HAEMOGRAM OF ADULT *Clarias gariepinus* EXPOSED TO CHRONIC LEVELS OF ROUNDUP

[1]GABRIEL, Ujagwung Ugwemorudong and [2]ERONDU, Ebere Sunday

[1]Department of Fisheries and Aquatic Environment, Faculty of Agriculture, Rivers State University of Science and Technology, PMB 5080, Port Harcourt, Nigeria.

[2]Department of Animal Science and Fisheries, Faculty of Agriculture, University of Port Harcourt, PMB 5383, Port Harcourt, Nigeria.

Corresponding Author: Gabriel, U. U. Department of Fisheries and Aquatic Environment, Faculty of Agriculture, Rivers State University of Science and Technology, PMB 5080, Port Harcourt, Nigeria. **Email:** ugwemg@yahoo.com **Phone:** +234 8032720882

ABSTRACT

Clarias gariepinus (mean weight, 850 ± 60 g) mean length, 38.75 ± 6.7 cm) were exposed individually to four concentrations of Roundup, containing 360g/l glyphosate in the form of 480g/l isopropylamine salt (1.5, 3.0, 4.5 and 6.0 mg/l) and a control (0 mg/l), five replicates per concentration for 70 days in static renewal bioassay to determine the effect of exposure on selected blood variables (red blood cells, RBC; packed cell volume, PCV; white blood cell (WBC). thrombocytes, reticulocytes absolute red cell indices- mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC), leucocrit. Exposure to chronic levels of Roundup caused a reduction in the values of PCV, HB, ESR, thrombocytes, WBC, reticulocytes, RBC, MCV and MCH relative to the control. Response of the blood variables among the treated group varied very widely and was not directly related to concentration of herbicide except in thrombocytes. There was a reduction in the values of the variables involved in oxygen transport (PCV, Hb, RBC) and absolute red cell values in some of the exposed fish. Thrombocytes appeared to be the most responsive variable as the value was drastically reduced from 1060 x 10^9 cells/l at 1.50 mg/l to 122.50 x 10^9 cells/l at 6.0 mg/l. It was only in the ESR that the value in the control (0.25 mmhr^{-1}) was significantly different from that of the treated groups (6.0 mmhr^{-1} at 6.0 mg/l). The regression lines of best fit for the relationship between the herbicide concentration and the various variables showed that the responses of the test fish to the herbicide varied as indicated by the curve types. The response of adult C. gariepinus to sublethal concentrations of Roundup suggest that the fish could be under intense physiological stress in the wild and culture conditions where the herbicide is applied at the recommended rate (liquid, 12l/ha; solid, 2.2 kg/ha) without the least effort to maintain the maximum recommended 0.6 mg/l in 1m water column after application.

Keywords: Roundup, Glyphosate, Haemogram, *Clarias gariepinus,* Ecotoxicology

INTRODUCTION

In recent years aquatic pollution problems associated with aquatic plants particularly those involving water hyacinth, *Eichhornia crassipes* have assumed an economic dimension. Aquatic weeds in general and water hyacinth in particular have been identified as a worldwide tropical problem (Petierse *et al.*, 1996). Water hyacinth infestation of our waterways has been identified as a serious economic problem in Nigeria, West Africa and indeed tropical countries (Akinyemiju *et al.*, 1988; Petierse *et al.*, 1996). Water hyacinth in Nigeria water bodies has probably become the most noxious weed deleterious to the Nigerian aquatic system (Olaleye and Akinyemiju, 2002). This is attested to by the amount of fund the government expends on its curtailment and control (NIFFR, 1995). Furthermore, the loss to the Nigerian economy because of infestation by the plant since 1983 has been estimated at $180m annually (World Bank, 1990). Methods of control already tried with limited success include mechanical control (Kusemiju, 1988), herbicidal control (Akinyemiju *et al.,* 1988) and the biological control by GTZ in the Kanji Lake (NIFFR, 1995).

