TOXICITY OF ROUNDUP (A GLYPHOSATE PRODUCT) TO FINGERLINGS OF *Clarias gariepinus*

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ABSTRACT

Acute static renewal bioassays were conducted on fingerling and adult of Clarias gariepinus (mean weight, 1.22 ± 0.6g; mean total length, 5.25 ± 1.25 cm) using the herbicide, Roundup (glyphosate). In the acute study, fingerlings were exposed in triplicate to 0.0, 14.0, 16.0, 18.0, 20.0 22.0, and 24.0 mg/l of the herbicide for 96 hours to determine general behavioural responses and specific responses (opercular beat frequency (OBF), tail beat frequency (TBF), lethal concentration (LC_{50}) and median lethal time (MLT_{50}). Exposed fish showed initial stress responses such as increased opercular ventilatory rate, dash and erratic swimming and gasping for air. The pattern of response of TBF to time and concentration of the herbicide was irregular, whereas that of OBF tended to decrease with increase in time, but increased with increase in concentration. Cumulative mortality values increased with exposure time. The effect of exposure time on the behavioural variables was: TBF ($p > 0.05$), OBF ($p < 0.001$) and cumulative mortality ($p < 0.01$) and that for concentrations were TBF ($p > 0.05$), OBF ($p < 0.01$) and cumulative mortality ($p< 0.001$). Interactions between time and concentration produced significant effect only in cumulative mortality ($p < 0.01$). The 96 hour LC_{50} of Roundup on the fish was 19.58 mg/l. The MLT_{50} decreased with exposure concentration with 24 mg/l killing half the exposed fish at 19.69 (17.41 - 21.94 CL) hours. Results from this study show that the herbicide cannot be described as having "low toxicity and being environmentally friendly" as suggested by the manufacturer, Monsanto.

Keywords: Roundup, Toxicity, Behavioural responses, *Clarias gariepinus*

INTRODUCTION

Glyphosate products are manufactured by Monsanto Company worldwide and marketed under a variety of trade names such as Roundup, Rodeo, Accord, Vision, Pondmaster, Landmaster and Touchdown among others in different parts of the world. Roundup, an organophosphate plays very important roles in fisheries management (Caffrey, 1994). It has been severally used in the control of aquatic weeds in Nigerian waters (Olaleye and Akinyemiju, 1995; 2002; Ezeri, 2002) indicating preference for it rather than other organophosphate herbicides. The aquatic ecosystem has been contaminated with pesticides including glyphosate products which may result from drift from aerial and ground applications despite established buffer zones and run-offs from agricultural fields (Perschbacher *et al.*, 1997). In the aquatic environment these may be toxic to several non-target organisms. The application rates for the various forms are liquid, 12l/ha and solid, 2.2

kg/ha. The recommended maximum level of glyphosate in 1m depth water after application is 0.6 ppm (Tooby, 1976). However, this may often be exceeded as the levels of the herbicide in the water column after application are not monitored. Glyphosate-based products are described by the manufacturer as pesticide of "low toxicity and environmental friendliness" (Cox, 2001). But results from several studies involving man and other organisms strongly suggest that the product can be toxic, producing a number of physiological changes in organisms and in some cases resulting in mortality depending on the level, duration and route of exposure (Cox, 2001).

Acute levels of glyphosate products and their mixtures have been shown to be toxic to early life stages of fishes, producing mortality and various abnormal behavioural changes which may be deleterious to the survival and reproduction of affected species (Abdelghani, *et al*., 1997; Cox, 2001; 2002). Toxicity of glyphosate, its products and/or surfactants on cold and water species like minnows (Beyers, 1995); salmons (Chapman, 1989; Wan *et al*., 1991) and trout (Mitchell *et al*., 1987) have been extensively investigated both in the field and under laboratory conditions. Other fish species studied included carp (Liong *et al*., 1988), *Tilapia* spp. (DeSilva and Ranasinghe, 1989) and mosquito fish, *Gambusia affinis* (Feei, 1987) and gold fish, *Carassius auratus* (Anton *et al*., 1994). These studies revealed that the responses of aquatic organisms to acute concentrations of glyphosate, its product and/or surfactants were variable possibly due to i) differences in the formulation of the herbicide, ii) level of mixture of component herbicides, iii) species-specific sensitivity and iv) condition of the media which are known to modify toxicity of the herbicides to exposed organisms. However, there is paucity of information on the toxicity of the agrochemical on important aquaculture species in the tropics.

