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Toxicity Of Methylene Blue On Nile Tilapia (Oreochromis Niloticus) Juveniles

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Abstract: *Methylene blue is used in fish culture to treat fish with fungal infection.So the aim of the study was to assess the level at which it could be toxic on Oreochromis niloticus (Linnaeus 1757) juveniles treated with methylene blue, thus,laboratory determinations of lethal concentration (LC_{50})through a static bio-assay test were performed in order to determine its sub-lethal effects on the test fish. The experiment was conducted for 96 h using O.niloticus of an average weight of 6.00±2.40 g in twelve aquaria with ten (10) fish each with a control without methylene blue solution. Water parameters (pH, DO, temperature, conductivity), proximate analysis and the histopathological study of the gills, spleens and liver of the test organisms were investigated. The LC_{50} was also determined using probit analysis. At the end of experiment, results revealed abnormal behaviors such as erratic swimming, hyperventilation, high rate of opercula movement, restlessness in the test fish at high concentrations (133, 166 and 199mg/l) of methylene blue solution. These observations as well as mortality records were dose- dependent. Gills, Liver and Spleens damages such as architectural loss, dissolution of nucleus, degeneration, necrosis were observed in fish exposed to high concentrations of methylene blue (133, 166 and 199mg/l).There were no significant differences in the values obtained for pH, Temperature, Dissolved Oxygen and conductivity after the 96 hours exposure except in 67 mg/l concentrations in conductivity and dissolved oxygen (DO).It was observed that the O.niloticus juveniles exposed to 133mg/l concentration of methylene blue had the highest percentage crude protein (59.30%) while those exposed to 100mg/l concentration had the lowest % CP (58.25%). The highest % lipid (13.50%) was observed at the lowest concentration (67mg/l) of methylene blue while the lowest lipid (12.00%) was observed in the highest concentration (199mg/l). The % Ash ranges from 18.00% (199mg/l)−20.00% (67mg/l), highest % moisture was 65% at concentration 100mg/l and lowest was 5.50% at concentrations 67, 133 and 166mg/l, while the LC_{50} was observed at 187mg/l.Normal situation was observed in all the examined tissues of fish from the control tanks; therefore the results of the study showed that methylene blue could be toxic to fish when use at high concentration to treat fish with fungal infections and diseases .*

Keywords: *Methylene blue, O. niloticus,Histopathology, Proximate analysis, LC_{50}.*

I. Introduction

The use of chemicals,fungicides, pesticides, insecticides, other agricultural and industrial compounds by farmers and fishery managers are very common.(Ayoola, 2008). The constant flow of their effluents into water bodies often leads to pollution and these can be detected at very high concentrations in the aquatic bodies (Mason,1991, Ayoola,2008, Olufayo and Alade 2012), thus their uncontrolled use may have negative effects and a long term environmental impact on natural aquatic systems (Camargo and Martinex 2007).Methylene blue ($C_{16}H_{18}N_3SCL$) has been used extensively as fungicides in fish cultureto treat fungi diseases.It is used as an alternative to malachite green, both are used in aquaculture to treat fungal infections of fishat early stages.(Drolet*et al.,* 2004), It is effective against some external protozoan such as *Chilodonella*, *Costia* and fungal disease,*Ichthyophthirius* (Drolet*et al.,* 2004), this compound as an oxygen transporter,converts methemoglobin to normal oxygen carrying component of fish blood, hemoglobin (Anderson 2002).It has been reported that methylene blue has interference with oxidation reduction processes in fish and other aquatic organisms (Anderson 2002).Due to repeatedand persistence applications of methylene blue in fish ponds when treating for infections and diseases, large quantities find their ways into the water bodies affect treatment. However, only very few reports have described its effects on *O.niloticus* juveniles.

Fish generally contain protein of very high quality and also has sufficient essential amino acids required by the body for growth and maintenance of lean muscle tissue (Speedy, 2003). *O. niloticus* is one of the most important fish cultured in Nigeria and this fish species was chosen for this study because of its economic importance and sensitive to toxicants (Olufayo and Nwanga, 2014 ,Omitoyin *et al.*,2006).The objective of the study was to determine the lethal concentration and toxic effects of methylene blue on histopathology of Nile tilapia (*O.niloticus)* juveniles in order know the safe concentration to be used when treating for fungal infections and diseases in fish .

