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# Effect of Ronstar® on the Hematological Parameters of the Freshwater Catfish *Clarias albopunctatus* Fingerlings

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***Authors' contributions***

*All authors participated actively in carrying out this work. The corresponding author NSO designed the work and did the literature search for the write-up. The corresponding author and the third author NA did the statistical analysis. Authors HU and NA collated the data. All the authors read and approved the final manuscript for publication.*

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## ABSTRACT

*Clarias albopunctatus* juvenile (mean weight 85.40±2.67 g) was exposed to sublethal concentrations of Ronstar (0, 0.2, 0.6 and 1.2 μg/l) in a static renewal bioassay system for 15 days. The hematological parameters of the fish were determined every 5 days. When compared with the control, the erythrocyte count (RBC), hemoglobin (Hb) and the hematocrit (Hct) were significantly reduced (P<.05) between the treatment groups. These values also differed (P<.05) within the treatment groups. The leucocyte counts increased with increasing Ronstar concentration and during the exposure. Compared with the control, significant (P<05) lymphocytosis, monocytopenia, neutropenia and reduced eosinophil were all evident in the treatment groups. The reduction in the erythrocyte count and hemoglobin are indications of anemia in the fish exposed to Ronstar. The assay of these parameters could be of immense value in establishing safe limits for pesticides in Nigerian waters as well as in the monitoring of fish health.

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## 1. INTRODUCTION

Increased industrialization and pesticide applications to boost agricultural production are some of the factors responsible for the indiscriminate discharge of potentially harmful compounds into the aquatic ecosystem. Such compounds as detergent, fertilizers, insecticides and herbicides are discharged either as effluents or runoff from the watershed. These contaminants affect the physico-chemical characteristics of the water bodies and hence affect the biochemistry and physiology of aquatic life therein. Pesticides affect the tissue enzyme activities of some aquatic organisms [1-5], impose oxidative stress [6-7] and alter the carbohydrate metabolism [8-11]. Pesticides are also known to affect the hematology of fish [12-17].

Currently, Ronstar is the herbicide of choice among paddy rice farmers in the rice fields of Adani-Omor axis in south eastern Nigeria. The runoff from these farm lands are washed into Ezu River. Thus, the extensive use of Ronstar in this area represents a likely threat to non-target organisms like fish.

In Nigeria, there is increasing body of literature on the impacts of pesticides on aquatic life particularly aquatic food organisms like fish [5,8,11,14-16,4]. In this study, we chose the *Clarias* species not only because of its wide distribution and abundance in Nigeria but also it has high economic and cultural values. Moreover, it is a good animal model for ecotoxicological research on account of it being hardy and withstanding severe stress. Since the hematological parameters of fish, are known to respond rapidly to alterations in water quality [10,17]. the purpose of this study was to investigate the hematological changes in *Clarias albopunctatus* exposed to sublethal concentrations of Ronstar.

## 2. MATERIALS AND METHODS

### 2.1 Fish Collection and Treatment

The samples of *Clarias albopunctatus* used in the study were caught from Anambra River at Otuocha, Nigeria. The fish was transported to our laboratory in two 50L plastic containers and acclimatized for two weeks before the commencement of the experiment. On arrival at the laboratory, the fish were put into 450L plastic tank and treated with 2% potassium permanganate solution as a disinfectant for thirty minutes. During the period of acclimation, the fish was fed 35% crude protein diet at 3% body weight every day at 8.00h. The acclimation tank was continuously aerated to maintain the dissolved oxygen level above 6.0mg/L in order to prevent anoxia.

### 2.2 Test Chemical

The commercial preparation of Ronstar® (CAS No 19666-30-9) [Rhone Poulenc] containing oxadiazon [2-tert-Butyl-4-(2,4-dichloro-5-isopropoxyphenol-delta 2-1,3,4-oxadiazoline-5-one] as active ingredient, was used for the study. The test concentrations (0.2, 0.6 and 1.2μg/L) were prepared from the commercial preparation containing 120 g/L oxadiazon by serial dilution.

