Haematological and histopathological alterations in juveniles of freshwater catfish, *Clarias gariepinus* (Burchell, 1822) exposed to paraquat

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Abstract

Paraquat, a chlorinated contact herbicide is mainly used to control weeds in agriculture. The use of this herbicide may lead to its excess being washed away into aquatic ecosystems during surface runoff thereby affecting non-targeted organisms including fish. This study examined the alterations in haematology and histopathology of fish exposed to sub-lethal concentrations of Paraquat. 100 juveniles of *Clarias gariepinus* (mean weight, 17.1±6.0g; mean total length, 14.0±1.4cm) were exposed in a 48 hour renewal bioassay to sub-lethal concentrations (0.00, 1.125, 2.25, 4.00 and 9.00mg/L) of paraquat for 14 days with 10 fishes per concentration in duplicates. Five fish samples from each concentration were used for the haematological study and the haematological parameters examined were; Packed Cell Volume (PCV), Haemoglobin (Hb), Red Blood Cell (RBC), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC) and White Blood Cells (WBC). There were significant decreases (p<0.05) in PCV (from 35.70±2.97 % in the control to 16.00±0.58 % at 9.00 mg/L), Hb (from 11.90±1.10 g/dl in the control to 4.50±0.09 g/dl at 9.00 mg/L), RBC (from 3.36±0.06 10^6/µL in the control to 1.70±0.03 10^6/ µL at 9.00 mg/L), MCV (from 105.90±9.30 fl in the control to 96.14±1.80 fl at 9.00 mg/L) and MCHC (from 33.40±0.40 g/dl in the control to 28.40±0.81 g/dl at 9.00 mg/L) values of the exposed fishes which was concentration dependent but, there was no significant difference (p>0.5) in the MCH values. The White Blood Cells showed significant (p<0.5) increase at 4.00mg/L (24217±2377 10^3/µL) from that of the control (15350±2828 10^3/µL). Platelets and Lymphocytes increased significantly (P<0.05) different from that of the control while Heterocytes decreased (from 29.30±3.50 % in the control to 24.00±4.10 % at 9.00mg/L) significantly (p<0.05) with increasing concentrations. The major histopathological alterations observed were: multilayered epidermis with polygonal cells at 4.00 mg/L, thickening of epidermal cells at 9.00 mg/L, congestion of dermal blood vessels and gill capillaries at 1.125 mg/L, sloughing of the secondary gill lamellae at 2.25 mg/L and necrosis of the gill epithelium at 9.00 mg/L. This study therefore showed that chronic exposure to sub-lethal concentrations of Paraquat can affect fish adversely. Most importantly, farmers should be made to consider these when using this herbicide in controlling weeds on farmlands.

Keywords: *Clarias gariepinus*; Paraquat; sub-lethal; alterations; haematology; histopathology.

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Introduction

Anthropogenic factors contaminate aquatic ecosystems thereby inducing alteration of different sorts in the physiological and biochemical status of animals (Gabriel *et al* 2009). These alternations are considered as adaptive mechanisms which allow the organism to cope with real or perceived stressors so that the normal homeostatic state could be maintained (Barton, 2002). The use of pesticides is a common practice in agriculture and these agrochemicals whether applied on farmlands or on water to kill water weed have negative effects on aquatic biota (Edori *et al* 2013). These pesticides may be applied in very low concentrations, yet in the long run these sub-lethal concentrations will prove to become lethal to organisms (Yuan *et al* 2004) by altering behaviour, feeding habit, reproduction rate, school groups (Murty, 1986) and may result in death in severe cases (Gabriel and Edori, 2010). It has been reported that pesticides available in the aquatic environment get accumulated in the body tissues of aquatic organisms which is eventually integrated into the food chain (Omitoyin *et al* 2006) and such residual effects get to destroy organs such as liver, kidney, gills, brain, muscles and genital organs or hinder them from performing their biochemical functions optimally (Edori *et al* 2013).

Paraquat (1,1-dimethyl-4,4-bipyridinium dichloride) is a nitrogen based compound used as herbicide to control weeds on farmlands in the tropics. Globally, it is one of the most widely used herbicides and comes next to glyphosphate. It is a quick-acting and non-selective

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herbicide, which destroys green plant tissue on contact and by translocation within the plant (Zeneca, 1996). It has been reported that for agricultural use, paraquat is marketed as gramozone in formulations of active ingredients ranging between 24-36% (Simeon *et al* 2013).