II. Materials And Methods

Two hundred juveniles of *O.niloticus* with the mean weight 6.00 ±2.40 g purchased from the Fish Farm of the Federal University of Technology Akure, Nigeria were transported to the Limnology Laboratory, Department of Fisheries and Aquaculture Technology for the toxicity tests.They were acclimated for 48hours in glass tanks (60 x 40 x 40 cm), well aerated prior to the toxicity test. These fish were not fed during the period of the experiment in order to minimize production of waste materials thereby reducing ammonia build up in the tanks.

Static toxicity test was run to determine the sub-lethal concentration (96 h LC_{50}) of methylene blue ($C_{16}H_{18}N_3SCL$) to *O. niloticus*. The test was conducted in15 l aquaria ,10 fish per aquarium ,containing methylene blue of different concentrations (67, 100, 133, 166, 199mg/l) .The control aquaria were without methylene blue; keeping all other conditions constant. The water temperature, dissolved oxygen (DO), conductivity, and pH were determined before and after dissolving the methylene blue and the values were within the optimum tolerance range of the test organisms. The number of dead fish were counted every 24 hours and removed from the aquarium as soon as possible. The mortality rate was determined and recorded at the end of the 96 h. The behavioral changes of *O. niloticus* juveniles immediately after the introduction of methylene blue were observed and the total number of mortality were recorded.

Histological Determination

At the end of the 96 h experiments, fish were dissected and their tissues (gills, liver and spleen) were collected and fixed in 10% formalin for 72 hours after which they were dehydrated in graded levels of ethanol, embedded in paraffin wax, sectioned, stained with haematoxylin and eosin dye for examination and photomicrographs.

Determination of Proximate Composition

After the 96 h exposure, the test fish were killed, sliced into 30g each and oven dried at a temperature of 105^{o}C for 24 hours. Dried samples of the fishes were milled and grinded separately; the fish was finely minced and thoroughly mixed. The crushed samples (10g) were kept in nylon packages and properly wrapped throughout the period of analysis to prevent dehydration and water intrusion. The protein, moisture, lipid and ash contents of the samples were determined according to AOAC (1990) method.

Statistical Analysis

Data collected were subjected to Analysis of Variance (ANOVA) while the median lethal concentration (LC_{50}) at 96h was determined using probitlogit analysis.

III. Results

Results obtained show that there were no significant differences between pH, temperature at the end of the experiment while some significant differences were observed in DO at concentration 133mg/L and conductivity in concentration 67mg/l (Table 1). It was observed that about 60% mortality was recorded at concentration 199mg/l, 50% mortality occurred at 187mg/l and less than 50% mortality were recorded at concentration 166mg/l and 133mg/l respectively (Table 2). The LC_{50}value was observed at 187mg/l for 96 h (Figure 1).This value reveals the 50% mortality recorded on the test organisms within the experimental period.
The behavior of fish in the control tanks were normal while erratic swimming, loss of reflex, restlessness and air gulping were observed in fish at higher concentrations of methylene blue solution. At 199mg/l, the exposed fish became very weak and high mortality was recorded (Table 2).

Table1: Effects Of Methylene Blue On The Physico-Chemical Parameters Of *O. niloticus* Juveniles

Methylene blue (mg/l)	Temp (^{O}C)	pH	DO mg/l	Conductivity
Control(0.00 mg/l)	24.95^{a}	6.78^{a}	6.24^{a}	18.08^{a}
67 mg/l	26.60^{a}	6.59^{a}	4.22^{a}	27.78^{ab}
100 mg/l	26.45^{a}	6.85^{a}	4.68^{a}	19.01^{a}
133 mg/l	26.60^{a}	6.90^{a}	5.10^{ab}	19.04^{a}
166 mg/l	26.60^{a}	6.50^{a}	4.88^{a}	19.26^{a}
199 mg/l	26.55^{a}	6.62^{a}	4.91^{a}	19.27^{a}