### 2.3 *In vivo* Exposure Study

A preliminary study was done which determined an LC50 of 0.02 mg/L. One hundred and eighty fish (mean weight 85.4±2.67 g) used in the study were divided into four groups of 45 fish per group. These concentrations represented 1/100, 1/33 and 1/16.6 of the 96hr $LC_{50}$ of 0.02 mg/L determined earlier as a preliminary part of the study. The fish in groups 1, 2 and 3 were exposed to 0.2 0.6 and 1.2 μg/L Ronstar, respectively. The fourth group was exposed to tap water only (0.0 μg/L) as the control. The quality of the tap water (total hardness, 121 mg/L as $CaCO_3$; alkalinity 66.8 mg/L; pH 7.8; conductivity 3.85 mS/m; dissolved oxygen 6.8 mg/L; temperature 28°C, ammonia, nil) were determined using APHA standard methods [18]. All the treatments were randomly subdivided into three replicates of fifteen fish per replicate. Each replicate group was kept in a 50L plastic aquarium containing 40L of test solution throughout the study period. A static renewal bioassay technique, in which all the test solutions were changed and renewed daily, was adopted. During the study period, the fish were fed 35% crude protein diet at 3% body weight daily at 8.00h after all the test solutions had been replaced.

## 2.4 Blood Collection and Hematological Assay

The blood was collected by cardiac puncture every five days using heparinized 2 ml hypodermic syringe [19]. On each sampling day, two fish from each replicate experiment were anaesthetized with MS222 and killed by inserting a knife through the frontal frontanelle. The hematocrit was determined by the microhematocrit method [20] while the cyanmethemoglobin method [21] was used to assay the hemoglobin concentration (g/dl). The erythrocytes were diluted with Turk's solution and the erythrocyte count ($x10^6/mm^3$) was determined using the improved microscope Neubauer counter. The leucocytes were diluted with Turk's solution and the leucocyte count (expressed as $x10^4/mm^3$) was also calculated using the neubauer microscope counter. All hematological morphological indices were determined as described by Dacie and Lewis [22]. The differential leucocyte counts were made on thin blood smears stained with Gemsa-Romanowsky stain [23].

## 2.5 Statistical Analysis

The data were analyzed using the one-way analysis of variance (ANOVA), followed by Fisher's Least (post-hoc) significant difference (F-LSD) test using Steel and Torrie method [24] at 95% significant level. The SPSS statistical software version 17.0 was used to analyze the result.

## 3. RESULTS

The effects of Ronstar® on the hematological parameters of *Clarias albopunctatus* are shown in Table 1. The hematological parameters of the control fish did not vary throughout the study. When compared with the control, the erythrocyte count decreased significantly ($p<0.05$) in the treatment groups with increasing Ronstar concentration and with duration. It also differed significantly ($p<0.05$) within the treatment groups. Generally, there was both concentration- and time-dependent decrease in the erythrocyte count in the treatment groups. At the end of the study the erythrocyte count had decreased by 20, 36 and 44% in the groups exposed to 0.2, 0.6 and 1.2 µg/L Ronstar, respectively.

The hemoglobin concentrations in *C. albopunctatus* exposed to the different concentrations of Ronstar were significantly ($P<0.05$) lower than the control and they differed also among the treatment groups ($P<0.05$). Significant ($P<0.05$) concentration- and duration-dependent decrease in hemoglobin concentrations were evident in the fish exposed to Ronstar. However, there was no significant difference ($P>0.05$) in the hemoglobin concentration of the fish exposed to 1.2µg/L Ronstar after ten days of exposure. At the end of the study, the hemoglobin concentration had decreased by 39, 41 and 63% in the fish exposed to 0.2, 0.6 and 1.2µg/L Ronstar, respectively.

When compared with the control, the hematocrit was significantly ($P<0.05$) reduced in the treated fish and there was also significant difference ($P<0.05$) in the hematocrit values among the treatment groups throughout the study. The hematocrit decreased by 60% in the fish exposed to 0.2 and 0.6µg/L Ronstar and 65% in the group exposed to 1.2µg/L Ronstar, respectively.

The hematological indices were also affected by Ronstar. After 5 days, the mean corpuscular volume (MCV) Fig. 1. increased in the treated fish when compared with the control but thereafter decreased with Ronstar concentration and duration. The mean corpuscular hemoglobin (MCH) Fig. 2. of the control fish was generally lower than in the treatment groups throughout the duration of the study. There was a significant ($p<0.05$) increase in the mean corpuscular hemoglobin concentration (MCHC) Fig. 3. in the fish treated with Ronstar. No significant ($p>0.05$) change in the MCHC value was observed in the fish exposed to 1.2µg/L Ronstar after day 10. The mean corpuscular hemoglobin (MCH) values were generally lower in the fish exposed to Ronstar than in the control.

