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Haematology of *Clarias gariepinus* exposed to Microcystin-LR

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ABSTRACT

A static bioassay experiment was conducted by exposing *Clarias gariepinus* to 200 μg/L and 400μ/L of MC-LR solutions for 14 days and 28 days to assess the haematological impacts such as estimation of red blood cells, white blood cell and Thrombocytes, haemoglobin percentage, haematocrit, and percentage changes. A dramatic reduction was observed in the RBCs in both experimental cases after 28 days; which can be attributed to haemolysis. Result showed significant impact of MC-LR on RBCs counts on duration (28 days) basis; regardless of the concentrations. The PCV after 28 days was significantly lower than that observed after 14 days, which was also significantly lower than that observed in the control set-up. The concentrations; particularly 400 μg/L MC-LR exerted significant stress on the fish. A general temporal increase was recorded in the total count of WBCs in both experimental cases. Results showed that MC-LR; particularly 400 μg/L caused significant haematological disruptions in the *C. gariepinus*, especially after 28 days of exposure i.e. 400 μg/L (28 days) > 400 μg/L (14 days) > 200 μg/L (28 days) > 200 μg/L (14 days). Due to the fact that natural aquatic ecosystems affected with algal bloom contain concentrations of MC-LR higher than the experimented levels, aquatic biota in affected water bodies are liable to suffer worse consequences than those observed in this experiment. This necessitates proactive measures on susceptible water bodies in order to forestall liable debilitating effects on the aquatic biota.

1. Introduction

Cyanobacteria (blue-green algae) thrive in nutrient rich and warm water bodies. Of recent, this algae has gained a lot of scientific research attention due to the fact that its bloom has been associated with myriads of health and ecological concerns. The algae contains cells which produce biotoxins. Among all the biotoxins produced by blue-green algae, Microcystins (mainly produced by *Microcystis aeruginosa*) have the highest eco-toxicological significance (Witeska, 2003). Sadly, extensive studies have shown that aquatic biota have high affinity for water containing strains of microcystis cells. The implication is that the biotoxin; which appears to be a part of aquatic biota diet (Bowen, 1982; Beveridge et al., 1993; Lopez and Costas, 1999; Mohamed and Carmichael, 2003; Stewart et al., 2008) is deleterious to the consumers. Further worrisome is the fact that Microcystins are very stable; because they are not broken down under atmospheric conditions (OEHHA, 2009).

Microcystin-LR (MC-LR) is the most toxic and most abundant of all Microcystin congeners; hence has attracted the highest ecotoxicological attention (OEHHA, 2009). While some schools of thought posit that microcystins are actually carcinogenic (IARC, 2006), some other experts have demonstrated experiments to show that microcystins are tumor promoters i.e. they are not carcinogenic but agents that stimulate the proliferation of cancerous cells (OEHHA, 2009). However, neither direct nor in-direct carcinogenicity negates their ecotoxicological significance. MC-LR can be classified as a hepatotoxin i.e. they inflict damages mainly on the liver through inhibition of protein phosphatase 1 and 2A, thereby increasing protein phosphorylation, ultimately resulting in necrosis and compromised liver architectural integrity (Malbrouck and Kestemont, 2006), apoptosis and intrahepatic hemorrhage (Hooser et al., 1989; Carmichael, 1994; Fawell et al., 1994; Hooser, 2000). Furthermore, the occurrence of microcystin has been associated with myriads of catastrophes ranging from hepatotoxicity to high death tolls; even in humans (Azevedo et al., 2002). For example, high incidences of liver cancer recoded in some settlements have been traced to mycrocystin-invaded rivers (Fawell et al., 1994). However, other studies have also demonstrated high toxicity of MC-LR to other organs than the liver. For example, Carps exposed to MC-LR elicited sever damage to renal proximal tubular cells and hepatocytes (Fawell et al., 1994). Severe liver damages were observed in juvenile carps exposed to 400 μg/kg body weight (b.w.) (Fischer and Dietrich, 2000), 40 μg/kg b.w. in adult carp (Carbis et al., 1996) and 4400 μg/kg b.w. in trouts (Wiegand and Pflugmacher, 2005). Damages to other organs i.e.