Glyphosate products are effectively used in the management of weeds in the aquatic environments (Fairchild *et al*., 2000; Kilbride and Paveglio, 2001). The products contain approximately 50 – 70 % inert ingredients/surfactants (polyoxymethylamines, POEA) that enhance the efficiency of the products (Hartzler, 2001). They bestow different toxicological characteristics to the products making them more toxic (Servize *et al*., 1987). Glyphosate products have been shown to be toxic to all life forms, producing various physiological alterations in exposed fish (Abdelghani *et al*., 1997; El-Gendy *et al.,* 1998; Ma and Liang, 2001). Roundup, a glyphosate product is a systemic and selective herbicide used to kill broadleaved, grasses and sedge species (USEPA, 2002).

Several studies involving exposure of fish species to herbicides/pesticides indicated that exposed fish species showed poor health status demonstrated by adverse effects on measured haematological variables (Santhakumar *et al*., 2001; Atamanalp and Yanik, 2002; Oluah and Nwosu, 2003). Haematological variables of some fish species manifested quick and measurable changes under exposure to toxicants such as tobacco leaf extracts (Agbon *et al*., 2002); crude oil (Gabriel *et al*., 2001), actellic (Mgbenka *et al*., 2003; Oluah and Mgbenka, 2004). Generally, reports on the effects of herbicides on the physiology of cultured fish species in the tropics are on the increase because of the extensive use of these chemicals to control weeds on farms and in the aquatic environment.

Information on changes in the blood parameters of cultured fish species exposed to glyphosate is very important in the management of these fish species in the phase of increasing contamination of the environment with herbicides. The present study assessed the effect of sublethal levels of Roundup on the blood of *C. gariepinus.*

MATERIALS AND METHODS

Forty adult *C. gariepinus* (mean weight, 850 ± 60 g) mean length, 38.75 ± 6.7 cm) were obtained from a private fish farm at Ede in Ogba/Egbema Ndoni LGA of Rivers State, Nigeria. They were transported in 70/ plastic trough whose mouth was covered by net by car to the Department of Fisheries (RSUST), Rivers State University of Science and Technology, Port Harcourt. On arrival the fish were transferred to a concrete tank (2.5 x 2.3 x 1.0m^3) that was half-filled with water. It was fed a 35 % crude protein diet at 5% total body weight. The daily ration was divided into two portions and each half fed to them at 0800 – 0930 hours and 1600 – 1800 hours respectively, for two weeks until the experiment commenced. One fish each was collected with dip net into 40l circular plastic aquaria (60l) during the acclimation for seven days. They were fed the same diet at one percent body weight. Food remains were siphoned out of the aquaria daily during each water exchange. A quarter of the water was exchanged at the 24th hr and half at the 48th hour and complete exchange was done on the third day. The aquaria were usually washed with a piece of foam, after which the water was removed and fresh water was replaced to the 30l mark.

Five fish were exposed individually to four concentrations of Roundup (1.5, 3.0, 4.5 and 6.0 mg/l) and a control (0 mg/l). After the preparation of the various solutions of the test solution, the fish were scooped with a dip net and each gently placed in the test aquaria. The mouths of the aquaria were covered with nylon net with a slit (opening) at the middle to prevent fish escape from aquaria. The slit was tied with twine. The twine was loosed during feeding and cleaning of aquaria, and exchange of test solutions after which they were tied again.

The fish was fed as in the acclimation period. A quarter of the solution was replaced on the 24^{th}, half on the 48^{th} hr and the whole volume on the third day. Spent solutions were disposed of by gently tilting the aquarium until the whole solution was poured out. Fresh solutions for replenishing were prepared the day the exchange was done. Blood samples were collected by cardiac puncture while the fish were restrained physically. Samples were preserved in EDTA embedded bottles. The samples were thereafter analysed for red blood cells (RBC), packed cell volume, (PCV) and white blood cell (WBC) (Blaxhall and Daisley, 1973), and for thrombocytes, reticulocytes, absolute red cell indices, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration, MCHC and leucocrit (McLeay and Gordon, 1977; Brown , 1980).

