herbicides. These surfactants alter the functional characteristics of affected biomembranes [14]. According to SERA [12], the contribution of POEA to the toxicity of formulation is more with regards to glyphosate in the mixture.

Pesticides are known to have varied effects on earthworms. For example, loss of biomass was reported in *Eisenia foetida* exposed to glyphosate, 2, 4-D herbicides, acetochlor and metamidophos [15-16]. Also,growth inhibition was observed in *E. foetida* treated with metolachlor and its isomers [17] and in *Aporrectodea caliginosa* treated with isoproturon [18].

Enzymatic activities in earthworms are also affected by both chemical pollutants and pesticides. ATPase and cholinesterase activities in *E. fetida* were inhibited by albendazole[19] and imidazolium ionic liquids [20]. On the contrary, increased activities of such enzymes as aldrin epoxidase, cytochrome P 450 monooxygenases and glutathione-S transferase were observedin *E. fetida* treated with acetochlor [21].Generally, information on mortality has remained one major parameter for evaluating the toxicity of pesticides in earthworms[15-16, 19,22-24]. Also, changes in the structural integrity of the walls of earthworms [1, 11 -13] are fast becoming good indices of herbicide toxicity.

Studies have shown that Roundup has adverse effects on non-target organism in the environment including man. In mammals, it affects the electron transport chain, enhances mitochondrial membrane permeability [25], inhibits cytochrome P450 and monooxygenase [26, 27-29]. The studies by Ololurnsogo et. al [29] showed that N-phosphonomethyl salt of glycine uncoupled the oxidative phosphorylation in the rat liver mitochondria and inhibited transhydrogenase activity and consequently, it caused impairment of mitochondrial functions [30].Based on changes on green fluorescent protein, Rekek et al.[31] reported that glyphosate-containing herbicide, TouchDown, promoted neurodegeneration in both dorsal nerve ring and ventral nerve as well as the shortening of retrovesicular ganglia in the nematode Caenorhabditis elegans.It has also been reported [15,32-34] that herbicides affect the histological integrity of the body walls of earthworms.

Marc et al [35] reported that Roundup inhibited protein synthesis and impaired cell cycle processes by delaying CDK1/ Cycling B activation. Also, Nora and Gilles-Eric [36] observed that Roundup inhibited succinic dehydrogenase, caused DNA fragmentation, pyknosis, altered membrane permeability and necrosis and apoptosis.

In Nigeria, thereis dearth of information on the effects of herbicides on earthworms and moreover,the use of earthworm in evaluating the ecotoxicological impacts of pesticides is not widespread. The objective of this study wastherefore, to investigate the toxicity of glyphosate-containing herbicide, Roundup to theearthworm *Nsukkadrilus mbae* as well as changes in its histology under laboratory conditions.

2 MATERIALS AND METHODS

2.1 Collection of Test Organisms and Experimental Design

Two hundred earthworms used in this study were collected from the Zoological garden and acclimatized for seven days before the commencement of the study. After acclimatization, one hundred and sixty five

(165) individuals were randomly divided into five treatment groups of 33 individualseach. Each treatment was further randomized into three replicates containing eleven (11) earthworms each.

Earthworms in treatments 1, 2, 3, and 4 were exposed to 13.0, 52.0, 117.0 and 207.0 mg/kg soil of glyphosate. The fifth group, a control, without glyphosate (Roundup) application, was also set up. All the concentrations were mixed thoroughly with topsoil samples that were sterilized by heating to kill any earthworm in the soil.

2.2 Preparation of glyphosate test concentrations

The commercial preparation of Roundup(glyphosate-containing herbicide)containing 480g/L of isopropyl amine salt (active ingredient) was used as the stock solution. This stock contains polyoxyethylenealkylamine (POEA) surfactant whose contribution to the toxicity of the commercial formulation is more with regards to glyphosate toxicity in the mixture [12].This commercial stock was used to prepare the various test concentrations (13.0, 52.0, 117.0 and 207.0 mg/kg soil).

2.3 Mortality Study.

The mortality in each groupwas recorded every 24h for a period of 96 h. The earthworms were confirmed dead when they remained immobile and motionless when pricked or touched with an object. The

percentage mortality was calculated using theAbbott method [37] for toxicity studies after correcting for natural (control) mortality.

2.4 Histological Studies

At the end of the toxicity tests, live earthworms from each treatment including the control were taken and washed with distilled water. The earthworms were transferred into jars containing agar gel and left for another 96 h to facilitate the removal of the sand content of the gut [19, 38] as the agar is easily eaten by the earthworm. Thereafter, they were cut into twohalves and put into a specimen bottle; fixed with Bouin's fluid for 12 h before subjecting it to histological procedures of embedding in paraffin wax, sectioning and staining with haematoxylin eosin for microscopic observation.