Results from several investigations involving the use of several fish species support the concept that toxicant-induced stress on organisms can be quantified by methods other than mortality (Saglio *et al*., 1996; Gabriel and Kparobo, 2002; Gabriel *et al.,* 2009). Hence, changes in fish behaviours can be used as a sensitive indicator of acute and sublethal toxicant exposure. Influence of toxicants on the opercular beat frequency, OBF and tail beat frequency, TBF (Ekweozor *et al*., 2001) and schooling, spawning and feeding behaviours in several fish species have been studied (Saglio *et al*., 1996; Saglio and Trijasse, 1998). Results from some studies involving the clariids seem to suggest that changes in OBF and TBF of fish exposed to various toxicants was directly related to toxicant concentration but inversely related to time of exposure (Oti and Ukpabi, 1999) and could be used to monitor the negative effect of herbicide application before mass mortality that may result from indiscriminate use of the herbicide.

Information on the lethal levels of Roundup on fingerlings and its effects on the behavioural changes such as tail and opercular beat frequency of *C. gariepinus* are virtually non-existent. Therefore the objectives of this research were to study the behavioural responses (tail and opercular beat frequencies per minute, TBF and OBF), lethal concentrations and median lethal time to death of fingerlings of *C. gariepinus* under exposure to acute Roundup®.

MATERIALS AND METHODS

The fingerlings of *C. gariepinus* (mean weight 1.22 ± 0.6g; mean total length 5.25 ± 1.25 cm) were obtained from a private farm, Comsystem, Kpite, Rivers State and transported in 25 litre jerry can to the Wet Laboratory, Department of Fisheries and Aquatic Environment, Rivers State University of Science and Technology, where they were distributed 60 fish per aquarium in four rectangular aquaria filled with 20 litre borehole water (dissolved oxygen, 0.01 ± 0.05 mg/l, pH- 7.5 ± 1.3; conductivity, 410 ± 20.4 µS/cm; total dissolved solid 400 ± 10.25 ppm). They were fed at one percent biomass, half at 0900 and 1600 hours for a week. Cleaning of the tanks and water exchange were done daily. Mortality during acclimation period was less than one percent. Mucus accumulation on the skin as well as gills and skin pigmentation were recorded.

Range finding test and trial runs were done according the methods in Reish and Oshida (1987) with some modifications. Twenty litres of each of the following concentrations: 14, 16, 18, 20, 22 and 24 ppm of Roundup containing 360 g/*l* glyphosate (in the form of 480g/*l* isopropylamine salt) and a control were prepared in triplicate in glass aquaria. Ten fish was randomly distributed into each of the tanks. The general behaviours, opercular beat frequency, OBF, tail beat frequency, TBF and mortality (%) were recorded at 12, 24, 48, 72 and 96th hour, respectively. The exposure lasted for 96 hours.