Means within the same column are not significantly different (P>0.05)

Table 2: BEHAVIOURAL CHANGES OF OREOCHROMIS NILOTICUS JUVENILES EXPOSED TO DIFFERENT LEVELS OF METHYLENE BLUE IN DEFINITIVE TEST

	24 HOURS					48 HOURS					72 HOURS					96 HOURS				
CONCENTRATION (MG/L)	67	100	133	166	199	67	100	133	166	199	67	100	133	166	199	67	100	133	166	199
ERRATIC SWIMMING	-	-	-	-	+	-	-	-	+	+	-	-	-	+	+	-	-	+	+	+
LOSS OF REFLEX	-	-	-	-	+	-	-	-	+	+	-	-	-	+	+	-	-	+	+	+
HYPER VENTILATION	-	-	-	-	+	-	-	-	+	+	-	-	-	+	+	-	-	+	+	+
CHANGES IN BEHAVIOUR	-	-	-	-	+	-	-	-	+	+	-	-	-	+	+	-	+	+	+	+
DISCOLOURATION	-	-	-	-	+	-	-	-	+	+	-	-	-	+	+	-	-	+	+	+
% MORTALITY	-	-	-	-	-	-	-	-	10	20	-	-	10	30	40	-	-	25	45	60

+ : Presence of specific observation

- : Absence of specific observation

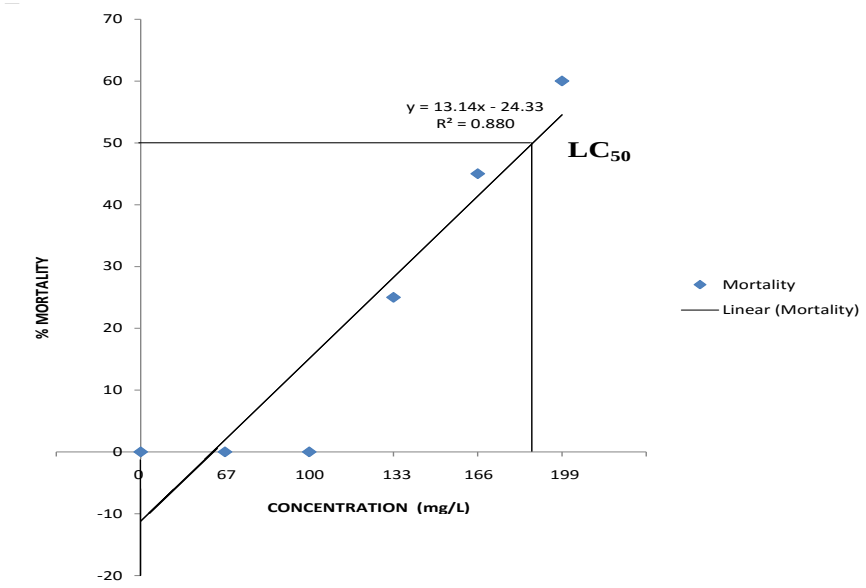

Fig. 1: LC_{50} OF *Oreochromis niloticus* JUVENILES EXPOSED TO DIFFERENT CONCENTRATIONS OF METHYLENE BLUE

Histopathological Observations

Gills

The gills of *O.niloticus* exposed to different concentration of methylene blue shows varying degrees of degeneration, vacuolation, inflammation of filament cells, and necrosis. Normal gill structures were observed in the control fish (Plate 1). It was observed that slight architectural loss occurred at concentration 67mg/l (Plate 2), vacuolation and degeneration were observed at concentrations 100mg/l and 133mg/l (Plate 3 and 4). inflammation of filament cells was also observed at concentration 166mg/l (Plate 5) while complete degeneration of lamellae was observed at concentration 199mg/l (Plate 6).