The use of haematological assessment in ecotoxicological researches, environmental monitoring and evaluation of aquatic animal health is gaining ground in the field of aquaculture. It is one of the most important and a reliable indicator of fish health status which is constantly influenced by nutritional and environmental factors (Hashemi *et al* 2017). Haematology is used as a guide in the diagnosis of many diseases and in evaluating the responses to therapy in both animals and humans (Solomon and Okomoda, 2012). It has been reported that changes in the haematological indices of fish can be used for assessing the effects of contaminants as fish blood might reveal conditions within the body of fish long before there is any outward appearance of symptoms (Seriani*et al* 2010; Ogamba *et al* 2014; Hashemi *et al* 2017).

Histopathological studies have proven to be a sensitive tool used in detecting direct effects of chemical compounds within target organs of laboratory experiments and is also reported to help establish casual relations between exposure to contaminants and various biological responses (Odo *et al* 2016). Fanta *et al* (2003) reported that the study of histopathology provides very important and useful data concerning changes in cellular or subcellular structure of an organ much earlier than external notification. One of the advantageous uses of histopathological biomarkers in environmental studies is that it allows the examination of target organs and the alterations found in these organs are easier to identify than functional ones. These alterations serve as warning signs of damage to the wellbeing of the organism (Odo *et al* 2016).

A number of researchers have reported different effects of paraquat on fish ranging from acute toxicity on *C. gariepinus* (Omitoyin *et al* 2006) to chronic toxicity on gill and liver electrolyte (Edori *et al* 2013), metabolic and enzyme parameters (Ogamba *et al* 2011), organ indices and haematology (Simeone *et al* 2013), blood plasma indices of *C. gariepinus* (Kori-Siakpere *et al* 2007; Seiyaboh *et al* 2013), *Oreochromis niloticus* (Fidelis, *et al* 2012) and benny fish *Mesopotami chthyssharpeyi* (Alizera *et al* 2012; Hashemi *et al* 2017). The choice of paraquat in this study was because of its quick action. It also ranks among the frequently used herbicides for control of weeds on land, which eventually find its way into aquatic ecosystem (Ye *et al* 2002). This study was therefore carried out to examine the effects of sublethal concentrations of paraquat on the haematology and histopathology of the freshwater catfish (*C. gariepinus),* a good and commonly used bioindicator of environmental pollution.

Materials and methods

Sources and acclimatization of fish

A total of 100 juveniles of the African catfish, *C. gariepinus* (mean weight, 17.1±6.0 g; and mean total length 14.0±1.4 cm) was obtained from a private fish farm in Ibadan, Nigeria, transported to the laboratory in well aerated plastic containers and kept in the 30L capacity rectangular tanks. These tanks were filled with dechlorinated tap water and fish were kept for 14 days to allow them acclimatize to environmental conditions. The holding medium was changed every three days. Fish were fed twice daily with commercial feed pellets at 5% body weight and uneaten food was siphoned out regularly to prevent pollution of the water.

Experimental design and preparation of test solution

Completely randomized experimental design was used in this experiment with five treatment levels and two replicates as described by (Simeon *et al* 2013). The test solutions for the experiment were prepared from the stock of paraquat (276 g/L) in the market. Four different concentrations of the herbicide were prepared and a control based on the already determined LC_{50} value of 18 mg/L (Omitoyin *et al* 2006). Nominal fractions of the reported LC_{50} which gave: 9 mg/L (1/2 of LC_{50}), 4 mg/L (1/4 of LC_{50}), 2.25 mg/L (1/8 of LC_{50}), 1.125 mg/L (1/16 of LC_{50}) and 0.00 mg/L (Control) were prepared and used as test solutions.

Sub-lethal test

A total of ten rectangular tanks of 30L capacity were used for the experiment and required concentrations of the herbicides were made up to the 10L mark. Ten fully acclimatized fishes (APHA, 1981) were introduced into the different test concentrations of the herbicide and observed for 14 days. The test solutions were renewed every 48 hours in order to maintain the requisite concentrations (OECD, 2002).

Physico-chemical parameter analyses

Physico-chemical parameters such as pH and temperature were monitored regularly throughout the period of the experiment using a digital pH meter with a glass thermometer.