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heart (Bes et al., 2001), bones (Bury et al., 1995) etc.; have also been recorded.

Water quality is a determining factor to the heath of fish; hence dynamics of fish populations (Rose et al., 1993). Therefore water bodies deserve relentless biomonitoring for perpetual aquatic ecosystem sustainability assurance. Bioassay experiments are so far one of the most viable tools in toxicity assessment of xenobiotics (Oshode et al., 2008). While haematological parameters are standard sine qua non in determining the sub-lethal concentrations of xenobiotics in fish (Witeska, 2003); which are the indices of their health status (Oshode et al., 2008). Differential count of blood cells is a reliable haematological index in studying the anthropogenic perturbations on aquatic environmental conditions (Goger and Sawant, 1989). Extensively, fish haematological parameters are often determined as an index of their health status (Oshode et al., 2008). There is dearth information on the haemotological effects of microcystins on fish. Although there has been enormous research efforts on the oxidative stress of microcystin exposed fish species (Jos et al., 2005; Isibor, 2017). Fish haematological studies of other toxicants have been profoundly promising (Aguigwo, 1998; Maheswaran et al., 2008), hence MC-LR does not appear to be an exception. Previous studies of red blood cell (RBC) of fish exposed to some heavy metals have indicated anemia. Interestingly, in some cases, particularly after short exposures, blood parameters such as haematocrit, RBC, mean corpuscular volume, and haemoglobin percentage declined substantially (Dethloff et al., 1999). The toxic stress and haematological effects of copper, lead, mercury and cadmium on the hematology of *C. gariepinus* have been enormously reported (Kori-Siakpere and Egor, 1999; Rogers et al., 2003; Kori-Siakpere et al., 2006; Maheswaran et al., 2008).

C. gariepinus has a very high commercial value in Africa; particularly in Nigeria. It remains the best choice of commercial fishing farms due to its toughness and relatively fast growth rate; hence appreciable cultivability. *C. gariepinus* cultivation contributes about 17% of over 6000 tonnes of annual fish production from all fisheries sectors in Africa (Awachie and Ezenwaji, 1998).

The present study was undertaken to evaluate some haematological effects resulting from the exposure of *C. gariepinus* to sublethal concentrations of MC-LR. The resulting data could be a useful tool in ameliorating the morbidity of MC-LR to fish population.

2. Material and methods

A static bioassay experiment was conducted for 14 and 28 days using *Clarias gariepinus* as test organisms. This was done in order to study the toxicity of MC-LR at concentrations of 200 μg/L and 400 μg/L on the fish.

2.1. Acclimatization of Clarias gariepinus

Healthy specimens of *C. gariepinus* were obtained from a viable fish farm within Ikpoba-Okha Local Government Area of Edo State, Nigeria. *C. gariepinus* was chosen for the study due to its ability to withstand stress and its high commercial value in Nigeria. A preliminary check was done on the health status of the fish to ascertain good general health conditions. Gender discrimination was avoided during selection of the fish to be recruited for the experiment. Afterwards, the screened individuals were acclimatized for 2 weeks in 3 different glass aquaria ($60 \times 48 \times 48$) cm^3 containing 96 L of non-chlorinated freshwater. Eight fish individuals were released into each aquarium. During the period of the acclimatization they were fed with artificial feed and ground shrimps harvested from an unperturbed section of Osse River, Ovia North East Local Government Area of Edo State, Nigeria. The size of the fish varied from 22.5–32.6 cm in standard length and 55.2–96.4 g in weight. Water in the aquaria was changed 24 hourly with uninterrupted aeration and photoperiod of 12–12 h light-dark cycle. The physico-chemistry of the water was maintained at temperature of 27 ± 2 °C, dissolved oxygen of 7.5 ± 0.6 mg/L and the hydrogen ion concentration (pH = 8) employing Electric Probe Hydro-lab water quality meter (HANNA HI 9813 GRO).