Liver

Histoligical examination reveals no observation changes in the control (Plate 7). Slight space formation was observed at concentration 67mg/l (Plate8). Degeneration with formation of pyknotic nuclei was observed at concentration 100mg/l (Plate 9). Cytoplasmic vacuolation in hepatocytes was observed at concentration 133mg/l (Plate 10). Vacuolation of hepatocytes were also observed at concentration 166mg/l (Plate 11).Damage of hepatopancreas which was characterized with loss of contact between hepatocytes and pancreocytes were observed at concentration 199mg/l (Plate 12).

Spleen

Normal features of spleen were observed in the control (Plate 13). It was observed that there was slight cellular changes in concentration 67mg/l (Plate 14). Vacuolation and space formation were observed in concentration 100mg/l (Plate 15). Degeneration and nucleus dissolution with vacuolation were observed in concentrations 133mg/l and 166mg/l (Plate 16 and 17). Dissolution of nucleus with loss of chromatin material was observed in concentration 199mg/l (Plate 18).

HISTOPATHOLOGICAL PLATES

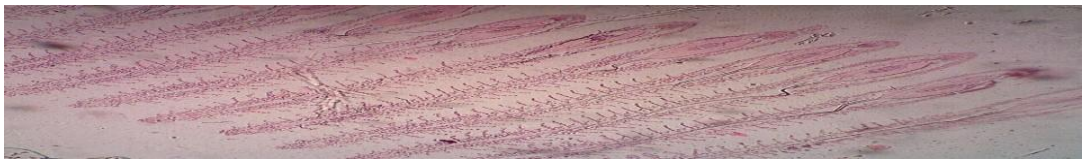

Plate 1: Control (0.00 mg/l) gills of O. niloticus juveniles in the control tank shows normal features (×400)

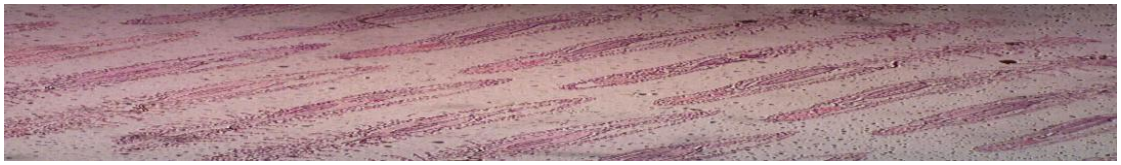

Plate 2: Gills of O. niloticus juveniles exposed to 67mg/l of methylene blue showing architectural loss with curling in the gill lamellae (×400).

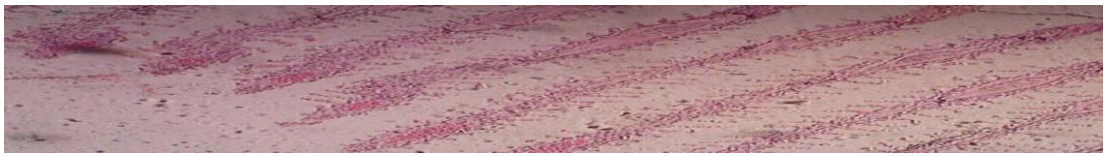

Plate 3: Gills of O. niloticus juveniles exposed to 100mg/l of methylene blue showing slight vacuolation and slight degeneration with ballooning dilation of the lamellae (×400).

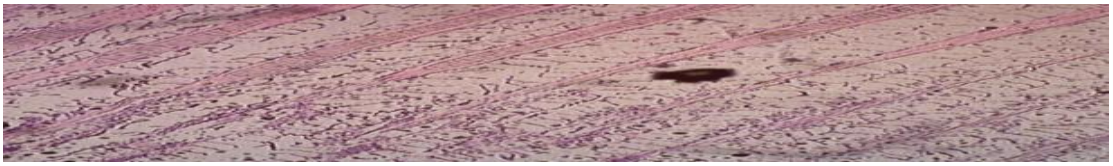

Plate 4: Gills of O.niloticus juvenile exposed to 133mg/l of methylene blue showing high level of degeneration and inflammation of filament cells (×400)