2.5 Statistical Analysis

The data obtained were analysed for differencesamong the treatment groups using one-way analysis of variance (ANOVA) at 95 % level of significance followed by FSLD post-hoc test. The LC_{50} was determined using probit analysis [39]

3 RESULT

3.1 Mortality

The result of the study showed that there was no mortality in the control group (Table 1). The mortality rate was least at the beginning of the study (24 h) and increased with exposure period.There was no significant difference (P = 0.05) in the mortality data in the groups exposed to 13 and 52 mg/ kg soil after 24 h.Similarly,the mortality in the earthworms exposed to 117 and 207 mg/ kg soil did not vary after 24 h h of exposure.There was a significant difference (*P* = 0.05) in the mortality of the earthworms between different treatment groups and within the same concentration at different exposure periods.The percentage mortality was 6.1 in the groups exposed to 13 and 52 mg/ kg soil after 24 h while the mortality rate was 9.1 in the group exposed to 117 and 207 mg/ kg soil.

Generally,the percentage mortality(Fig.1) increased withincreasingglyphosate concentration and duration of exposure in the treatment groups.duration of exposure in each treatment. After 96 h, the percentage mortalities were 36.4, 57.6, 75.8 and 84.8 in the groups treated with 13, 52, 117 and 207 mg/ kg soil, respectively.There wasa significant difference (*P* = 0.05) in the mortality of the worms exposed to glyphosate except after 24h.The percentage survival (Table 2) was highest at 24h interval and least after 96h. The survivalrate of *N. mbae* in the various groups did not vary after 24h and 48 h (*P*= 0.05) but was significantlydifferentafter 72 h and 96h (*P*= 0.05).

The probit plots of the mortality data are shown in Figs 2-5. The mean lethal concentration (LC_{50}) values of glyphosate were 12.69 mg/Kg soil (x^2 = 0.4), 10.4 (x^2 =0.91), 9.46 (x^2 = 6.84) and 10.49mg/Kg soil(x^2 =5.21) at 24, 48, 72 and 96h, respectively. The LC_{50} values at 48 and 96h intervals did not differ (P =0.05) but was different at 24 and 72 h (*P* =0.05).The plots of best fit of the probit transformed data (linear plots) showed that there was high degree of positive correlation (r = 0.9879) between the parameters.

3.2 Histopathology

There are no visible alterations in the histology of the body wall of *N. mbae* in the control (Plate 1). The earthworms exposed to different concentrations of glyphosate developed varied histological changes (Plates 2-5). The most prominent changes included sequestration of the circular and longitudinal muscles. Also, there was generalized cellular cytolysis, tissue vacuolization and necrosis as some of the cells had no nuclei. Degenerative zones in the cuticle were noticeable as well as tissue erosion.Similarly, the chloragogenous layer was vacuolated and the cuticle was ruptured. The circular muscle layer was separated from the longitudinal muscle layer in some areas. There were also noticeable cytoplasmic and nuclear alterations in the epidermal cells and those of both circular and longitudinal muscles.

4 DISCUSSION

The result showed that glyphosate was very toxic to the earthworm at 72h (LC_{50} 9.46mg/Kg soil). Earlier studies showed that the toxicity of pesticides to earthworms varied depending on the species, pesticide and the test concentrations. Joanna and Aleksandra [40] reported that the LC_{50} of Bofix 260EC and Glifocyd 360 SL to *Eisenia foetida* were 4.17mg/dm^3 and 320mg/dm^3, respectively. The LC_{50} of 2, 4, 6-trinitrottoluene to earthworm *Eisenia* sp was 364.9 mg/Kg [22] while LC_{50} values in the range of 115.6-275.3 and 29.5-228.6mg/kg when *E. fetida* was exposed to acetochlor and methamidophos [16] respectively. Also, the 96h LC_{50}values of atrazine and chlophrifos to *N. mbae*and *Peronyx excavatus* were 7.23mg/kg soil and 122 mg a.i /Kg, respectively[22,41]. The LC_{50} values calculated in this study after 48h, 72h and 96h are comparable with the LC_{50} of 10mg a.i/kg reported for *Eseinia andrei* exposed to carbofuran [24]. On the other hand, Joanna and Aleksandra [40] reported a higher LC_{50} of 160 and 320 mg/dm^3 of Glifocydfor the earthworms *Dendrobaena veneta* and *E. fetida*, respectively.Also, the reported LC_{50} of glyphosate formulation in this study is comparable to acute LC_{50} of 8.0% reported whenC. elegans was treated with glyphosate formulation TouchDown.[31]. It is also comparable to>10 mg/L 48 h TL_{50} of glyphosate report by the US Environmental Protection Agency [42]for Atlantic oyster.Data on the mortality showed that the percentage mortality after 96h was highest (84.8%) in the group treated with 52mg/Kg soil of glyphosate and least in the group treated with 13mg/Kg soil). Oluah *et.al.*[41] reported that the

percentage mortality of *Nsukkadrilus mbae* exposed to 0.4, 0.8, 3.0 and 9.0 mg/kg soil of atrazine were 37.8, 73.3, 77.8 and 80%, respectively. Also, Correia and Moreira [15] reported 100% mortality in earthworm exposed to 500 and 1000mg/kg 2, 4-D herbicide. Similar high mortality[43]of 92.5% and 65% were observed when *E. foetida* was exposed to endosulfan (20.1 l/ha) and carbofuran (20kg/ha), respectively. On the contrary, Correia and Moreira [15] reported no mortality in the earthworm exposed to varying concentrations of glyphosate.