2.2. Microcystis aeruginosa collection and MC-LR extraction

With the aid of a plankton net (20 μm mesh size) algal samples were collected from the surface of an algae-invaded section of Jemaison River in Delta State, Nigeria. 250 ml dark bottles with lids were properly rinsed with distilled water and used to collect algal samples and then kept in a cooler. They were then transported immediately to the laboratory for analysis.

In the laboratory, culture of the algal samples were centrifuged in the 250 ml bottles; in batches at 3500 rpm for 2 h to concentrate the algal cells into a pellet. The cell pellet was then lyophilized for 48 h using a freeze-dry system (Xie et al., 2005; Rinta-Kanto and Wilhelm, 2006). The lyophilized cells were extracted three times with 10 ml 0.1 M acetic acid and 20 ml of a mixture of methanol: chloroform (1:1 v/v). The cell suspension was sonicated in an ultrasound bath for 15 min, then stirred for 30 min at room temperature, and then centrifuged again at 4500 rpm for 15 min. Over 90% of MC-LR was identified in the cyanobacterial cell extracts analyzed using a Varian 9012 equipped with a Varian ProStar 330 Diode Array Detector. Solutions for MC-LR treatments were prepared for the aquariums by dissolving 1 mg of MC-LR in 0.5 ml ethanol and diluting to 200 and 400 μg/L using aquarium water. The concentration of ethanol in all treatments was 0.05%, and a treatment of 0.05% ethanol was used as a vehicle control while aquarium water was the negative control.

2.3. Exposure of Clarias gariepinus to MC-LR

Using the method demonstrated by Pandey et al. (2005), the calculated LC_{50} of MC-LR for the fish was estimated to be 1200 μg/L in 12 days. Feeding was discontinued on commencement of the experimental regime. 8 replicates of *C. gariepinus* were kept separately in three 96 L capacity aquaria. The first contained non-chlorinated freshwater, the second contained 200 μg/L MC-LR solution while the third contained 400 μg/L MC-LR solution. After 14 days, 4 replicates were collected from each aquarium for comparative haematological analysis. The remaining 4 individuals were collected from the aquaria after 28 days for further analysis.

2.4. Haematological analysis

Blood samples were collected from both the control and experimental fish retrieved after 14 and 28 days. The blood samples were taken by puncturing the posterior caudal vein using ethylenediamine tetra acetate (EDTA) as anticoagulant (Rose et al., 1993). 2 ml of blood sample was decanted in heparinzed bottles for determination of blood parameters. The microhaematocrit method described by Snieszko (1960) was used to determine the packed cell volume (PCV/ haematocrit). Haemoglobin concentration was measured with Haemoglobin test kit using the cyanmethemoglobin method (Kori-Siakpere and Egor, 1999). Blood samples were diluted with 0.85% NaCl solution i.e. 1 (blood): 5 (NaCl solution). Blood cells were counted within 5 of 25 chambers of a Neubauer haemocytometer and the mean value was calculated (Goger and Sawant, 1989; Schmitt et al., 1999). The counting was done under an Olympus Vanox Research Microscope (Mag 60),

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Model 230485. The derived haemoglobin percentage was calculated using standard formula as described by Jain (1986).