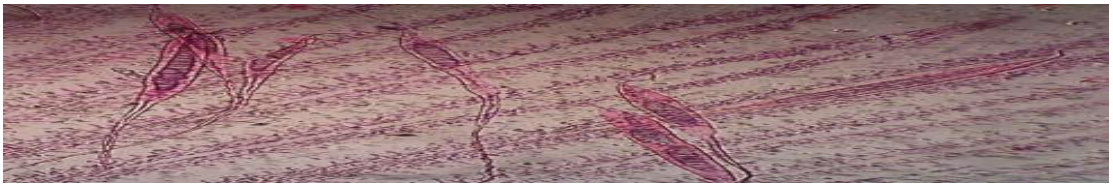

Plate5: Gills of O. niloticus juveniles exposed to 166mg/l of methylene blue showing high level of degeneration and inflammation of filament cells (×400).

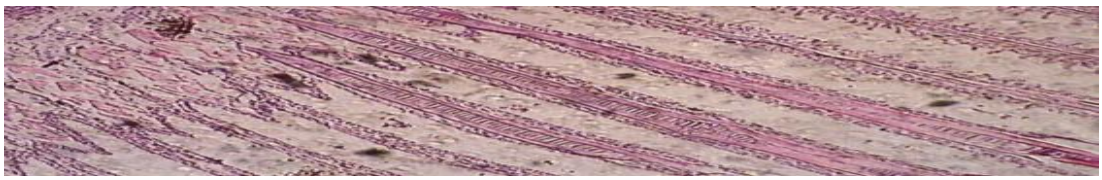

Plate 6: Gills of O. niloticus juveniles exposed to 199mg/l of methylene blue showing complete degeneration of lamellae and necrosis (×400).

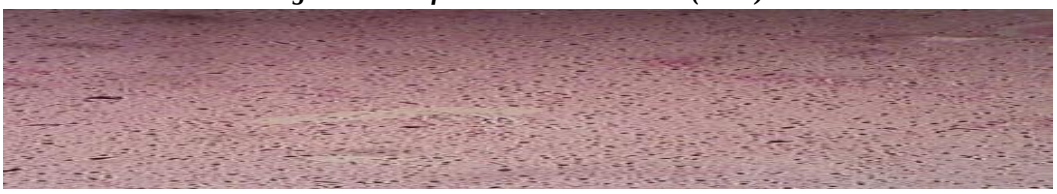

Plate 7: Liver of O. niloticus juveniles in the control tank shows normal features (×160).

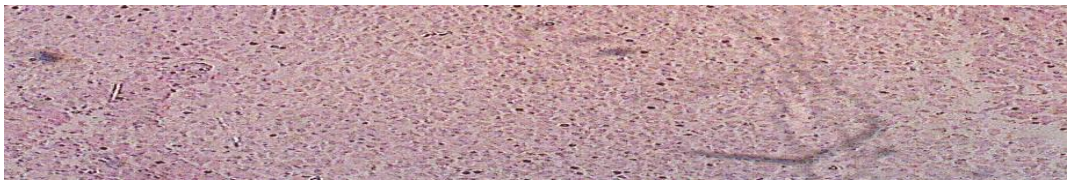

Plate 8:Liver of O. niloticus juveniles exposed to 67mg/l of methylene blue showing changes in the normal architecture of the liver (×160).

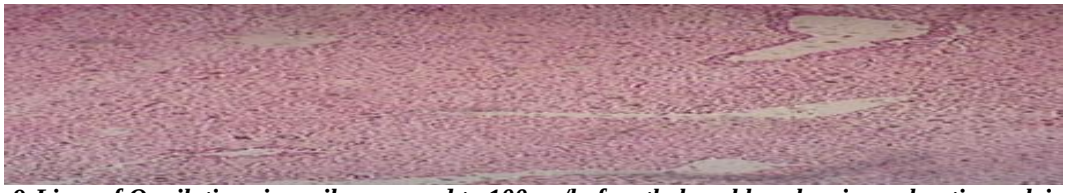

Plate 9:Liver of O. niloticus juveniles exposed to 100mg/l of methylene blue showing pyknotic nuclei with slight degeneration (×160).