RESULTS

There was a reduction (p>0.05) in PCV and Hb values in the treated group below their respective control; PCV, 31.00 ± 1.47%; Hb, 10.30 ± 0.47g/l (Table 1). The lowest values of the two variables were recorded at 3.00 ppm. The ESR value in the treated group were much higher the control, 0.25 ± 0.20 $mmhr^{-1}$ with that at 6.00 ppm raised 24x that of the control (Table 1). Thrombocytosis was recorded at 1.50 and 3.00 ppm and thrombocytopaenia occurred at 4.5 and 6.00 ppm) were recorded in the exposed fish relative to the control. The value decreased with increase in toxic concentration (Table 1). Leucocytosis was elicited by the toxicant at 4.50 ppm but leucopaenia at the other concentrations with the lowest value at 3.00 ppm relative to the control, 23.50 ± 7.24 x 109 cells/l (Table 1). Roundup causes a reduction in the leucocit value at the lower levels, 1.50 - 3.00 ppm, whereas the reverse was the case at the levels in comparison to the control, 1.25 ± 0.25%). There was a reduction in the number of immature RBC (reticulopaenia) in all the exposure concentrations (Table 1). Roundup caused erythrocytopaenia (p>0.05) particularly with similar values at 1.50 – 4.50 ppm. The values of the red cell indices MCV, MCH and MCHC were widely variable (p>0.05) within the treated group and between the control and treated fish without any relation with the toxicant concentration (Table 1). MCH values in the treated fish were lower than the control, 33.37 ± 1.71 pg, similar to what recorded in MCV and MCHC except at 1.500 ppm (Table 1). The regression lines of best fit for the relationship between the herbicide concentration and the various variables showed that the responses of the test fish to the herbicide varied as indicated by the curve types (Table 2).

DISCUSSION

Erythrocytopaenia, reduced haematocrit and Hb recorded in exposed fish were indications that sublethal levels of Roundup caused anaemia in the exposed fish. Similar observations have been made in fish species exposed to single or combinations of these herbicides (Finlayson and Fagella, 1986; El-Deen and Rogers, 1992; Annune and Ahuma, 1998; Babatunde *et al*., 2001; Agbon *et al*., 2002). Common carp and channel catfish exposed to molinate, a rice herbicide for 12 and 28 days respectively, were anaemic particularly at the 28^{th} day. Grass carp exposed to diquat chloride (2 and 53 mg/l) for 12 – 168 days (El-Deen and Rogers, 1992), *O. niloticus* exposed to paraquat for 96 hours Babatunde *et al.* (2001) and large mouth bass exposed to Vision (a glyphosate product) and glyphosate (D'Silva and Winter, 1997) showed marked changes (reduction or increase) in some of the blood variables such as Hb and PCV. In some of these studies changes were obvious in about two hours and were neither time nor concentration dependent. El-Deen and Rogers (1992) demonstrated that, white catfish, *Ictalurus catus* and common carp sampled from field basins receiving molinate and thiobencarb had normal range of blood values probably due to the low levels of the herbicides compared to the level in their laboratory study. Other studies by Janz *et al.* (1991) and Mitchell *et al.* (1987) showed that Roundup and Vision did not produce significant changes in the blood

Table 1: Haemogram of adult *Clarias gariepinus* exposed to chronic levels of Roundup for 70 days

Conc. of Roundup (mg/l)	PCV (%)	Haemoglobin (g/dl)	ESR (mm/hr)	Thrombocytes ($x10^9$ cells/l)	White blood cells ($x10^9$ cells/l)
0.0	31.00±1.47a	10.30±0.47a	0.25±0.25b	370.00±10.80b	48.72±15.24a
1.5	25.00±5.52ab	8.18±0.49ab	5.25±0.85a	1060.00±383.42a	42.75±8.07ab
3.0	19.75±5.15b	5.88±1.20b	3.00±1.63ab	672.50±60.14ab	23.50±7.24b
4.5	23.00±3.79ab	6.33±0.52ab	3.75±1.65ab	265.00±55.15b	65.08±9.42a
6.0	28.50±2.34ab	8.23±0.73ab	6.00±2.48a	122.50±31.00a	46.00±3.72ab