Data obtained from the experiments were subjected to ANOVA using Statistical Package for the Social Sciences, SPSS version 15 and differences among means were separated by Duncan Multiple Range test at 0.05%. The dependent variables in the trials (OBF, TBF and cumulative mortality) were regressed on concentration of the toxicant to obtain the regression lines of best fit for predicting the values of the dependent variables with changes in that of the independent with Microsoft Excel®. Correlation analysis was used to determine the degree of association among the dependent and independent variables. Lethal concentrations (LC_{50}) values for the 24, 48, 72 and 96 hour and the median lethal times (MLT_{50}) for the various concentrations of herbicide were done with Probit Analysis (Finney, 1971). Safe concentration of the herbicide at the various time intervals were obtained by multiplying the lethal concentration by a factor, 0.1 (EIFAC, 1983). The interaction effects of the behavioural responses (TBF and OBF) with exposure duration and concentrations of the herbicides were presented graphically.

RESULTS

On introduction into the toxicant the fish showed initial hyper-excitability, stress responses such as increased opercular ventilatory rate, dash and erratic swimming and gasping for air within the first two hours. As exposure time increased before death occurred they "hung" on the surface of the solution gulping air, fell steadily to the aquaria bottom. This was usually followed by dash swimming. This sequence was repeated several times before the fish lost balance, lay flat on the bottom (exertion), tail beat stopped, followed by cessation of opercular movement and then death (non-response to tactile stimuli).

The pattern of response of TBF/min. to time of exposure and concentration of the herbicide was irregular, whereas that of OBF tended to decrease with time, but increased with increase in concentration (Figures 1 and 2). OBF differed with the time and concentration of exposure ($p < 0.001$), but TBF did not ($p > 0.05$). The various concentrations of the herbicide produced significant changes only in the OBF ($p < 0.001$), but not in the TBF, $p > 0.05$ (Table 1). OBF appeared to be more responsive to the treatment than TBF. Tail beat stopped long before opercular ventilation ceased, then followed by death (Figures 1 and 2).

Interactions between the exposure time and concentration of the herbicide did not produce marked changes in the OBF and TBF ($p > 0.05$). Differences in the responses of TBF to the interactive effects of the various concentrations of Roundup and time of exposure were narrowest at the 48th and 96th hours, but vary widely between the 24th and 72nd hours (Figure 1). For the OBF it was narrowest at the 24th hour, became increasingly wider from the 48th to the 72nd hour, and decreased at the 96th hour (Figure 2).

Normal probability plots (lines of best fit) for predicting the values of the dependent variables (OBF, TBF and mortality, %) on the fixed variables (time of exposure and concentration of Roundup) showed the relationship were very variable (Table 2). Correlation analysis showed that OBF/min. was negatively correlated with time ($r = -0.32$, $p < 0.01$), TBF/min. ($r = -0.06$, $p > 0.05$) and cumulative mortality ($r = -0.31$, $p < 0.01$); but it is positively correlated with concentration of Roundup ($r= 0.05$, $p > 0.05$). TBF/min. was negatively correlated with time of exposure ($r = -0.03$, $p>0.05$), concentration of Roundup ($r = -0.20$, $p < 0.05$) and cumulative mortality ($r= -0.19$, $p < 0.05$).

Table 1: Tail and opercular beat frequency (TBF and OBF) and cumulative mortality of fingerlings of *C. gariepinus* exposed to various concentrations of Roundup for 96 hours

Variable	Time of exposure (hours)				
	12	24	48	72	96
TBF/min.	5.45 ± 2.73[b]	15.49 ± 4.41[ab]	7.87 ± 4.41[ab]	21.12 ± 5.22[b]	4.49 ± 5.40[b]
OBF/min.	113.50 ± 7.23[a]	113.10 ±7.60[a]	115.23 ±7.23[a]	10.79 ± 8.99[a]	65.15 ± 9.15[b]
Cum. mortality	6.67 ± 6.86[e]	37.227 ± 31.21[d]	56.11 ± 36.16[c]	63.89 ± 32.39[b]	73.33 ±29.31[a]