This study showed that exposing earthworm to glyphosate resulted in alterations in the microstructural integrity of the earthworm. This histological changes observed in this study are similar to those reported when the *N. mbae* was treated with atrazine [41]. Similar structural changes in both the circular and longitudinal muscles of *Lumbricus* sp in polluted soils [44] and in *E. foetida* treated with malathion [34]. Loss of chromatin [45] and swollen nuclei in the chloragocytes [46] were observed in earthworm *Pheretima posthumus* treated with glyphosate and carbaryl. Testicular tissue damages, pyknotic cells, cytolysis and tissue vacuolations found in this study were also documented in *E. foetida* exposed to sublethal malathion [34,47-48] and in *N. mbae* exposed to atrazine [41].

Earlier studies with higher animals have demonstrated that glyphosate-containing herbicides cause DNA fragmentation, chromosomal damage and aberration [49,35,36] inhibition of mitochondrial function, increased membrane permeability[28-30] and neuronal damage[31,34]. Similar reports [32, 53] indicated that there are distortions in the microtubular and DNA structures in male reproductive system of the earthworm *Eudichogaster kinneari* and *Eisenia sp*due to dimethioate and benomyl treatment. The rupturing of the earthworm cuticular layer and the alterations in the muscular architecture in the glyphosate-exposed *N. mbae* could be regarded as indications of glyphosate toxicity. Such histological changes could predispose the animal to impaired intracellular transport mechanisms, dysfunctional biochemistry and physiology. The net effect would be the development of necrotic conditions which are indicative of osmoregulatory imbalance. These would lead to cell death- ultimately manifesting at theorganismal level.

Furthermore, It is known that pesticides inhibit acethylcholinesterase in animals [50-51] thereby preventing the hydrolysis of acetylcholine. This would result not only in the stimulation of cholinergic nerves but also the GABAnergic and dopaminergic neurons leading to decreasing muscle control [52] and indeed overall body weakness observed in the *N.mbae* that survived glyphosate formulation exposure in this study.

5 Conclusion

This result showed that glyphosate affects the structural integrity of the earthworm thereby impacting on the biochemical and physiological functions in the organism. The observed histological effects of glyphosate on *N.mbae* are good biological indicators of stress and osmoregulatory disorder and could thus be used as histological endpoints of pesticide toxicity in ecotoxicological studies involving earthworms.

Acknowledgement

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Declaration of interest

The authors have no declaration of interest to make on this work.

Table 1. Mortality of earthworm *Nsukkadrilusmbae* treated with glyphosate.

Concentration (mg/kg soil)	Duration of Treatment (hours)			
	24	**48**	**72**	**96**
Control (0.0)	0^{a1}	0^{a1}	0^{a1}	0^{a1}
13.0	2.0^{b1}	6.0^{b2}	9.0^{b3}	12.0^{b4}
52.0	2.0^{b1}	7.0^{c2}	11.0^{c3}	25.0^{c4}
117.0	3.0^{b1}	8.0^{d2}	19.0^{d3}	19.0^{d4}
207.0	3.0^{b1}	10.0^{e2}	12.0^{e3}	28.0^{e4}

Value in the same column with the same superscript(lower case) are not significantly different (p = 0.05) between different concentrations within the same exposure duration. Values with different numeric superscripts differ significantly (P=0.05) between different exposure periods within the same concentration.

Table 2: Percentage survival of *Nsukkadrilusmbae* exposed to glyphosate herbicide.

Concentration (mg/kg soil)	24hrs	48hrs	72hrs	96hrs
Control	100	100	100	100
13.0	93.9	81.8	72.7	63.6
52.0	90.9	69.7	42.4	24.2
117.0	93.9	78.7	66.7	42.4
207.0	90.9	75.8	66.7	12.1