Fish blood collection and analysis

Blood for analysis was collected from 5 fish samples per concentration from the treated and control groups at the end of 14 days exposure period. The fish was then placed on its back and the caudal fin held in position to avoid mobility. Blood was taken at the caudal vein within the caudal peduncle region using heparinized syringe and needle. 1 to 2 ml of blood was taken from fish in each tank for haematological analysis. The blood was temporarily kept in labeled heparinized bottles (Akinwade *et al* 2004) at 0^{o}C before the analysis. Blood

from the various treatments were analysed for the following haematological parameters: packed cell volume (PCV), heamoglobin (HB), red blood cell count, white blood cell count and differentials using standard haematological procedures (Blaxhall and Daisley, 1973). Packed cell volume (PCV) was determined in microhematocrit tubes after centrifuging for five minutes. Haemoglobin (Hb) was determined by the cyanomethaemoglobin method. Total white blood cell (WBC), red blood cell (RBC) and platelets were determined manually with a Neuauerhemacytometer using Natt-Herrick's solution as a diluent. The differential counts (leucocrit, lymphocyte and neutrophils) were examined by dropping well mixed blood film on clean microscope slides and allowed to dry for 24 hours. The slides were fixed in methanol and then stained with Grumwall-Giemsa stain. The count of the cells was done noting the different cell type and their percentage occurrence. The red blood cell (RBC) indices; mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC) and mean corpuscle volume (MCV) were calculated using methods described by Seiverd (1983) as shown below:

Mean corpuscular haemoglobin (MCH)

$$= \frac{\text{Haemoglobin (HB)}}{\text{RBC count}} \times 10$$

Mean corpuscular volume (MCV)

$$= \frac{\text{PCV}}{\text{RBC count}} \times 10$$

Mean corpuscular haemoglobin concentration (MCHC)

$$= \frac{\text{Haemoglobin (Hb)}}{\text{PCV}} \times 100$$

Histopathological preparation and wxamination

After the exposure period of 14 days, 2 fish samples were selected at random from each of the test concentrations and control groups for histopathological investigations. The skin, gill and liver were collected and immediately fixed in Bouine's solution, dehydrated in ascending grades of alcohol, cleared in xylene, embedded in paraffin wax and sectioned at 4-7 µm. The slides were stained with haematoxylin and eosin and examined microscopically (Osman *et al* 2010).

Statistical analysis

One way Analysis of Variance (ANOVA) was used to test for significant difference between means (SPSS 17) and Duncan Multiple Range Test (DMRT) was used to test for significant difference between treatments when $p<0.05$.

Results

The results of the Packed Cell Volume (PCV), haemoglobin (Hb), red blood cell RBC), mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) showed significant decreases ($p<0.05$) with increasing concentration of Paraquat as compared with the control groups (Table 1), but results of the mean corpuscular haemoglobin (MCH) did not show any significant difference ($p>0.05$). The result of the White Blood Cell and the differentials of *C. gariepinus* exposed to different concentrations of paraquat showed significant increases ($p<0.05$) in White Blood Cell (WBC), Platelets and lymphocytes with increasing concentrations compared with the control. Heterocytes had values that increased but not significantly different ($p<0.05$) from the other

Table 1. Red blood cell and red cell indices of juveniles of *C. gariepinus* exposed to different concentrations of paraquat for 14 days.

Concentration mg/l)	PCV (%)	Hb (g/dL)	RBC (10^6/µL)	MCV (fL)	MCH (pg)	MCHC (g/dl)
0.00 (Control)	35.70^{a}±2.97	11.90^{a}±1.10	3.36^{a} ±0.06	105.90^{a}±7.30	35.40^{a}±2.80	33.40^{a}±0.40
1.125	29.30^{b}±0.70	9.50^{b} ±0.40	3.37^{a} ±0.10	87.04^{ab}±1.90	28.10^{ab}±0.64	32.24^{a}±0.65
2.25	27.00^{b}±0.58	8.60^{bc}±0.12	3.21^{a} ±0.41	86.50^{ab}±10.90	27.60^{ab}±3.60	31.75^{ab}±0.70
4.00	18.70^{c}±1.33	6.00^{c} ±0.74	1.53^{b} ±0.10	122.10^{c}±5.20	38.80^{a}±3.09	31.71^{ab}±1.80
9.00	16.00^{c}±0.58	4.50^{c} ±0.09	1.70^{b} ±0.03	96.14^{b} ±1.80	27.30^{ab}±0.30	28.40^{b}±0.81

Means in the same column with the same superscript are not significantly ($p<0.05$) different.