2.5. Statistical analysis

Experimental data and those of control were statistically analyzed by means of analysis of variance (ANOVA) using the mean ± standard error. Significance was set at $P < 0.05$. All analysis was performed using SPSS software (version 13.0).

$$\text{Total blood cells in blood sample} = \frac{C}{N \times V} \times \frac{V_2}{V_1}$$

where C = number of cells counted, N = number of chambers counted, volume of chambers, V_2 = volume of diluted sample, V_1 = volume of original sample.Percentage change in blood parameters was calculated thus;

$$\% \text{ Change} = \frac{\text{New value} - \text{Initial value}}{\text{Initial value}} \times 100$$

3. Results and discussion

3.1. Haematological analysis

The total count of Red Blood Cells (RBCs) observed in the both experimental (200 and 400 μg/L) *C. gariepinus* after 14 days was significantly higher than that of the control; which was significantly higher than that exposed for 28 days ($P < 0.05$). On the 14th day, the RBC count increased to $11.3 \pm 0.32 \cdot 10^6$ mm^{-3} of blood in fish exposed to 200 μg/L MC-LR, while it increased to $13.2 \pm 0.12 \cdot 10^6$ mm^{-3} of blood in those exposed to 400 μg/L, compared to $9.2 \pm 0.31 \cdot 10^6$ mm^{-3} of blood in control (Fig. 1). After 28 days, the total RBCs count had dropped drastically to 6.7 ± 0.62 and $7.1 \pm 0.87 \cdot 10^6$ mm^{-3} of blood in the 200 and 400 μg/L experiments respectively. This implies that both doses earlier stimulated proliferation of RBCs which later reduced dramatically. The dramatic reduction of the RBCs in both experimental cases after 28 days can be attributed to haemolysis. Result showed that duration (28 days) of exposure had significant impacts on the total count of RBCs; regardless of the concentrations of MC-LR. Maitra and Nath (2014) observed a similar drop in RBCs of air breathing lungfish (*Heteropneustes fossilis*) exposed to urea; on duration and concentration basis. Similar upward trend (after 7 days), followed by a downward trend (after 14 days) was observed in the RBC count of urea-exposed *Heteropneustes fossilis*. However, the current observation is at variance with that of urea-exposed *H. fossilis* in terms of haemoglobin percentage i.e. an upward trend was observed with increased concentration and exposure duration (Maheswaran et al., 2008). Insignificant change in haemoglobin percentage on concentration and duration basis (Fig. 2) in the current study is at variance with the findings of Maheswaran et al. (2008). The coincidence between a drastic drop in RBCs and insignificant change in haemoglobin percentage can be attributed to the destruction of the RBCs by MC-LR; thereby releasing the haemoglobin, culminating in reduction of the former and stability in the concentration of the latter.

As for the analysis of the haematocrit (PCV) of the experimental fish (Fig. 3), evidences of anaemia were apparent on concentration and exposure duration basis; only in fish exposed to 400 μg/L. There was a significant reduction in the packed cell volume in the fish exposed to 200 μg/L of MC-LR after 14 and 28 days. As for those exposed to 400 μg/L, the PCV after 28 days was significantly lower than that observed after 14 days, which was also significantly lower than that observed in the control fish. The concentrations; particularly 400 μg/L MC-LR significant stress on the fish physiology. This implies that a water body containing MC-LR concentration as high as the illustrated concentrations are liable to disrupt the physiological equilibrium of the biota therein. This result conforms to the trend of haematocrit observed in *Oreochromis niloticus* exposed to sub-lethal concentrations of aluminium (Bhagwan and Bhikajee, 2000) and that observed in nickel-exposed *C. gariepinus* fingerlings (OEHHA, 2009).

A general temporal increase in WBCs count was recorded in both experimental cases (Figs. 4 and 5). After 14 days of exposure to MC-LR (Fig. 2), significant increases were recorded in the Eosinophils (200 and 400 μg/L), Manocytes (200 and 400 μg/L), Neutrophils (400 μg/L) and Thrombocytes (400 μg/L). This is an indication that there was a significant response of the immune system of the *C. gariepinus* after 14 days of exposure; particularly to 400 μg/L MC-LR. The total count of WBCs ranged from a minimum of $17.5 \cdot 10^9$ mm^3 (Lymphocytes) to maximum of $44.2 \cdot 10^9$ mm^3 (Neutrophils) in the 14 days exposure, while the range was