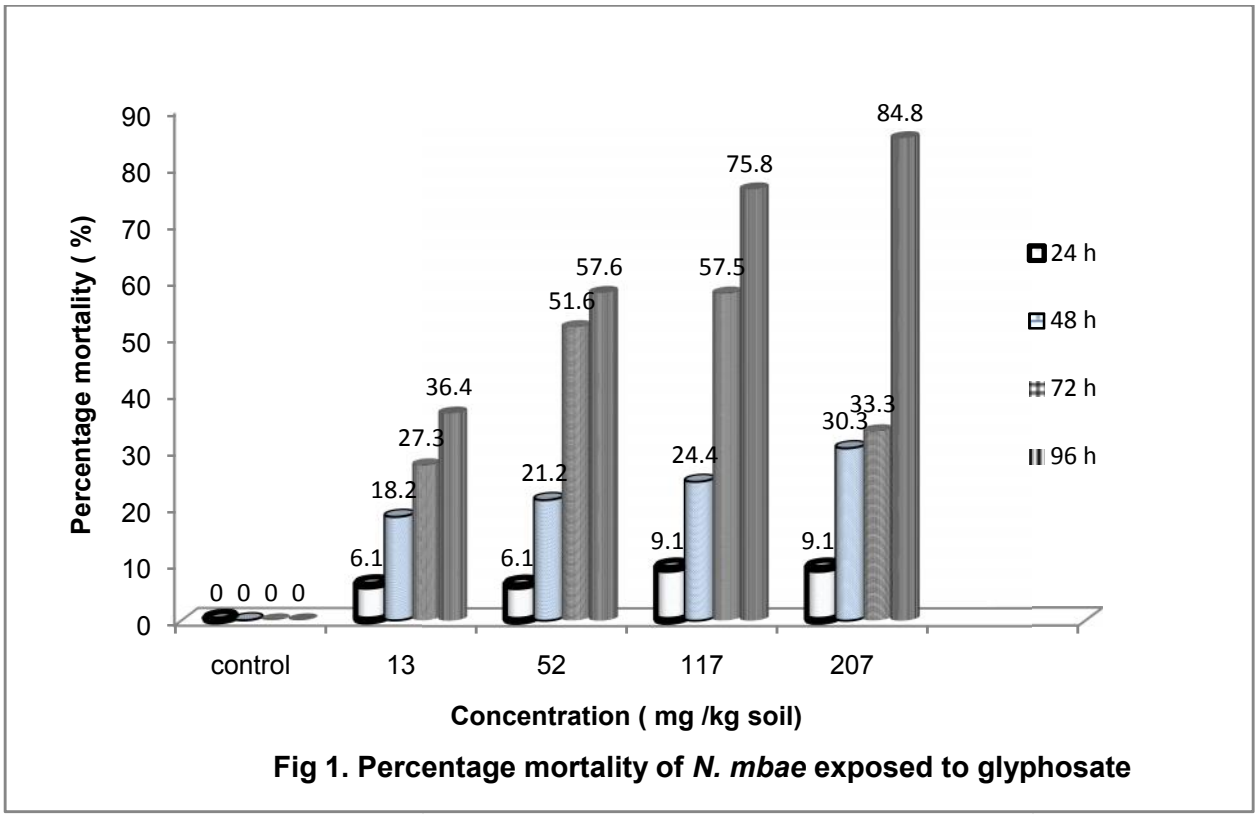

Fig 1. Percentage mortality of *N. mbae* exposed to glyphosate

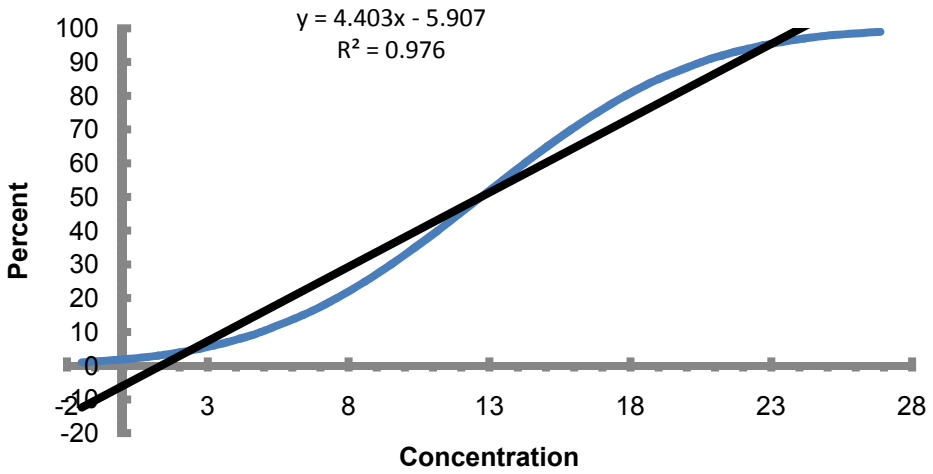

Fig.2 Probit plot (sigmoid) of *N. mbae* exposed to glyphosphate for 24 h and the plot of best-fit (linear) of the probit transformed data.