Table 2. White blood cell count and differentials of juveniles of *C. gariepinus* exposed to different concentrations of Paraquat for 14 days.

Concentration (mg/L)	WBC (10^3/µL)	Platelets (%)	Lymphocytes (%)	Heterocytes (%)	Monocytes (%)	Eosinophil (%)
0.00 (Control)	15350^{a} ±2828	76000^{a} ±42981	64.70^{a} ±2.90	29.30^{a}±3.50	1^{a}±0.30	4^{a}±0.30
1.125	14067^{ab} ±3530	168667^{bc} ±20299	60.70^{c} ±3.39	34.30^{c}±3.80	3^{a}±0.90	3^{a}±1.00
2.25	15500^{ab} ±1515	148667^{c} ±19690	67.70^{ab} ±6.40	25.30^{ab}±5.90	3^{a}±0.88	3^{a}±0.88
4.00	24217^{b} ±2377	176333^{b} ±51992	68.30^{ab} ±4.40	26.30^{b}±3.72	2^{a}±0.30	4^{a}±1.20
9.00	18300^{c} ±2775	79000^{a} ±31549	70.70^{b} ±4.30	24.00^{ab}±4.10	3^{a}±0.30	2^{a}±0.70

Means in the same column with the same superscript are not significantly ($p<0.05$) different.

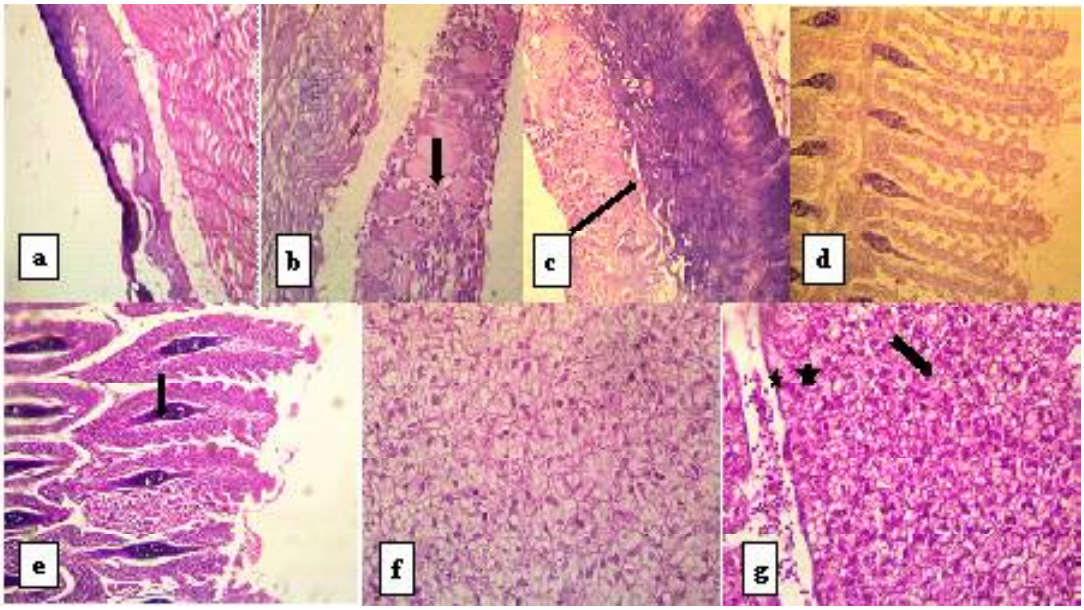
concentrations and the control. Results of Monocytes and Eosinophils showed values that were not significantly different (p>0.05) from the control group with increasing concentrations (Table 2).

Histopathological examinations of the skin, gill and liver of *C. gariepinus* exposed to various concentrations of paraquat showed alterations as presented in Table 3 and Plates 1(a-g).

Table 3. The degree of severity of histopathological aberrations across different concentrations in *C. gariepinus* exposed to Paraquat for 14 days.