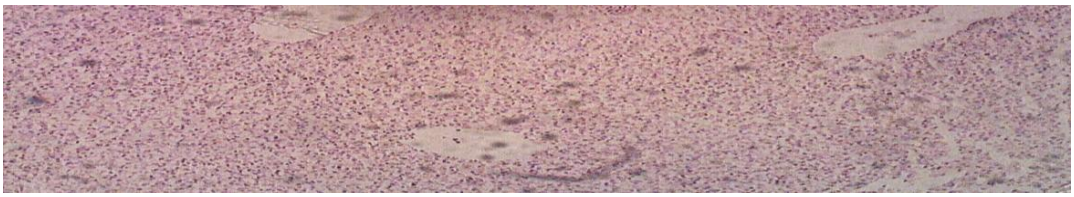

Plate 10:Liver of O. niloticus juveniles exposed to 133mg/l of methylene blue showingcytoplasmic vacuolation in hepaocytes (×160).

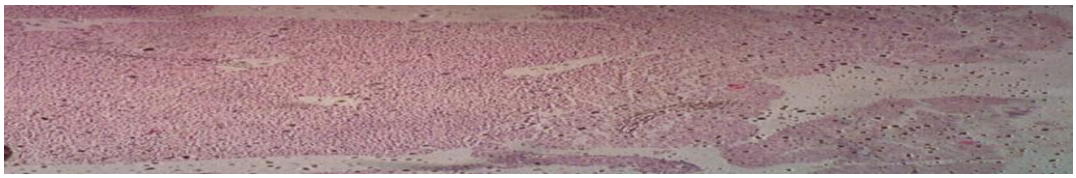

Plate 11:Liver of O. niloticus juveniles exposed to 166mg/l of methylene blue showing vacuolation, pyknotic area and alteration in the hepatocites (×160).

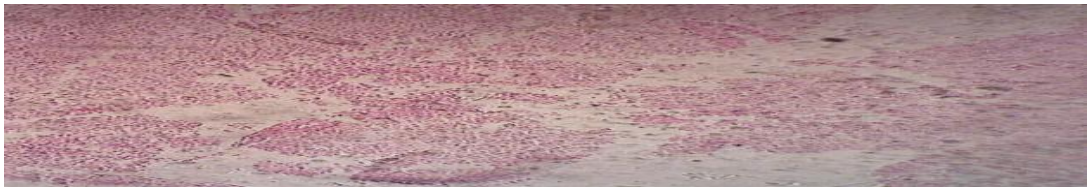

Plate12: Liver of O. niloticus juveniles exposed to 199mg/l of methylene blue showing damage of hepatopancreas characterized by loss of contact between hepatocyte and pancreocyte (×160).

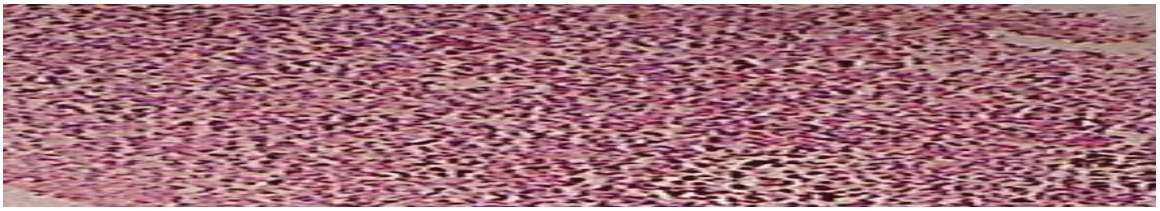

Plate 13: Spleen of O. niloticus juveniles in the control tank shows normal features (×160).

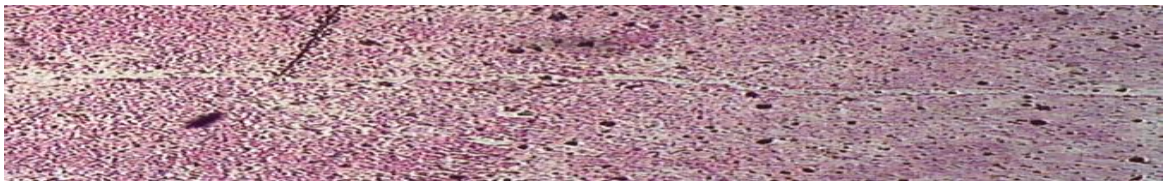

Plate 14:Spleen of O. niloticus juveniles exposed to 67mg/l of methylene blue shows slight cellular changes, vacuolation but dense nucleus (×160).