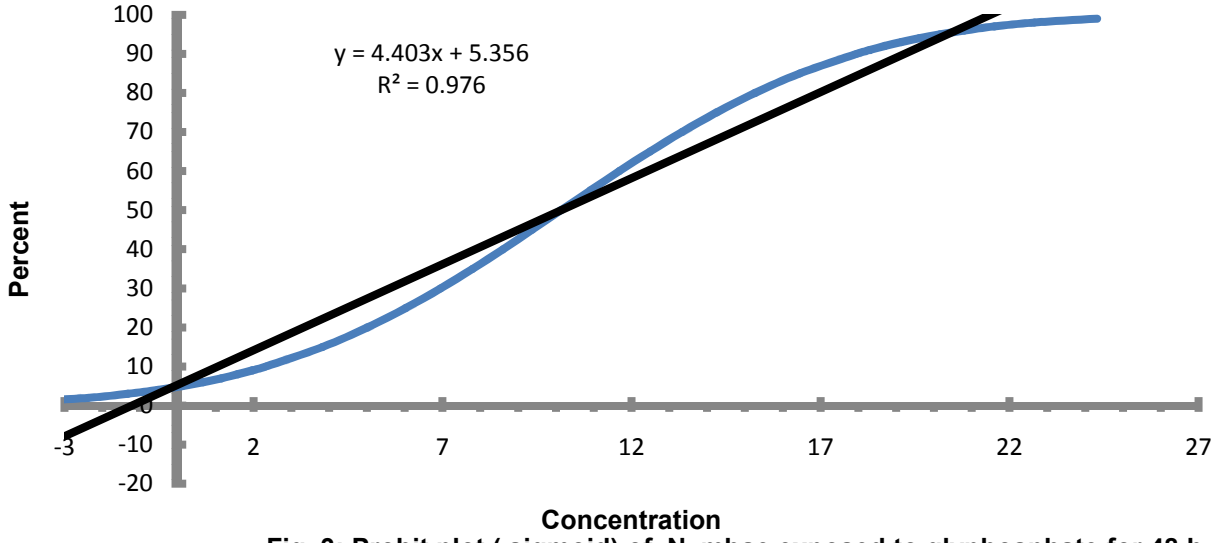

Fig. 3: Probit plot (sigmoid) of N. mbae exposed to glyphosphate for 48 h and the plot of best-fit (linear) of the probit transformed data.

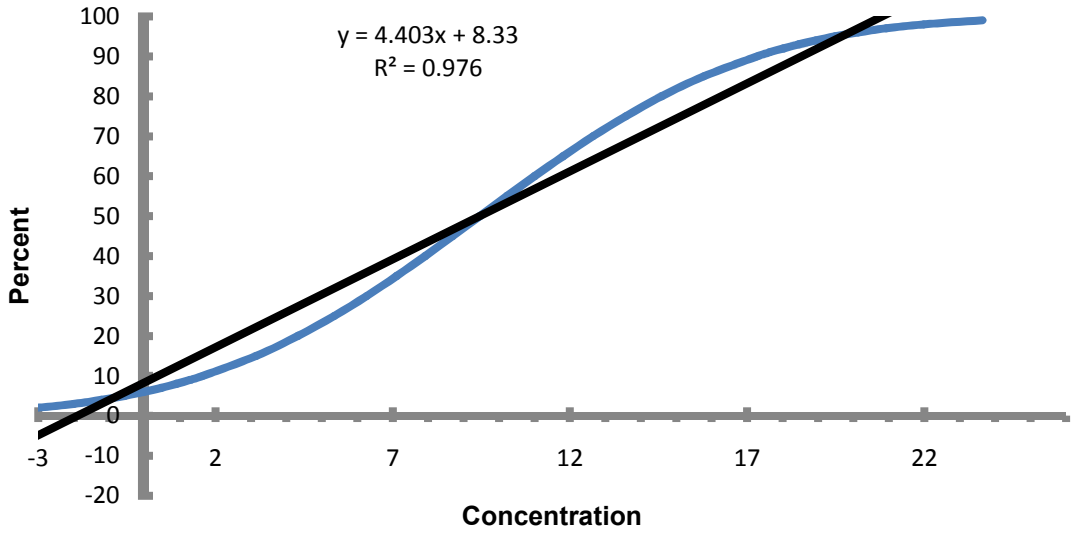

Fig.4 Probit plot (sigmoid) of *N. mbae* exposed to glyphosphate for 72 h and the line of best-fit (linear) of the probit transformed data.