Organs	Histopathological aberrations	Concentrations (mg/L)				
		0.00	1.125	2.25	4.00	9.00
Skin	Degeneration of epidermal cells	-	+	-	-	-
	Congestion of dermal blood vessels	-	++	-	-	-
	Multi-layered epidermis with ballooning degeneration	-	-	+	-	-
	Multi-layered epidermis with large abnormal polygonal cells	-	-	-	++	-
	Thickened epidermis with densely packed collagen fibres	-	-	-	-	+++
Gills	Thickening of primary and secondary gill lamellae	-	+	-	-	+++
	Congestion of gill capillaries	-	++	-	-	-
	Sloughing of secondary gill lamellae	-	-	+	++	-
	Necrosis of gill epithelium	-	-	-	-	+++
Liver	Vacuolar changes of hepatocytes	-	+	++	-	-
	Hepatocellular necrosis	-	-	++	-	-
	Large binucleate and dividing hepatocytes	-	-	-	-	++
	Congestion of blood vessels	-	-	-	-	++

Key: (–) =Nil/Absent, (+) = Mild, (++) = Moderate, (+++) = Severe.



Plates 1(a-g). Photomicrographs of histopathological alterations in *C. gariepinus* exposed to different concentrations of Paraquat. (a) Epidermal cells of *C. gariepinus* in the control group with no visible lesion x 400. (b) Epidermal cells of *C. gariepinus* at 4mg/L with a multilayered epidermis and very large polygonal cells (arrow) x400. (c) Epidermal cells of *C. gariepinus* at 9 mg/L with a thick epidermis and dermis of densely packed collagen fibres (arrow) x400. (d) Gills of *C. gariepinus* in the control group with normal gill lamellae x400. (e) Gills of *C. gariepinus* at 9 mg/L with necrosis (arrow) on the epithelium of gill lamellae x400. (f) Liver of *C. gariepinus* in the control-group with normal hepatocytes x400. (g) Liver of *C. gariepinus* at 9 mg/L with congestion of blood vessels (star) and dividing cells (arrow) x400.

Discussion

The data obtained from this study showed that sub-lethal concentrations of Paraquat had adverse effects on the juveniles of *C. gariepinus* as indicated by the alterations in haematological parameters. The significant decrease (p<0.05) in the values of Packed Cell Volume (PCV), haemoglobin (Hg), Red Blood Cell (RBC), Mean Corpuscular Volume (MCV) and Mean Corpuscular Haemoglobin Concentration (MCHC) observed in this study was in accordance with the findings of (Patnaik and Patra, 2006) who studied the haemapoietic alteration in *Clarias batrachus* induced by Cabaryl; Kori-Siakpere

et al (2007) who studied the acute haematological effects of sublethal levels of paraquat on *C. gariepinus* and Hashemi *et al* (2017) who investigated the toxicological effects of paraquat on the haematological parameters of *Mesopotami chthys sharpeyi*. However, the non-significant ($p>0.05$) reduction in the MCH values observed in this study was not in congruence with those of Kori-Siakpere *et al* (2007); Alizera *et al* (2012), and Hashemi *et al* (2017). The alterations indicate that exposed fish suffered from anemia induced by the herbicide. This is an indication of disruptive effects of paraquat on erythropoietic tissues as well as cells viability (Kori-Siakpere *et al* 2007). It is also possible that paraquat adversely suppressed fish osmoregulation which may finally result in dilution of blood (Kori-Siakpere *et al* 2007; Hashemi *et al* 2017). Similar observations have been previously reported by other researchers for some pesticides such as diazinon (Padash-Barmchi *et al* 2010; Svobodova, *et al* 2001), Dichlorvos (Benarji and Rajendranath,1990), malathion (Khattak *et al* 1996), and trichlorphon (Tavares *et al* 1999). Erythrocytes and their hemoglobin contents are responsible for oxygen transportation within the body and low number of red blood cells or insufficient amount of their haemoglobin content could influence energy balance of the body. In this case fish may suffer from oxygen deficiency (Koprucu *et al* 2006), which eventually prohibits its normal growth. Moreover it seems that reduction in red blood cells is a key factor which could be responsible for reduction in productivity (Kang *et al* 2003). The initial decrease observed in the values of WBC, could be an indication that the herbicide on introduction, interferes with the organism's immune system, at which point the fishes are more susceptible to bacterial or fungal infections (Alizera *et al* 2012). The white cells play a role in fish immunology and the subsequent increase observed could be attributed to several factors such as increase in thrombocytes, lymphocytes or squeezing of leucocytes in peripheral blood (Kori-Siakpere *et al* 2007). Increase in white cell counts has thus been reported to be a protective response to stress (Kori-Siakpere *et al* 2007; Alizera *et al* 2012).