Variable	Concentration of Roundup (mg/l)						
	0.0	4.0	16.0	18.0	20.0	22.0	24.0
TBF/min.	15.73 ±4.41[a]	5.79 ±4.41[a]	16.46 ±4.41[a]	9.00 ±4.63[a]	7.04 ±5.92[a]	0.00 ±0.00	0.00 ±0.00
OBF/min.	96.71 ±7.60[bc]	123.27 ±8.00[ab]	108.58 ±8.00[a-c]	100.93 ±8.00[a-c]	73.42 ±10.19[c]	124.72 ±16.99[ab]	136.99 ±16.99[a]
Cum. Mortality	0.00 ±0.00	22.67 ±12.80[d]	24.00 ±24.43[d]	32.67 ±19.81[c]	50.67 ±37.70[b]	77.33 ±36.15[a]	77.33 ±37.89[a]

Means with the same superscript in the row are not significantly different (p>0.05)

Table 2: Regression lines of best fit for the prediction of the values of OBF/min., TBF/min. and cumulative mortality of *C. gariepinus* exposed to acute levels Roundup for 96 hours

Dependent Variable	Independent variable	Prediction equation	Curve type	r^2
TBF	Time	$Y=2.8415e^{0.221x}$	Exponential	0.9828
TBF	Concentration	$Y=0.0017x^2 +0.0192x$	Power	0.9442
OBF	Time	Y=23.314Ln(x) +16.325	Logarithmic	0.9186
OBF	Concentration	Y=25.117Ln(x) +19.269	Logarithmic	0.9812
Mortality	Time	Y=0.513x	Linear	0.9922
Mortality	Concentration	$Y=0.0021x^2 + 0.2451x +4.4278$	Polynomial	0.9722

Where x = independent variable, y= dependent variable

Table 3: Lethal concentrations and associated 95% confidence limits of Roundup to *C. gariepinus* fingerling exposure to Roundup for 96 hours

Time (hours)	Lethal Concentration	Safe concentration	Probit model estimation equation
24	LC_{50}- 20.81 (19.58-22.48)	2.08	y= -4.73+0.23x
24	LC_{90}- 26.44 (24.45-31.47)	2.64	
48	LC_{50}-18.50 (16.67-19.40)	1.85	y= -5.18+0.29x
48	LC_{90}-22.54 (20.88-26..09)	2.25	
72	LC_{50}-17.11 (16.30-17.84)	1.71	y= -5.30+0.31x
72	LC_{90}-21.44 (20.59-23.31)	2.14	
96	LC_{50}-15.88 (14.99-16.64)	1.59	y= -5.06+5.06x
96	LC_{90}-19.91 (18.97-21.42)	1.20	

Where y=dependent variable, x= independable variable

Cumulative mortality of exposed fish was very variable relative to the concentration of the herbicide (Figure 3).The cumulative mortality differed with the time of exposure ($p < 0.01$), concentration of toxicant ($p < 0.001$) and interactions between exposure duration and herbicide concentration ($p < 0.01$, Figure 3). Exposed fish produced copious amount of mucus on the gill and skin which appeared to be concentration-dependent in exposed fish with