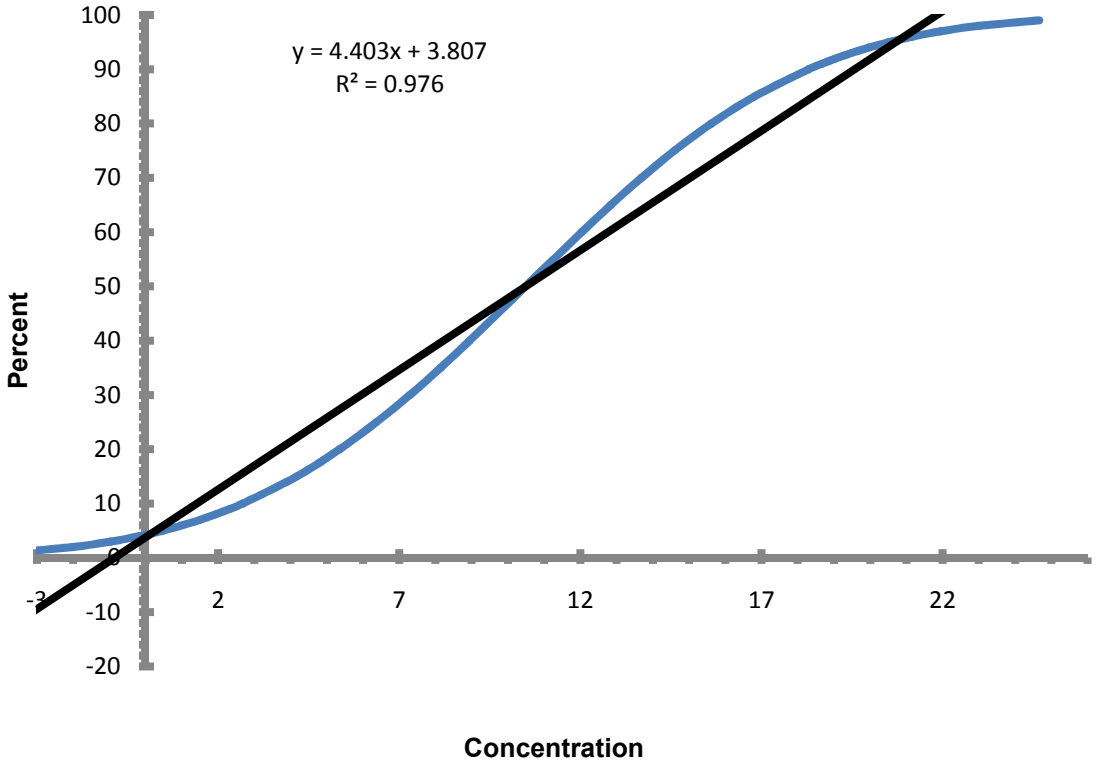

Fig. 5. Probit plot (sigmoid) of *N. mbae* treated with glyphosphate for 96 h and the line of best-fit (linear) of the probit transformed data.

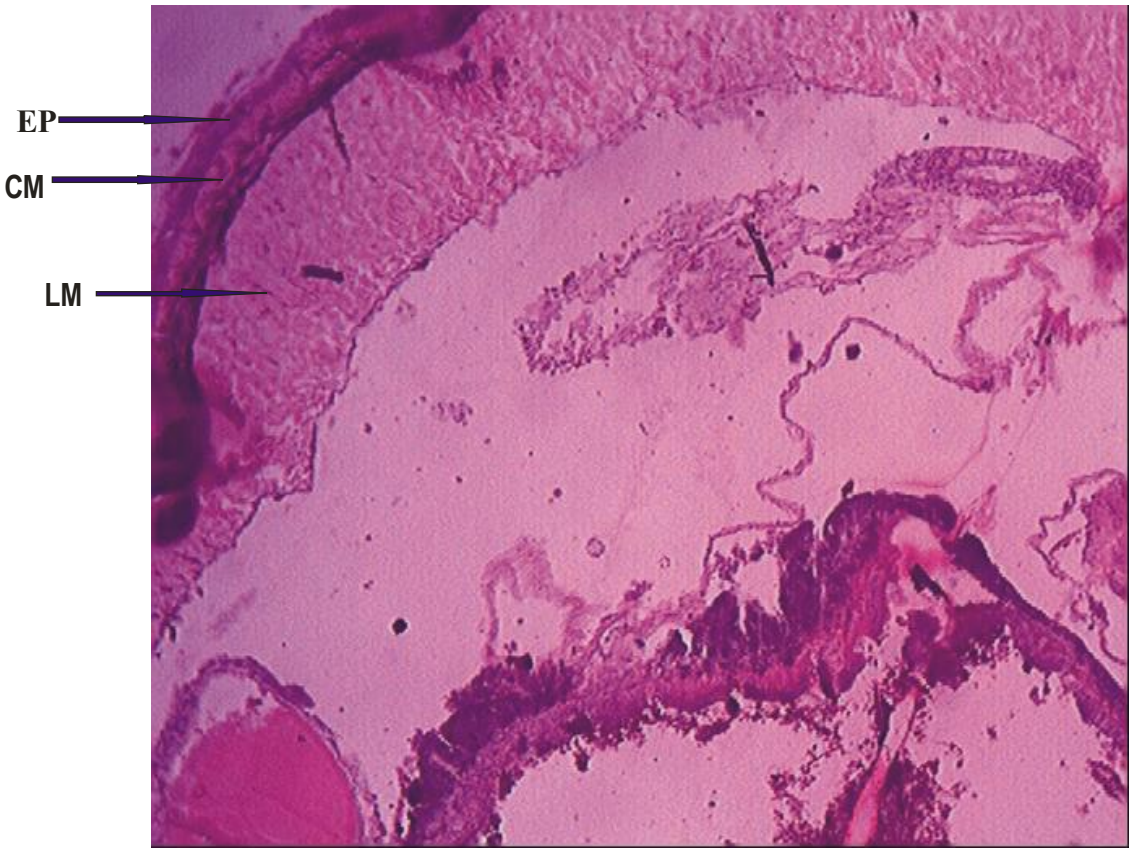

Plate 1. Control earthworm showing normal tissue organization (X100)