	Leucocrit (%)	Reticulocytes ($x10^9$ cells/l)	Red blood cells($x10^{12}$ cells/l)	Absolute red cell values		
				MCV (fl)	MCH (pg)	MCHC (%)
0.0	1.25 ±0.25ab	0.63 ±0.03a	3.20 ±0.11a	96.78 ±1.86a	33.37 ±1.71a	33.25 ±0.35a
1.5	1.00 ±.20b	0.53 ±0.04b	2.53 ±0.19ab	99.63 ±2.50a	31.38 ±0.47ab	33.68 ±0.41a
3.0	1.13 ±0.31b	0.40 ±0.7b	2.53 ±0.39ab	92.28 ±2.21ab	30.78 ±0.61ab	32.88 ±0.25a
4.5	2.50 ±0.65a	0.50 ±0.11ab	2.53 ±0.51ab	90.18 ±1.69ab	26.68 ±2.76b	29.78 ±0.50a
6.0	2.00 ±0.41ab	0.58 ±0.05ab	3.00 ±0.25ab	95.08 ±0.71ab	31.68 ±0.49ab	33.30 ±0.29a

PCV-Packed cell volume, ESR-Erythrocyte sedimentation rate, MCV-Mean corpuscular volume, MCH-Mean corpuscular haemoglobn, MCHC-Mean corpuscular haemoglobin concentration. Figures with the same alphabets in the same column are not significantly different ($p<0.05$)

Table 2: Regression lines of best fit for the prediction of the values of the various blood variables of *C. gariepinus* on the concentrations of Roundup after exposure for 70 days

Dependent variable	Prediction equation	Curve type	r^2
Packed cell volume	$y= 4.8659Ln(x) + 7.5784$	Logarithmic	0.9086
Haemoglobin	$y= 0.003x^2 +0.028x +5.5361$	Polynomial	0.9402
Erythrocyte sedimentation rate	$y= 2.5543Ln(x) -5.7314$	Logarithmic	0.9828
Thrombocytes	$y= 103.95e^{0.0262x}$	Exponential	0.9871
White blood cell	$y= 9.2985x^{0.4168}$	Power	0.943
Leucocrit	$y= 0.7673e^{0.0126x}$	Exponential	0.9137
Reticulocytes	$y= 0.1075Ln(x) + 0.129$	Logarithmic	0.9789
Red blood cell	$y= 2.3378e^{0.0032x}$	Exponential	0.798
Mean cell volume	$y=0.89.061e^{0.0012x}$	Exponential	0.9952
Mean cell haemoglobin	$y=2.8699Ln(x) + 20.103$	Logarithmic	0.9839
Red cell haemoglobin concentration	$y=1.7917Ln(x) + 25.987$	Logarithmic	0.9353

x=independent variable, y= dependent variable

variables of coho salmon. Lack of response in the blood of exposed fish could be due to differences in the exposure concentration, which may be less than the threshold concentration or the blood sampling may have been done at a point when the haematological responses have not been fully elicited or after the exposed fish may have adapted to the toxicant stress.