The leucocyte count increased significantly in the treatment groups when compared with the control ($p<0.05$). The leucocytosis was both concentration- and duration-dependent and differed within the treatment groups ($p<0.05$). The subpopulations of leucocytes identified in the study Table 1. are the lymphocytes, monocytes (agranulocytes), neutrophil and eosinophil (granulocytes). Lymphocytes were the most abundant species of leucocyte in the peripheral blood in both the control and treatment groups. It increased not only with duration of exposure but also with Ronstar concentrations. When compared with the control values, there was

significant (P<0.05) neutropenia, monocytopenia and decreased eosinophils in the Ronstar-treated fish with increasing concentrations and duration of exposure.

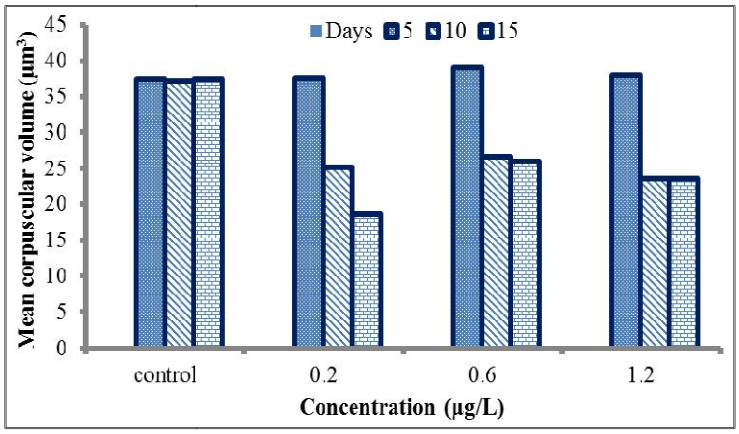


**Fig. 1. Change in the mean corpuscular volume of C.albopunctatus exposed to Ronstar®**

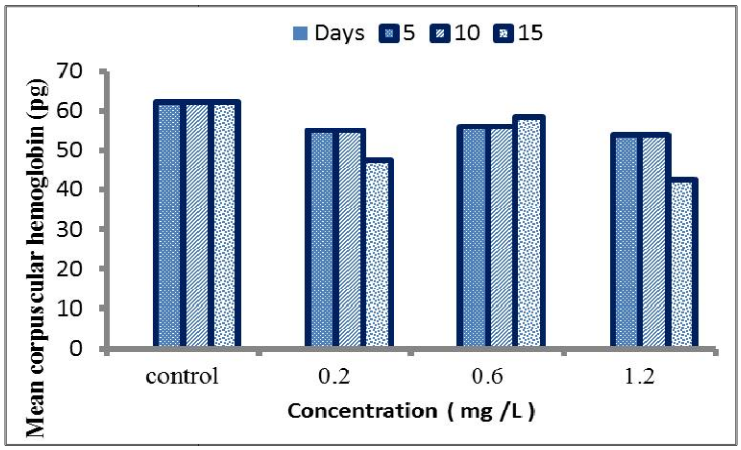


**Fig. 2. Changes in the mean corpuscular hemoglobin of C.albopunctatus exposed to Ronster®**

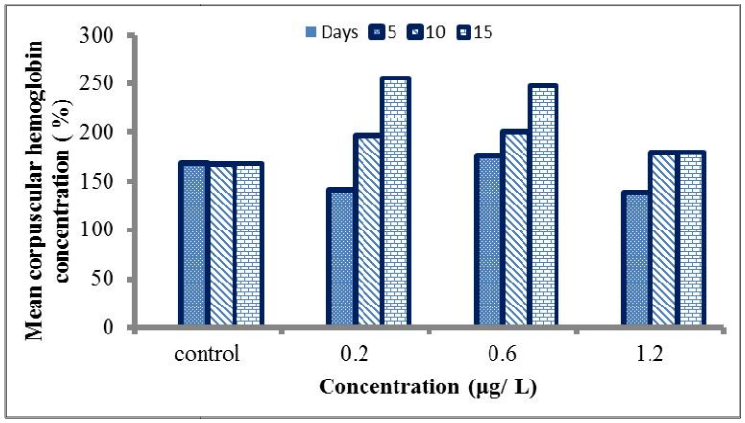


**Fig. 3. Changes in the mean corpuscular hemoglobin concentration of C. albopunctatus exposed to Ronstar®**

**Table 1. Changes in the hematological parameters of *Clarias albopunctatus* exposed to sublethal concentrations of Ronstar®**