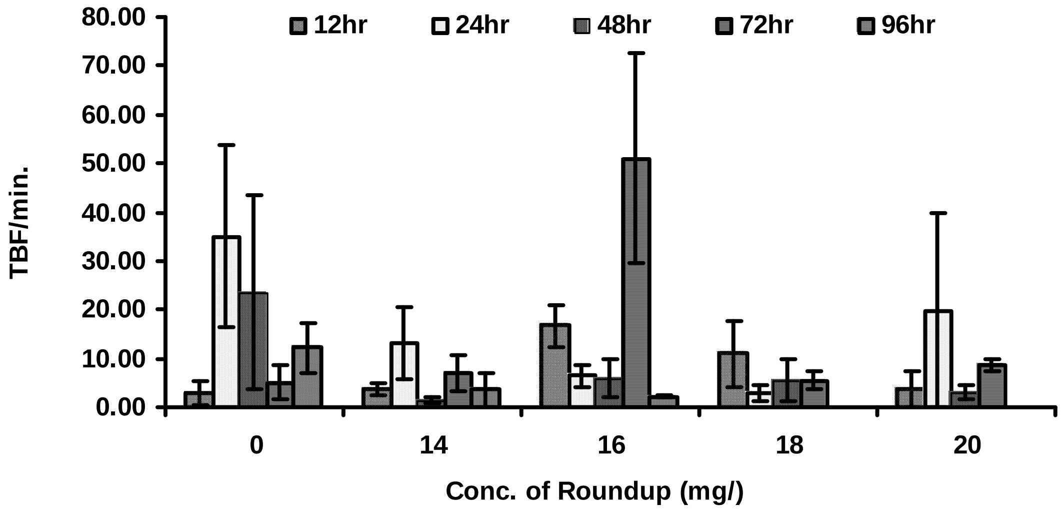

Figure 1: Tail beat frequency (TBF) of fingerlings of *C. gariepinus* exposed to various concentrations of Roundup for 96

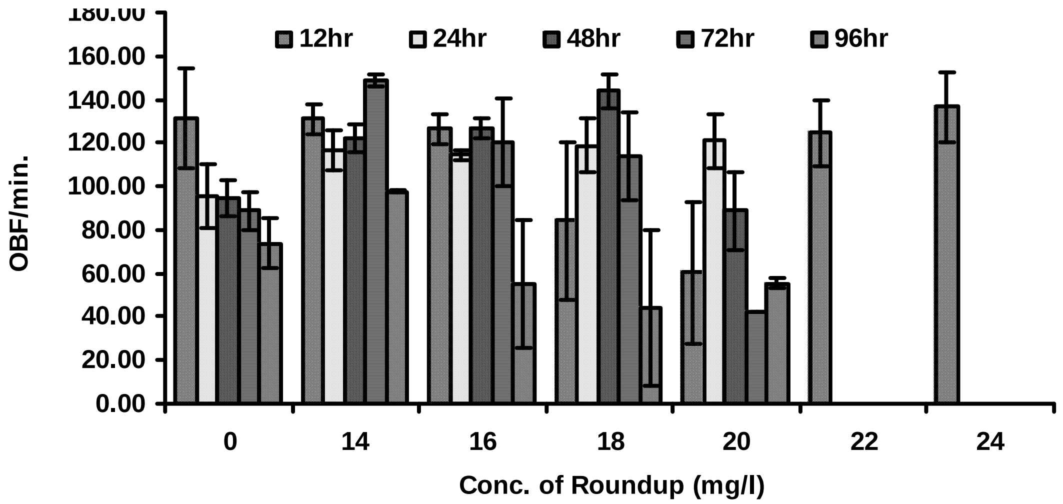

Figure 2: Operculum beat frequency (OBF) of fingerlings of *C. gariepinus* exposed to various concentrations of Roundup for 96