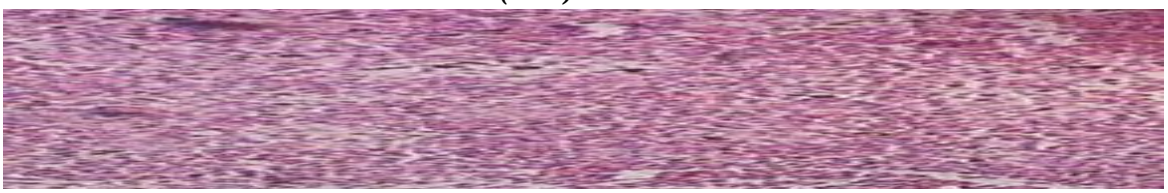

Plate 15:Spleen of O. niloticus juveniles exposed to 100mg/l of methylene blue shows space formation and vacuolation in the cells (×160).

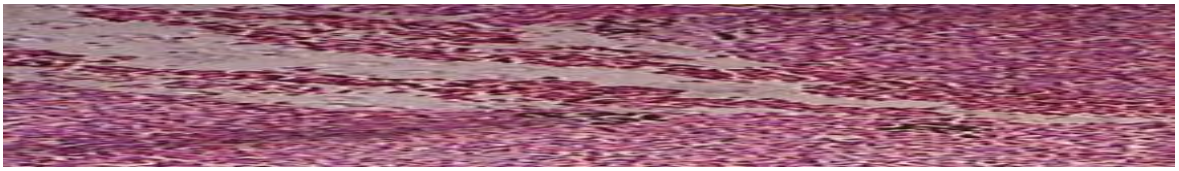

Plate 16: Spleen of O. niloticus juveniles exposed to 133mg/l of methylene blue shows degeneration of the spleen and high dissolution of the nucleus (×160).

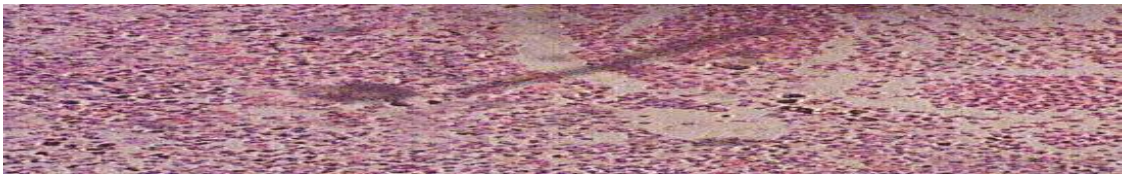

Plate 17:Spleen of O. niloticus juveniles exposed to 166mg/l of methylene blue shows high level of vacuolation and dissolution of nucleus (×160).

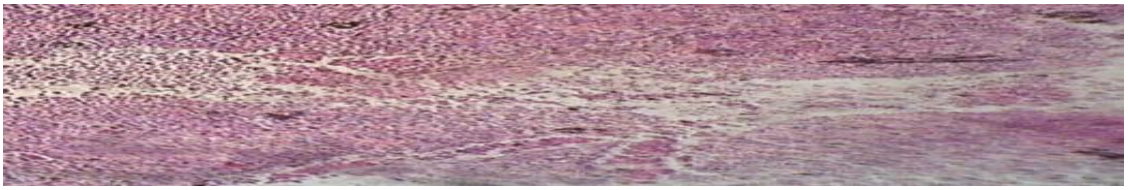

Plate18: Spleen of O. niloticus juveniles exposed to 199mg/l of methylene blue shows total dissolution of nucleus with loss of chromatin material (×160).