| Parameters | Duration ( Days) | Concentration ( µg/L) [1] | | | |
|---|---|---|---|---|---|
| | | Control | 0.2 | 0.6 | 1.2 |
| Erythrocyte ( $x10^6/mm^3$) | 5 | 2.67±0.44 $^{a1}$ | 2.39±0.12 $^{b1}$ | 2.1±0.14$^{c1}$ | 1.80±0.40 $^{d1}$ |
| | 10 | 2.67±0.44$^{a1}$ | 2.31±0.18 $^{b1}$ | 1.87±0.20 $^{c2}$ | 1.48±0.30 $^{d2}$ |
| | 15 | 2.67±0.44$^{a1}$ | 2.14±0.21$^{b2}$ | 1.69±0.72$^{c3}$ | 1.48±0.30$^{d2}$ |
| Hemoglobin (g/dl) | 5 | 16.8 ±1.27 $^{a1}$ | 13.2±1.15 $^{b1}$ | 12.3±1.07 $^{c1}$ | 9.7±1.14 $^{d1}$ |
| | 10 | 16.8±1.27 $^{a1}$ | 11.8±1.09 $^{b2}$ | 10.0±1.34 $^{c2}$ | 6.9±1.04 $^{d2}$ |
| | 15 | 16.8±1.27$^{a1}$ | 10.2±1.01$^{b3}$ | 9.9±0.42$^{c3}$ | 6.3±0.21$^{d3}$ |
| Leucocyte ($x10^4/mm^3$) | 5 | 4.05± 0.25 $^{a1}$ | 10.35±1.44$^{b1}$ | 11.05±1.60$^{c1}$ | 12.15±1.54 $^{d1}$ |
| | 10 | 4.01±0.25 $^{a1}$ | 15.6±1.80 $^{b2}$ | 16.2±1.29$^{c2}$ | 18.45±1.50 $^{d2}$ |
| | 15 | 4.50±0.25$^{a1}$ | 16.0±1.62 $^{b3}$ | 17.3±1.84$^{c3}$ | 18.45±1.50 $^{d3}$ |
| Hematocrit | 5 | 10.40± 0.25$^{a1}$ | 9.0±0.10$^{b1}$ | 7.0±0.05 $^{c1}$ | 7.0±0.02 $^{c1}$ |
| | 10 | 10.20±0.04 $^{a1}$ | 6.0±0.9 $^{b2}$ | 5.0±0.02 $^{c2}$ | 3.50±0.22 $^{d2}$ |
| | 15 | 10.10±0.04$^{a1}$ | 4.0±0.12$^{b3}$ | 4.0±0.11 $^{c3}$ | 3.50±0.22 $^{d2}$ |

*[1] Values with different alphabetic (lower case) superscripts in a row differ significantly between different concentrations and within the same exposure period. Values with different numeric superscripts in column differ significantly between different exposure periods within the same concentration. Results are expressed as mean± standard error of mean*