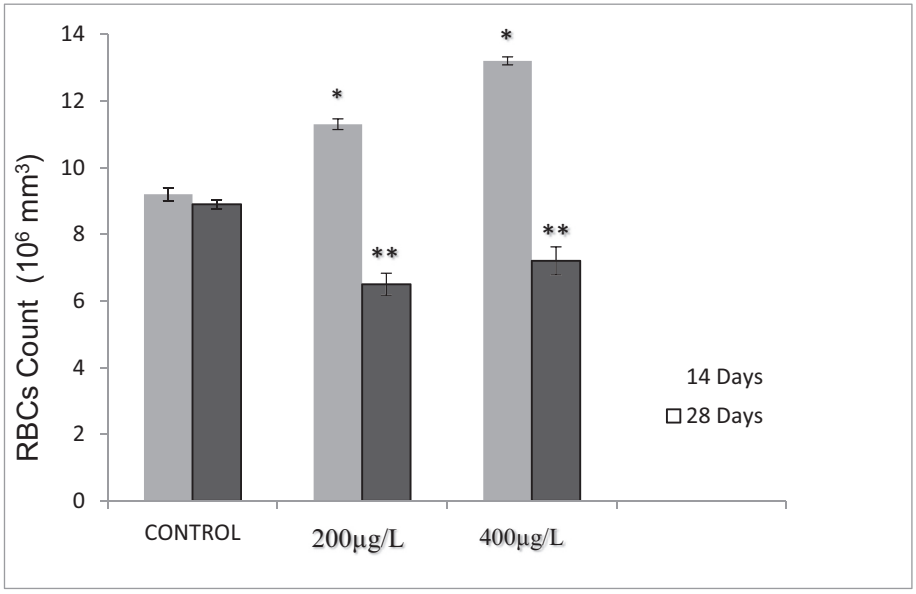

Fig. 1. RBC count of *C. gariepinus* exposed to different concentrations of MC-LR for 14 days and 28 days. * = significant difference at $P < 0.05$. Sample size = 4.

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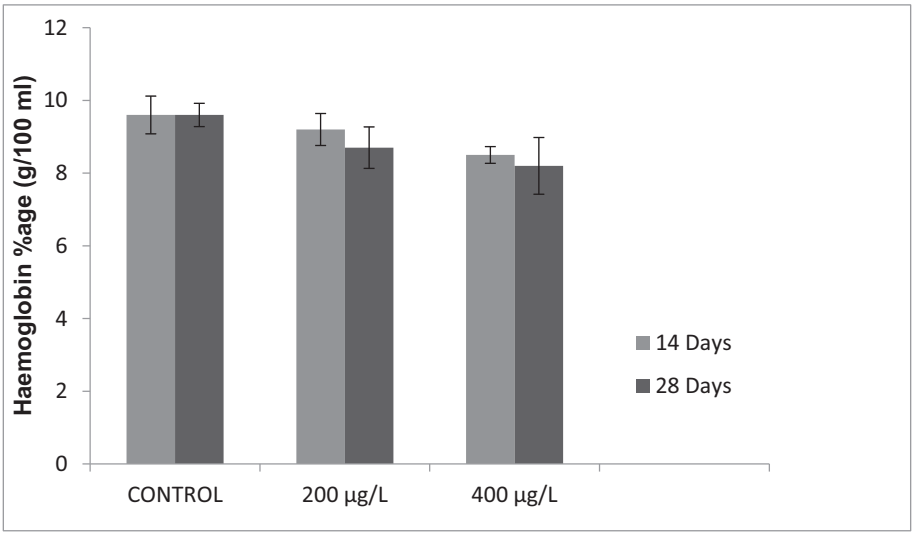

Fig. 2. Haemoglobin percentage of *C. gariepinus* exposed to different concentrations of MC-LR for 14 days and 28 days. Sample size = 4.