IV. Discussion

Organisms exposed to toxicants usually exhibit changes in opercula rate movements which is a sensitive indicator of physiological stress (Ayoola,2008, Olufayo and Alade, 2012). The irregular opercula rate movements ,different behavioral activities , erratic swimming, loss of reflex, discoloration observed in this study agreed with the findings of Dede and Kaglo (2001), Adewoye *et al.*, (2005) ,Olufayo and David (2010) and Ayoola, (2008) who reported various physical changes in behavioural activities of *O.niloticus* exposed to copper sulphate , paraquat herbicides ,indigofera dye and glyphosate respectively. These observations also were similar to the findings of Abalaka and Auta, (2010) who reported on *O.niloticus* exposed to trichloroform, Omitoyin*et*

al., (1999) reported similar observation in *Sarotherodon galilaeus*(Tilapia) fingerlings exposed to piscicidal plant extracts of *Tetrapleura tetraptera*. The response of the test organisms to the various concentrations differ according to the level of concentrations present in each treatment. It showed that methylene blue is toxic to the fish at very high dosage.

This behaviour displayed by *O. niloticus* used for this study also agreed with the findings of Oyedapo and Akinduyite (2011) who reported that when concentration of the toxic substance is higher than what the homoeostasis of the fish can control, it results into fast rate of opercula movement, this may cause physical damages to the skin, liver and gill of the fish and may also result in death (Ayoola, 2008).

Histopathological alterations observed in this study could be used as indicators of effects of methylene blue on *O.niloticus.* Various studies have shown that exposure of fish to toxicants such as agricultural wastes ,industrial effluents,herbicides and other pollutants resulted in several pathological changes in the fish tissues (Abbas and Ali, 2007, Olufayo and Alade, 2012 ,Mohamed 2009).

The histological response of the fish at various concentrations of the methylene blue showed some level of alterations such as degeneration, vacuolation, inflammation of filament cells and necrosis when compare with the control group, these were similar to the findings of Abd El-Gawad (2002) who exposed *O.niloticus*to different concentrations of lead acetate and zinc sulphate, and Mobarak and Sharaf (2011) who reported similar histopathological changes of gill of *Poecilia latipinna e*xposed to lead acetate.

Proximate composition of *O.niloticus* juveniles exposed to methylene blue revealed that Crude Protein varied from 59.80±0.85-58.25±1.20, Lipid: 13.50±0.71-12.00±0.00, Ash: 20.00±1.41-18.00±0.00, Moisture: 6.50±0.71-5.50±0.71 and NFE: 5.05±0.21-2.00±0.42.The percentage of crude protein showed no significant difference in protein present in the control experiment and the fishes exposed to different concentrations of methylene blue. Although there is a slight decrease in the protein percentage across all the treatment and this reduction corresponds with the findings of Abdel-Tawwab *et al*., (2007) who reported that copper toxicity lead to reduction in the protein content of fishes which may be due to increased utilization of protein for energy when exposed to stressful condition.

However the lipid content showed significant difference across all the treatments. This could also be due to the effect of the methylene blues on the fish or as a result of utilization of protein and oil for energy due to increased activity to avoid the polluted water. Similar observation was made by Martinex *et a*l.,(2004) who said that *Prochilodus lineatus* can use protein and fats for energy.

The variation observed in the proximate composition could be attributed to variation in the concentration of methylene blue exposed to the fish which is in relation with the findings of Adewoye and Omotosho, (2004) which shows that the proximate composition of fish is affected by the presence of pollutants in the aquatic environment.

The LC_{50}values vary widely depending on fish species, environment and the test conditions (WHO,1994) . Some Researcher suggested that the exact calculation of LC_{50} is valid only for substances that pose a lethal concentration of 1 and 5000mg/kg (Orsine *et al.*2012) while Larini, (1997) recommended a limit of 2000 mg/kg for LC_{50} test. The present study showed that 96h LC_{50} value of methylene blue was 187mg/l, this value was within the concentration ranges reported in the previous studies.

V. Conclusion

The result obtained in this investigation is a clear indicator that methylene blue is toxic to fish at concentrations higher than 100mg/l and it could lead to high mortality of fish in the culture system when they are treated with overdose of methylene blue.

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