The anaemic condition of exposed fish may have resulted from the destruction of red cells or disruption of erythropoiesis in the haematopoietic tissues such as the spleen and head kidney (Miale, 1982), haemodilution (Sampath *et al.*, 1993) - a condition in which water is added to the plasma to reduce the concentration of Roundup in the blood, destruction of intestinal wall thereby disrupting the absorption of vitamin B_{12} necessary for RBC production in the bone marrow (Seiverd, 1983). The latter is confirmed from the pathology of the small intestine which shows severe necrosis of the mucosa. Under such conditions it was observed that the bone marrow produced fewer erythrocytes which are usually large and well filled with haemoglobin. However, they are internally defective breaking down at an early stage. The red blood cells in this study had increased volume (macrocytic) and weight of haemoglobin with low haemoglobin concentration as shown by the values of MCV, MCH and MCHC which are characteristics of pernicious anaemia. Similar observation was made by Mgbenka *et al*. (2003) in *C. albopunctatus* exposed to gammalin 20. The anaemic state occasioned by the herbicide has great implications for the fish with respect to oxygen exchange and transport which may be greatly impaired. Impairment of oxygen exchange and transport may greatly affect the overall physiology and metabolism of the fish.

According to Hall and Malia (1984), the number of reticulocytes (immature RBC) is an index of red blood cell production by bone marrow and as such is one of the most valuable observations in diagnostic haematology. All exposed fish suffered reticulocytopaenia. Reticulocytopaenia, erythrocytopaenia and thrombocytopaenia observed in the fish at higher concentration of the herbicide are characteristic of aplastic pernicious anaemia. Seiverd (1983) noted that aplastic pernicious anaemia is a severe disease of the blood which is characterized by the failure of the bone marrow to produce the normal number of red cells, white cells and platelets. The author observed that this could result from the actions of drugs, chemicals and radiation. Hence, chronic level of the herbicide may have interfered with the haematopoeitic function of the bone marrow and head kidney of *C. gariepinus* thereby causing a reduction in the number of reticulocytes produced.

Changes in the leucocrit values may indicate haemodilution or haemoconcentration. Acute exposure to crowding, elevated temperature, and pulp mill effluents caused significant rise in the leucocrit values of two cold water fish species, rainbow trout, *Salmo gairdneri* and coho salmon, *Oncorhynchus kisutch* (McLeay and Gordon, 1977). Also Das (2003) showed that *Labeo rohita* exposed to rapid temperature increase and maintained for 72 hours suffered same fate as those reported above. However, results from this study and that of McLeay and Gordon (1977) did not reveal any concentration- or time- dependent relationship in the response of this variable to the stress factors. Interestingly, the range of values in this study fall within that reported by McLeay and Gordon (1977) and Das (2003) for the fish species they studied.

Blood of *C. gariepinus* was both thrombocytopaenic as well as thrombocytotic relative to concentration of the herbicide. The changes in the number of circulating thrombocytes had the most responsive direct relationship with the level of the herbicide. Such sensitivity has been reported in *Channa punctatus* and *H. fossilis* under the stress of starvation and heavy metal poisoning (Mahajan and Dheer, 1983; Banerjee, 1998). Thrombocytosis was reported in *H. fossilis* exposed to malachite green (Musa and Omoregie, 1999). However, thrombocytopenia was produced in brown and rainbow trout by crowding (Pickering and Pottinger, 1987) and *Notopterus notopterus* exposed to toxaphene (Binesh and Shukla, 1998). Hall and Malia (1984) associated thrombocytopaenia in man with decreased production from the bone marrow and increased splenic sequestration. For this phenomenon, Hoffbrand *et al*. (2002) identified failure of thrombocyte production as the most common cause and that in man it is usually a part of generalized bone marrow failure. This failure Hall and Malia (1984) claimed could result from exposure to chemicals. On the other hand, they observed that thrombocytosis may result from increased and effective production of thrombocytes which is most frequently associated with or secondary to inflammatory response, iron deficiency and drugs.