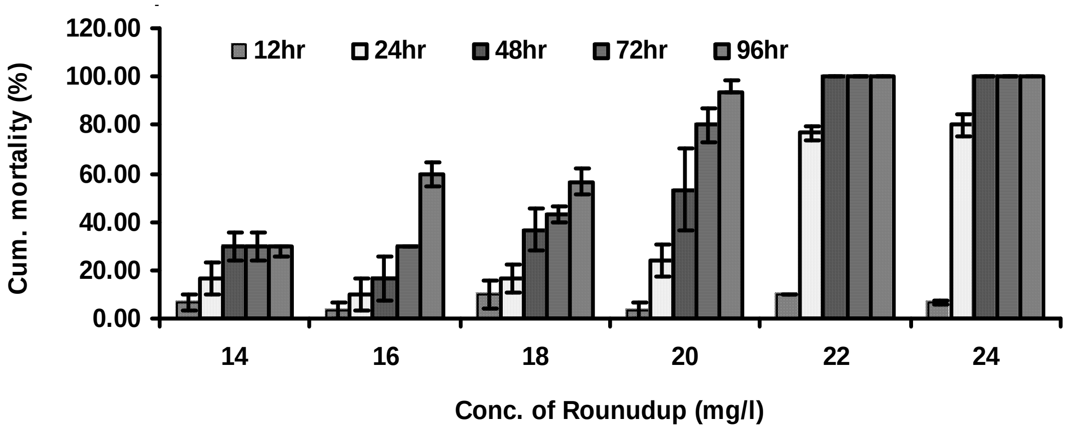

Figure 3: Percentage cumulative mortality of fingerlings of *C. gariepinus* exposed to various concentrations of Roundup for 96 hours

minimal amount on the control group. Pigmentation of the skin of the fish was not noticed in any of the exposure concentrations.

The 24, 48, 72 and 96 hour LC_{50} and associated 95% confidence limits of the herbicide concentrations shown in Table 3 indicated that the range of the values between the 24 hour and 96 hour LC_{50} (4.93 mg/l) was very narrow. Safe concentrations of Roundup to fingerlings of *C. gariepinus* were very low (2.08 mg/l for 24 hour and 1.59 mg/l for 96 hour). The time it took for half of the exposed fish to die at the various exposure concentrations decreased with time (Table 4) with the highest concentration (24 mg/l) killing half of the exposed fish at about one sixth the time it took for 14 mg/l of the herbicide.

DISCUSSION

Besch (1975) identified four main phases in the responses of fish to toxicants: the contact phase (brief period of excitability), exertion (visible avoidance characterized by fast swimming, leaping, and an attempt to jump out of the toxicant), loss of equilibrium and lethal (death) phase, when opercular movement and response to tactile stimuli cease completely. Similar trends in these behavioural responses was reported in clariids (Gabriel *et al*., 2009) as was noted in the exposed fish. The as abnormal responses for example, hyperactivity, increased ventilatory rate, dash and erratic swimming, increased surfacing among others may have increased the energy demand for metabolism beyond normal, leading to fatigue and stress (Arunachalam and Palanichamy, 1982). The stressful behaviours of the fish suggest that they may have suffered respiratory impairment due to the effect of the herbicide on the gills and general metabolism. Oxygen exchange across the gills might have been impaired as evidenced by the disruption of the structure of the gills and increased mucus production by mucus cells in exposed fish. These observations were in line with those of previous workers who assessed the acute effects of *Thevetia peruviana* and *Azadarichtha indica* on *Hyperopsis bebe occidentalis* (Oti and Ukpabi, 1999), gammalin 20 on *Aphysiomen gairdneri* (Ofojekwu *et al.,* 1982) and cassava leaf extract on *C. anguillaris* (Agwuigwo, 1998). The workers attributed these behaviours to nervous disorder elicited by the toxicants.

The response pattern of tail beat frequency of exposed fish with respect to exposure duration and concentration of the herbicide differed from the findings of Ekweozor *et al*. (2001), Gabriel and Kparobo (2002) and Onusiriuka and Ufodike (1994) on *C. gariepinus, O. niloticus*, *Heterobranchus bidorsalis* and *Clarias* hybrid exposed to fertilizer effluents, gramoxone, and bark of sausage plant and Akee apple, and the floral part of Akee apple, respectively. In these studies the values of TBF appeared to increase with time and concentration of the toxicant contrary to what was observed in this study possibly due to the mode of action of the toxicant in the exposed fish. The generally low and less variable values of the TBF recorded in this study may suggest that the fishes were less sensitive to the herbicide when compared to the response of *C. gariepinus* exposed to cypermethourin (pyrethouroid) possibly due to its mode of action and size of fish (Gabriel and Kparobo, 2002). Variable response and cessation of TBF before OBF, and subsequent death may indicate the trend in available metabolic energy. This appears to be the usual trend in the responses of *C. gariepinus* to acute concentrations of toxicants (Onusiriuka and Ufodike, 1994; Ekweozor *et al*., 2001; Gabriel and Kparobo, 2002). OBF appeared to be a more responsive variable in measuring behavioural toxicity in comparison to TBF in *C. gariepinus* exposed to Roundup. The trend in OBF in this study and several others is that it is usually raised, peaked and then fell with time for the various concentrations of the toxicants tested (Onusiriuka and Ufodike, 1994; Agwuigwo, 1998). However, variations between the trends in this study and others could possibly be accounted for by differences in the fish species, life stage and mode of action of the toxicant. Thomas and Rice (1975) observed that increased opercular rate may result from decreased efficiency in oxygen uptake, transport or increased metabolic rate. To cope with stress caused by exposure to the herbicide, the fish may have increased its TBF and OBF concurrently, but the latter particularly to increase the rate of water flow over the gills to enhance oxygen uptake from the water (Gabriel and Kparobo, 2002).