**Table 2. Changes in the differential leucocyte count in *C. albopunctatus* exposed to Ronstar®**

| Lecucocyte species ( % ) | Concentration ( µg/L) | Duration of exposure ( days) [1] | | |
|---|---|---|---|---|
| | | 5 | 10 | 15 |
| Lymphocyte | Control | 51.0±3.6$^{a1}$ | 51.4 ± 2.6$^{a1}$ | 51.0 ±1.30$^{a1}$ |
| | 0.2 | 60.0±2.7$^{b1}$ | 61.0±3.5 $^{b2}$ | 89 ± 4.26 $^{b3}$ |
| | 0.6 | 62 ± 1.6$^{c1}$ | 67 ± 3.4 $^{c2}$ | 71 ± 4.5 $^{c3}$ |
| | 1.2 | 73 ± 4.2$^{d1}$ | 80.0 ±4.1 $^{d2}$ | 85 ± 2.0 $^{d3}$ |
| Monocytes | Control | 9.0 ±1.5$^{a1}$ | 9.4 ± 0.6$^{a1}$ | 8.8 ±1.11$^{a1}$ |
| | 0.2 | 5.0 ±0.6 $^{b1}$ | 4.0 ± 1.02$^{b1}$ | 6.0 ±0.2$^{c2}$ |
| | 0.6 | 4.0 ± 0.4$^{b1}$ | 6.0 ±1.22$^{b2}$ | 6±1,10$^{b2}$ |
| | 1.2 | 6.0 ± 0.7$^{c1}$ | 5.0±1.10$^{c2}$ | 6± 1.54$^{c1}$ |
| Neutrophil | Control | 20.0±3.1$^{a1}$ | 20.4±1.3$^{a1}$ | 20.0±2.7$^{a1}$ |
| | 0.2 | 20.0±3.1$^{a1}$ | 12. ±23$^{b2}$ | 10.1±1.5$^{b3}$ |
| | 0.6 | 12.0±.4$^{b1}$ | 10.0±25$^{c2}$ | 9.0±1.26$^{c3}$ |
| | 1.2 | 10.0±1.2$^{b1}$ | 6.0±1.13$^{d2}$ | 5.0±1.14$^{d3}$ |
| Eosinophil | Control | 5.0±0.21$^{a1}$ | 5.0±0.14$^{a1}$ | 5.8±0.68 $^{a1}$ |
| | 0.2 | 4.0±0.20$^{b1}$ | 4.0±0.78$^{b1}$ | - |
| | 0.6 | 3.0±0.02$^{c1}$ | 4.0±0.63$^{b2}$ | - |
| | 1.2 | 1.0±0.01$^{d1}$ | 2.0±0.05$^{c2}$ | - |

*[1] Values with different alphabetic (lower case) superscripts in a row differ significantly between different concentrations and within the same exposure period. Values with different numeric superscripts in a column significantly between different exposure periods within the same concentration. Results are expressed as mean± standard error of mean*

## 4. DISCUSSION

Fish responds quickly to changes in the quality of its environment and such changes affect the hematology [14-15,25-27]. The physiological and biochemical responses in fish to such environmental alterations are varied [5,8,28-29] and this makes it difficult to fully understand and elucidate the adjustment mechanisms involved. This study showed that the sublethal concentrations of Ronstar had adverse effect on the blood parameters of *Clarias albopunctatus* as there were reductions in the hemoglobin, hematocrit and erythrocyte count in the fish. These observed effects were dependent on both the concentration of Ronstar and the duration of exposure. This is consistent with the effect of monocrotophos on *Anabas testudineus* [13] and

the effect of ammonium sulphate and detergent on *Labeo umbratus* [30]. Similar reductions in these blood parameters were reported in *Clarias albopunctactus* exposed to gammalin 20 [14] and in *Oreochromis niloticus* treated with gammalin 20 and Actellic [8] as well as in the tench, *Tinca tinca* treated with potassium nitrate [31]. Similar concentration- and time-dependent decrease in hemoglobin was reported in *Prochilodus lineatus* and in *Rhamdia quelen* exposed to the herbicide clomzone [32-33]. It is in agreement with the work of Ada [34] when *Oreochromis niloticus* was exposed to paraquat. The decrease in these blood parameters are indications of anemia and hemoglobin biosynthetic impairment. The mechanisms by which Ronstar induce this anemia in the fish are varied. Santhakumar et al. [13] reported that monocrotophos provoked erythropoietin inhibition in fish which adversely affected the maturation of erythrocytes in *Anabas testudineaus*. Furthermore, pesticides have been reported to impose oxidative stress in animals including fish [6-7,35-38] that promotes membrane lipid peroxidation and protein denaturation. Thus, the observed anemia in this study could be due to the effect of oxidative stress that accentuated hemolysis.

The decreasing values of MCV with exposure time and increased concentrations of Ronstar are indication of erythrocyte shrinkage [38-39] due to osmoregulatory imbalance. The decreased MCV in this study with the concomitant decrease in MCH and MCHC with increasing Ronstar concentration and duration of exposure are good indications of reduced hemoglobin biosynthesis.