The histopathological examination carried out in this study showed histological alterations in the skin, gill and liver of C. *gariepinus* in response to different sublethal concentrations of Paraquat. The skin of *C. gariepinus* showed normal thin epidermis and dermis with collagen fibres with no noticeable lesion in the control groups. However, the multiple foci degeneration of the epidermal cells at 1.125 mg/L concentration may have been as a result of loss of intercellular connections between the outer layers of cells of the epidermis. Also, the congestion of dermal blood vessel observed in the skin of fish at 1.125 mg/L concentration could have been as a result of initial cellular responses to stimuli caused by chemicals that bring about constriction of blood vessels. The multi-layered epidermis containing abnormal polygonal cells and multi foci tinged material observed at 4 mg/L concentration are suggestive of myxoid (accumulation of mucus-like substance) on the skin. The thick epidermis observed at 9 mg/L concentration is suggestive of hyperkeratosis in the fish, a kind of skin condition characterized by warty growth which may have been the effect of prolonged exposure to the chemical. All of these alterations to an extent could explain the reason(s) why manufacturers of these chemicals clearly state in their instruction or guidelines of usage that the chemical should not be brought in contact with the skin (eyes, hands and legs).

Photomicrographs of gill of *C. gariepinus* in the control-group showed normal structure of primary and secondary gill lamellae with no visible lesion but, proliferative thickening of the primary and secondary gill lamellae, congestion of gill capillaries and necrosis of gill epithelium resulted with increased concentration of the chemical. These observed changes in the gills could be as a result of the fact that gills remain in close contact with the external environment and are particularly sensitive to changes in the quality of water thereby becoming primary target organs of the contaminant (Osman *et al* 2010). The cellular damages observed in the gills in term of epithelium necrosis can adversely affect the gas exchange and ionic regulation (Osman *et al* 2010). The observed changes in the gills are in line with earlier reports of paraquat's acute toxicity on the gills of juveniles of *C. gariepinus* by Omitoyin *et al* (2006) and toxicity of other chemicals such as 2,4-D Amine on gills of *C. gariepinus* (Makinde *et al* 2015), Cyperdicot and Vitamin E supplementation on *C. gariepinus* (Odo *et al* 2016) and glyphosphate on some selected neotropical fishes (da Cruz *et al* 2016).

Photomicrographs of liver of *C. gariepinus* in the control-group showed centrally placed nucleus, normal hepatocytes with no vacuolar changes. The widespread vacuolar change of hepatocytes observed at concentration of 1.125 mg/L is suggestive of the fact that the liver responds quickly to slight changes in its environment especially when it relates to pollutants. Hepatocellular necrosis and congestion of blood vessels observed with increasing concentration of paraquat could be attributed to the direct toxic effects of pollutants on hepatocytes, since the liver is the principal organ responsible for detoxification in vertebrates generally and in fish particularly (Soufy *et al* 2007). Large hepatocytes having clear cytoplasmic vacuoles with no vascular changes, observed at 4 mg/L concentration could have been as a result of the liver cells detoxifying and quickly recovering to accommodate effects of pollutants as it increases. The observed vacuolization of hepatocytes could indicate an imbalance between the rate of synthesis of substances in the parenchyma cells and the rate of their release into the circulation (Osman *et al* 2010). The binucleated hepatocytes and the hepatocytes undergoing mitotic division observed at 9 mg/L concentration are suggestive of regenerating liver cells in response to increased pollution. The results are in agreement with those observed

by other researchers who studied the effect of different pollutants on fish liver (Ptashynski *et al* 2002; Fanta *et al* 2003; Osman *et al* 2010).

Conclusion

Findings from this study have been able to establish the fact that, exposure of juveniles of *C. gariepinus* to chronic sublethal concentrations of paraquat, can induce various alterations in haematology and histopathology, which is dependent on the concentration and period of exposure. These observed alterations can negatively affect the fish by hampering growth, reproduction, immunity and survival of the fish in its natural environment as well as in cultured or controlled conditions. Therefore, farmers and the generality of the people who often use these chemicals in the environment should be made to consider these deleterious impacts especially on non-target organisms like fish which constitute important biota of aquatic ecosystems.

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