Results from this study indicate that Roundup depending on concentration may elicit thrombocytosis or thrombocytopaenia in *C.*

gariepinus. At low concentrations (1.5 and 3.0 mg/l) it led to increased number of circulating thrombocytes, but at higher levels (4.0 and 6.0 mg/l) it might have caused bone marrow failure leading to reduction in thrombocytes production. The implication of thrombocytopaenia for the exposed fish is that in case of any injuries, bleeding and clot retraction time may increase leading to death. Erythrocyte sedimentation rate is the rate of fall of RBC per unit time. Sublethal levels of Roundup caused a rise ($p<0.05$) in the ESR value of the fish. Similar results were obtained in *C. gariepinus* exposed to sublethal levels of Akee apple, *Bligha sapida* and sausage plant, *Kigelia africana* (Onusiriuka and Ufodike, 2000). Exposure to Roundup may have altered (raised) the levels of plasma proteins leading to rouleaux formation and agglutination (leading to increased cell mass) and decreased plasma viscosity probably due to haemodilution and hence raised ESR values in exposed fish above that of the control. Brown (1980) identified size of erythrocytes, plasma composition, and mechanical and technical factors as main factors influencing ESR, but plasma as the most important factor. According to her, in normal blood, red cells are negatively charged and repel each other. But under certain conditions such as diseases and exposure to chemicals, plasma proteins (fibrinogen and globulin) may be altered causing rouleaux formation leading to larger mass and increased sedimentation rate. Agglutination of red cells due to changes in red cell surface also lead to rouleaux and resulting in larger cell in larger cell mass and increased ESR. Rouleaux and aggregation are affected mainly by the levels of fibrinogen, $\alpha_{1,\,2}$ globulin increasing as their levels are raised in the blood.

Changes in circulating WBC in fish are characteristic of exposure to culture stress and xenobiotics (Ellsaesser and Clem, 1986; Omoregie, 1998; Lohner *et al*. 2001). These studies show that exposure to environmental stress could cause leucocytopaenia or leucoytosis in fish. In this study the changes were not concentration related. Reports from works by Prasad *et al.* (1987), Omoregie, (1998) and Musa and Omoregie (1999) showed a concentration-dependent change in the WBC of *C. gariepinus* and *O. niloticus* exposed to crude oil and its water soluble fraction, and malachite green, respectively. *C. albopunctatus* subjected to sublethal levels of two insecticides (gammalin 20 and actellic 25EC) had reduced RBC, PCV, Hb and significant leucocytosis (Mgbenka *et al*., 2003; Oluah and Mgbenka, 2004). Variation in the WBC values of these fishes could be accounted for by the exposure level and time, mode of action of the chemicals and species-specific responses. However, depending on the level of exposure, Roundup may have either challenged of inhibited the defense mechanism of the fish, hence the variations in the value of WBC.

The variable responses of the various blood variables to the herbicide described by various curve types in this study suggests that the exposure concentrations of the herbicide were too low to have elicited some level of direct responses from the variables studied. It could also be that such a response may have occurred earlier and subsided due to adaptation to toxicant stress before the blood samples were collected for analysis. This may be related to the threshold concentration, defined by Webster's Third International Dictionary as "the point at which a physiological or psychological effect begins to be produced". Cairns (1992) pointed out the difficulty of finding a well-defined rate change in a dose response curve in ecotoxicology study. The results obtained from this study appeared to be the most common way blood variables respond to xenobiotics as observed from other studies (Onusiriuka and Ufodike, 2000; Lohner *et al*., 2001). Hirubec (2001) observed that confusing and conflicting haematological data have been published due to species and test methodology differences. Despite these obstacles, the author opined that haematological variables are important diagnostic tools in detecting sublethal conditions affecting production performance if some basic guidelines are followed. He noted that adherence to the guidelines will drastically reduce the differences in results often reported. These guidelines include reduction in stress of capture, appropriate blood handling and sample preparation, appropriate analytical method, generation of normal databases for each species, information on how specific blood values are altered with specific stress factors.

The response of adult *C. gariepinus* to sublethal concentrations of Roundup suggest that the fish could be under intense physiological stress in the wild and culture conditions where the herbicide is applied at the recommended rate (liquid, 12*l*/ha; solid, 2.2 kg/ha) without the least effort to maintain the maximum recommended 0.6 mg/l in 1m water column after application.

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