According to Agbede *et al.* (2000), a layer of mucus (glycoproteins, preteoglycans and proteins) forms the interface between the skin of the fish and the external environment in addition to scales in scaly fish. The layer is continuously replenished by mucus secreting cells and the rate can increase in response to infection, chemicals or physical irritants. The greater amount of mucus on the skin and gill of exposed fish relative to the control is could be a strategy to protect itself against injury. But this has grave implications particularly for the gills as it impairs oxygen uptake. Increased mucus as a result of increased activity of mucus cells was reported in *C. gariepinus* on exposed to *Datura innoxia* root (Ayuba and Ofojekwu, 2002) and *O. niloticus* exposed to cadmium (Annune *and* Ahuma, 1994).

Lack of skin pigmentation in Roundup-exposed fish contradicts the results obtained from the results obtained in hybrid clariid under acute concentrations of cassava mill effluents (Oti, 2000) and bark of *Thevetia peruviana* (Oti, 2003) and *Chrysichthys nigrodigitatus* exposed to gammalin 20 (Oti, 2003). Lagler *et al.* (1977) reported that the dark colour of fish skin is due to the presence of melanin pigment in the chromatophores of the skin. The pigments are moved towards the periphery by melanin stimulating hormone (MSH) particularly under the influence of irritants such as toxins (Novale, 1959). It seems that the herbicide did not elicit this response in the skin of *C. gariepinus*, hence the absence of such pigmentation.

The mortality pattern, lethal concentrations and MLT_{50} show that increasing concentration of the herbicide caused an increased disruption of the physiology of the fish with time to the point where they could not maintain homeostasis leading to a break down of the whole system, followed by death. The threshold concentration causing 100% mortality in this study was 22 mg/l which is lower than that reported for other toxicants tested on any of the clariid species (Onusiriuka and Ufodike; 1994; Agwuigwo, 1998; Ayuba and Ofojekwu, 2002; Oti, 2002) suggesting that it may be more toxic than other tested toxicants. Half of the exposed fish (50%) were killed by 15.88 mg/l of herbicide in 19.69 hours, hence the herbicide can be classified as being slightly toxic (Helfrich *et al.*, 1996). It means that it is more toxic to *C. gariepinus* than 2,4-D, 96 hour LC_{50} 151.67 mg/l (Macaulay, 2005) an organophosphate commonly used singly or in combination with Roundup in the control of aquatic weeds.

The results strongly suggest that the herbicide is not as "harmless and environmentally friendly" as was claimed by Monsanto, the manufacturer of the product. Besides, in the wild where the agro-chemical is indiscriminately used the impact of the exposure stress caused by the herbicide, may be protracted, following the survivors throughout life and may affect various aspects of their lives. This was demonstrated in fry of Japanese medaka (*Oryza latipes*) exposed to sublethal concentrations of endosulfan (Gormley and Teather, 2003) in which reproduction at the adult stage was severely affected.

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