EP = Epidermis, CM = Circular muscle , LM= Longitudinal muscle

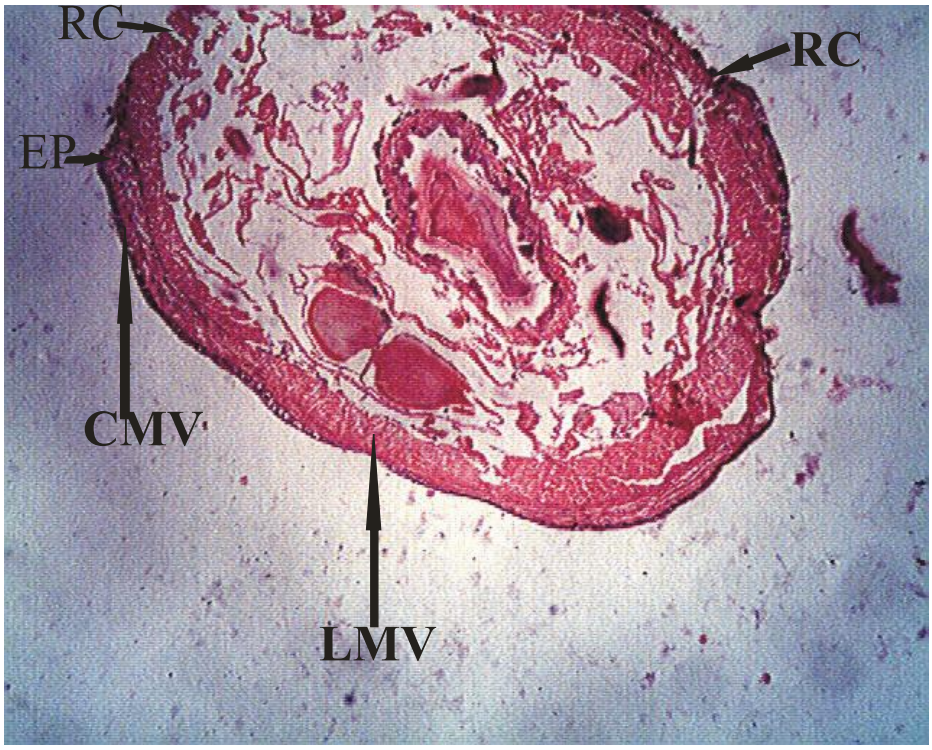

Plate 2. Section of N. mbae exposed to 13 mg/ Kg glyphosate for 96 h (x 100)
EP = Epithelium, CMV= Vacuolized circular muscle,
LMV - Vacuolized longitudinal muscle, RC = Rupturing cuticle.