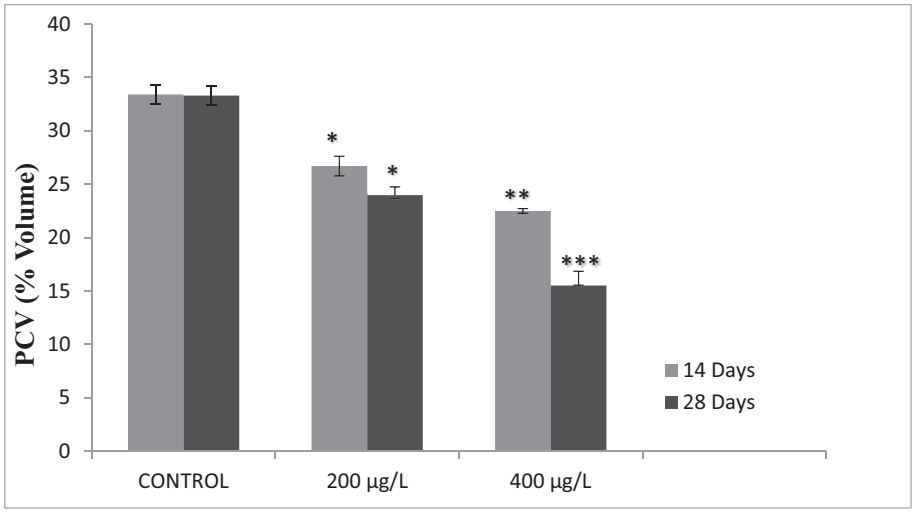

Fig. 3. Haematocrit (PCV) of *C. gariepinus* exposed to different concentrations of MC-LR for 14 days and 28 days. * = significant difference at $P < 0.05$. Sample size = 4.

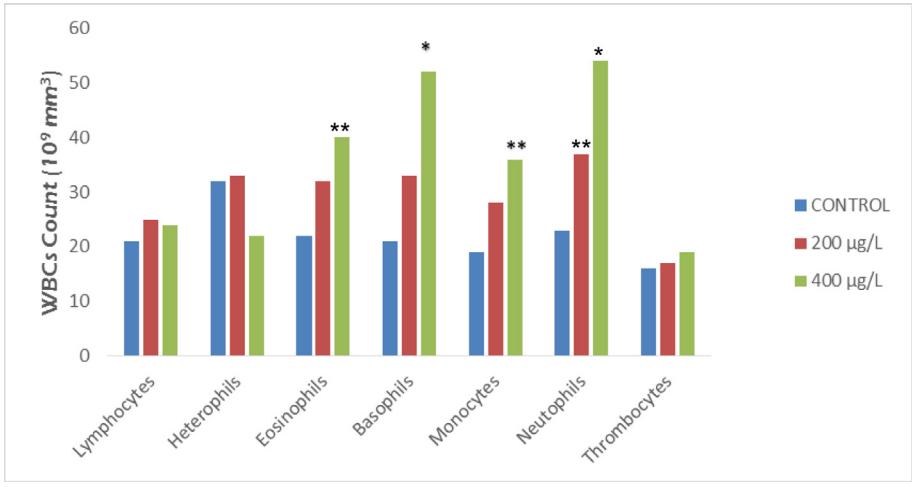

Fig. 4. WBCs count of *C. gariepinus* exposed to different concentrations of MC-LR for 14 days. * = significant difference at $P < 0.05$. Sample size = 4.