The fact that the erythrocyte count decreased with increasing Ronstar concentration and duration of exposure suggested that Ronstar may have caused impaired erythropoiesis in the fish. Gordon et al. [40] reported that a normal kidney is necessary for optimum production of erythropoietin that controls erythropoiesis in vertebrates. Reddy et al. [41] reported that the damage of kidney tubules in *Sarotherodon mossambicus* exposed to herbicide, diuron, resulted in reduced erythropoietin formation and consequent decrease in erythrocyte count. Histological changes were reported in the kidney of *C. albopunctatus* exposed to Ronstar [42]. Thus, in this study, the observed reduction in the erythropoietic activity in the Ronstar-exposed fish may be due to impaired erythropoietin production in the kidney by Ronstar. This thereby reduced the number of hemopoietic stem cells that could have differentiated into erythroblasts. This led to erythroblastopenia and indeed anemia in the fish since there was no noticeable compensatory erythropoiesis. Earlier studies showed that pesticides cause damage to or clogged the gill filaments [15,42] thereby impairing oxygen transport across the gill. This condition may have induced hypoxia in the fish with accompanying osmoregulatory imbalance that manifested in the decreased MCV values.

The extent of the effect of the surfactant on the observed changes in the hematological parameters of *C. albopunctatus* is not known. The studies of Isomaa et al. [43] and Vives et al. [44] indicated that such anionic amphiphiles might have minimal effect, as they tend to protect the erythrocytes from hemolysis rather than predisposing them to it. The significant reduction in the erythrocyte count and hemoglobin concentration observed in this study suggested that at such sublethal concentrations, Ronstar induces hypoxia, which limited the amount of oxygen would be carried in the blood to the tissues.

Leucocytosis has been reported in fish exposed to different pollutants as increased leucocyte count was reported in *Aphanius despair* [28] and *Oreochromis aureus* [20] exposed to sublethal mercury. Similar leucocytosis was observed in *Oreochromis mossambicus* exposed to copper [26] and clariids exposed to cadmium [27] and brewery wastewater [11]. Other studies also showed that pesticides and herbicides stimulate leucocyte production in fish as leucocytosis was found in *Labeo* exposed to metaystox [29] and in *Anabas testudineus* treated with monocrotophos [13]. Similar leucocytosis was found in clariids exposed to gammalin 20 and actellic [14-15] as well as in *Lymphocytosis fossilis* exposed to insecticides [12]. Thus, increased leucocyte production could be regarded as a normal response of fish to stress due to these chemical species.

Differential count showed that the lymphocytes were the dominant agranulocyte in the fish while the neutrophils were the dominant granulocytes. Lymphocytosis was also reported in Clariid species exposed to insecticide and brewery wastewater [16,45] as well as *Oreochromis* exposed to copper [26]. Thus, the observed leucocytosis and the concomitant lymphoctyosis are in indication that Ronstar induced other negative reactions in the fish, leading to the stimulation of the immunocompetent cells. There was also noticeable neutropenia in the fish

following exposure to Ronstar which was in agreement with the observation made in sturgeon (*Huso huso)* treated with diazinon [46] and in carp exposed to permethrin [47]. From the study, it could be inferred said that the impairment of erythropoiesis and hemoglobin biosynthesis, represent part of the physiological mechanisms through which Ronstar exert its effect on the fish. The significant decrease in the mean corpuscular hemoglobin concentration (MCHC) signified that Ronstar caused both microcytic and hypochrcomic anemia in the fish.

## 5. CONCLUSION

Generally, the result showed that sublethal concentrations of Ronstar led to wide range of changes in the hematological parameters of the fish. The study showed that Ronstar caused both concentration and time-dependent decrease in erythrocyte count, hemoglobin and hematocrit values. Based on the data, these parameters could be useful biomarkers for routine monitoring of the effects of pesticides on both cultured and wild fish as they could be early warning indicators of pollution or altered water quality parameters. In summary, it is plausible to suggest that the observed anemia reported in this study could have developed through the inhibition of both erythropoiesis and hemoglobin synthesis on the one hand or by provoking oxidative stress and osmotic imbalance on the other that predisposed the fish accelerated hemolysis. The result of the study could gainfully be applied regulatory policy development with a view to setting safe limits for pesticides in Nigerian water bodies so as to minimize environmental pollution.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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