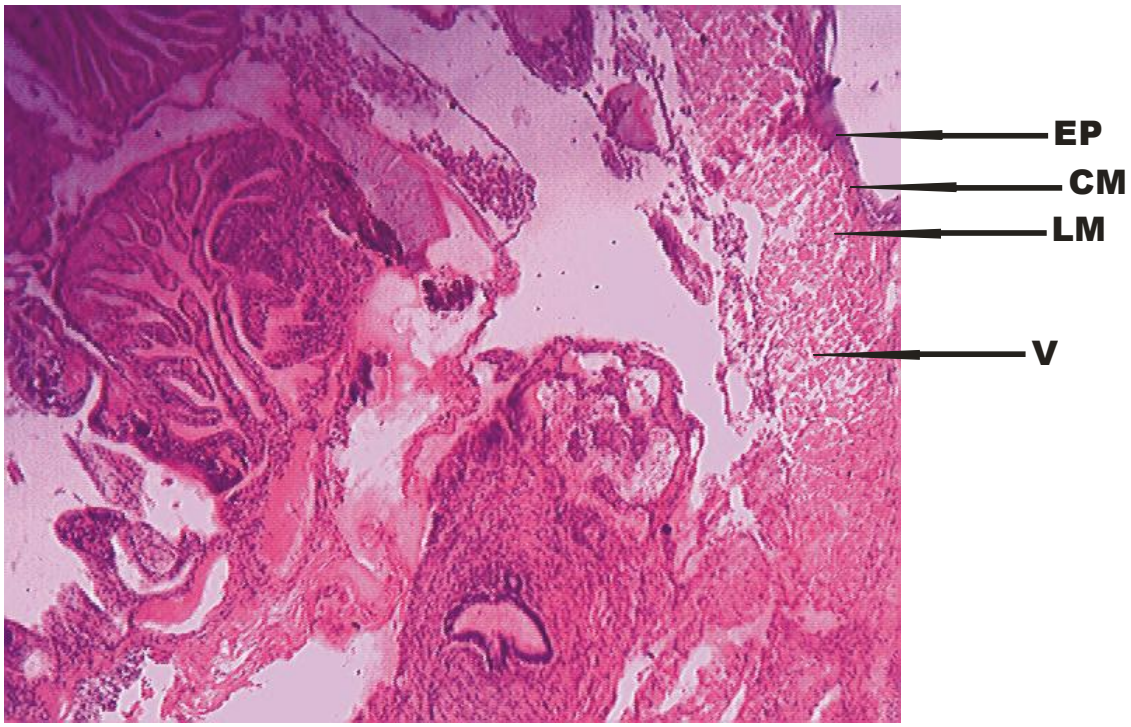

Plate 3: Section of N.mbae exposed to 52mg/Kg glyphosate for 96h

EP= Epithelium; CM= Circular muscle; LM= Longitudinal muscle; V= Vacuolated longitudinal muscle.

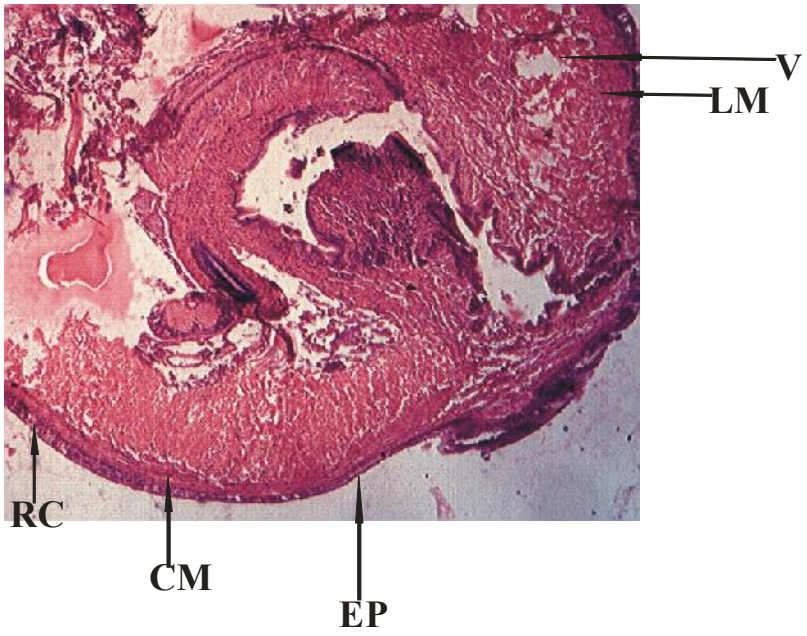

Plate 4. Section of N. Mbae exposed to 117 mg /Kg glyphosate for 96 h (x 100)
RC -= Rupturing cuticle, CM = Circular muscle, LM = Longitudinal muscle
V = Vacuolation of the muscle layer

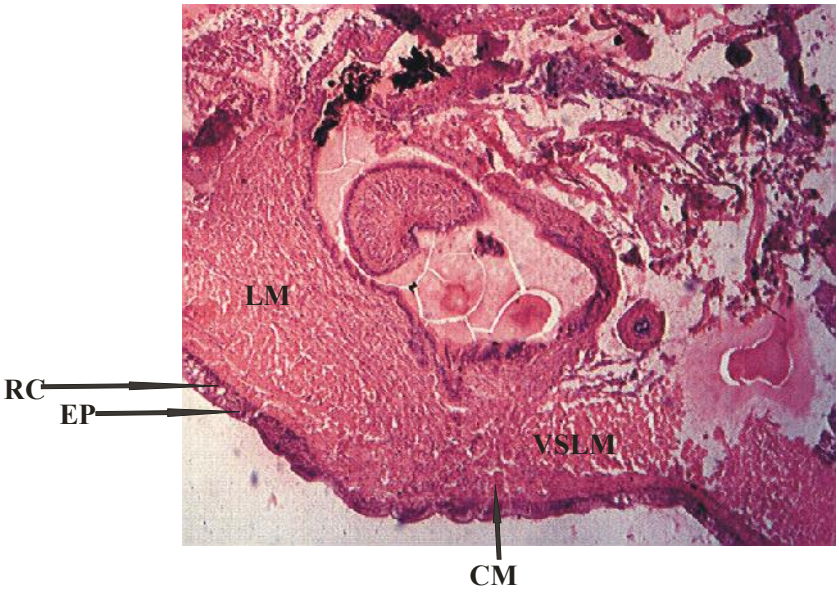

Plate 5. Section of *N mbae* exposed to 207 mg/ Kg glyphosate for 96 h(x 100)

RC = Rupturing cuticle, EP = Epidermis, CM = Circular muscle, LM = Longitudinal muscle, LM = Longitudinal muscle, VSLM= Vacuolized and sequestered longitudinal muscle

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