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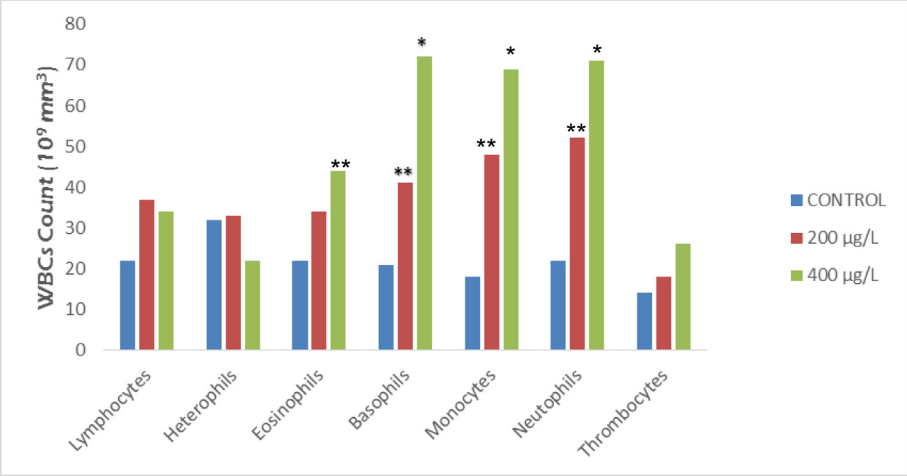

Fig. 5. WBCs count of *C. gariepinus* exposed to different concentrations of MC-LR for 28 days. * = significant difference at $P < 0.05$. Sample size = 4.

$23.3 \cdot 10^9$ mm^3 (Heterophils) to $72.4 \cdot 10^9$ mm^3 (Basophils) in the 28 days exposure. The temporal heterogeneity of immune response in the fish conforms to the results obtained in the analysis of the RBCs count.

After 28 days, significant differences occurred in the counts of Basophils, Manocytes and Neutrophils between the 200 µg/L and 400µ/L experimental fishes (Fig. 3). Generally, significant increases were recorded in the counts of Basophils (400 µg/L), Manocytes (400 µg/L), Neutrophils (400 µg/L), and Thrombocytes (200 and 400 µg/L); which were all significantly higher than the Basophils (200 µg/L), Manocytes (200 µg/L) and Neutrophils (200 µg/L); which were higher than the rest experimental cases ($P < 0.05$). This indicates that 400 µg/L of MC-LR had most significant impacts on the immune system after 28 days in all the cases, except in the case of the Thrombocytes; where 200 µg/L exposure triggered significant response. Increase in exposure duration and concentration was accompanied by increase in eosinophils, basophils, monocytes and neutrohpils in zinc-exposed *C. gariepinus* and *O. niloticus* (Annune et al., 1994), copper-exposed *Oncorhynchus mykiss* (Vosyliene, 1996), mercuric chloride-exposed *Clarias batradrus* (Maheswaran et al., 2008), and urea-exposed *Heteropneustes fossilis* (Maitra and Nath, 2014); to mention a few.

The graphical results only represent an illustration of the scenario at a glance. The variations in the blood parameters in response to exposure to MC-LR; in both concentrations and duration perspectives are a vital tool in assessment of the actual level of stress inflicted on the subject fish. There is need for a baseline information on the extents of impacts different concentrations and durations of exposure to MC-LR has on the haematological equilibrium of the fish. Extrapolations from these facts hold insights to corresponding trends in higher or lower case scenarios in aquatic systems. The need for haematological extrapolations therefore necessitates actual percentage changes in these haematological indices.

In the fish exposed to 200 µg/L MC-LR, significant percentage increases were observed in the Basophils (57.1%) and Neutrophils (60.9%) after 14 days of exposure. After 28 days of exposure to the same concentration, significant percentage increases were observed in the Lymphocytes (68.2%), Eosinopils (54.5%) and Basophils (95.2%). These recorded significant differences are indications of stimulation of the immune system by MC-LR (Table 1); hence empirical evidences of stress in the fish. Furthermore, highly significant percentage increases observed in the Monocytes (166.7%) and Neutrophils (136.4%) of fish exposed to 200 µg/L for 28 days are indications that the MC-LR toxicity is directly proportional to the duration of exposure (Maitra and Nath, 2014).

Table 1
Percentage change (increase or decrease) in blood parameters on concentration and duration basis.

Parameters	200 µg/L MC-LR		400 µg/L MC-LR	
	14 days	28 days	14 days	28 days
Haemoglobin %age	2.1	−23.4	−9.4	−5.6
Red blood cells	22.8	−27	43.5	−19.1
PCV	−20.1	−27.3	−32.6	−51.4*
Lymphocytes	19	68.2*	14.3	54.5*
Heterophils	3.1	3.1	−31.3	−31.3
Eosinophils	45.5	54.5*	81.8*	100*
Basophils	57.1*	95.2*	147.6**	242.9**
Monocytes	47.4	166.7**	109.5**	283.3**
Neutrophils	60.9*	136.4**	134.8**	222.7**
Thrombocytes	6.3	28.6	18.8	85.7*

Values are presented in Percentage (%). * = 50–100%; represents significant increase, while ** = >100%; represents highly significant increase.

On the other hand, fish in the aquarium containing 400 µg/L MC-LR elicited significant percentage increase in eosinophils (81.8%), while highly significant increases were observed in the monocytes (109.5%), basophils (147.6%) and neutrophils (134.8%) after 14 days of exposure. On the basis of 28 days exposure to 400 µg/L, significant percentage increases were observed in the lymphocytes (54.5%), eosinophils (100%) and Thrombocytes (87%). While highly significant increases occurred in the basophils (242.9%), monocytes (283.3%), and neutrophils (222.7%). Exposure to biotoxins generally culminate in increase leucocytes; a condition of stress referred to as leukemia. Similar haematological observations occurred in fish exposed to organophosphorous insecticides (Vasait and Patil, 2005), copper (Singh et al., 2008), phenol (Patnaik and Patra, 2006; Swift, 1978) and urea (Maitra and Nath, 2014).

It is noteworthy that there was a significant percentage decrease in PCV (-51.4) of the 400 µg/l experimental fish after 28 days. The results of this study is at variance with the significant increase in RBC count observed in zinc-exposed *Clarias gariepinus* (Annune et al., 1994). Variability of results in these different studies can be attributed to species-specificity and toxicant-specificity (Ololade and Oginni, 2010).

Results showed that MC-LR; particularly 400 µg/L caused significant haematological disruptions in *C. gariepinus*, especially after

28 days of exposure i.e. 400 μg/L (28 days) > 400 μg/L (14 days) > 200 μg/L (28 days) > 200 μg/L (14 days). In previous study, MC-LR elicited disrupted hatching, developmental malformations and liver damage in embryo and larvae of carp, trout, and zebra fish (Malbrouck and Kestemont, 2006). Immune response of Tilapia fish to MC-LR in a time-dependent manner has also been documented earlier (Jos et al., 2005).

Besides the haematological perturbations microcystins inflict on biota, a major worrisome fact is that microcystins may also be bioaccumulated along food chain; up the pyramid of biomass, through alimentation. Fishes are at a relatively higher risk due to the fact that they are at the top of aquatic food chain and gather up the loads of the biotoxin from all organisms at their lower trophic level. As a result, fish in an aquatic environment affected with microcytins stand a chance of accumulating microcytins higher than the levels adopted in this experiment and humans who consume such fish stand even higher exposure chances. Any River diagnosed to have concentration of microcytins up to 400 μg/L requires an urgent attention. However, results of this research might not be replicable in other species as previous findings have shown high variability. This therefore necessitates deterministic research on the bioaccumulation factors (octanol/water partition coefficients) in the tissues of edible fish species.

4. Conclusion

Microcystin-LR of 400 μg/L, followed by 200 μg/L inflicted observable haematological disruptions in *C. gariepinus* for the periods of exposure. Often cases natural aquatic ecosystems affected with algal bloom contain concentrations of MC-LR higher than the experimented levels (OEHHA, 2009). This is a prognostic of worse consequences in natural scenarios than the observations in this experiment. This therefore necessitates immediate remedial measures on affected water bodies in order to forestall liable debilitating sub-lethal effects on the aquatic biota.

Conflicting interests

No conflicting interests exist.

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