ACUTE TOXICITY OF ZINC OXIDE NANOPARTICLES ON BLOOD CELL MORPHOLOGY, HAEMATOLOGY AND HISTOPATHOLOGY OF *HETEROBRANCHUS LONGIFILIS*

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ABSTRACT

This study investigated the toxicity effect of lethal concentrations of zinc oxide nanoparticles (ZnO-NPs) on the blood morphology, haematology and histopathology of Heterobranchus longifilis. The fish were assessed in a semi-static bioassay for 96 hours in five different concentrations (0.00, 60.00, 80.00, 100.00, 120.00 and 140.00 mg/l) of ZnO-NPs. The 96-hour LC_{50} was estimated as 100 mg/l. At the end of the experiment, blood samples were collected for morphological and haematological assays, while the fish was sacrificed to remove the gill and liver for histological bioassay. Results showed that the levels of haematological parameters such as red blood cell (RBC), haemoglobin (HB) and packed cell volume (PCV) decreased significantly ($p<0.05$) compared to the control. The levels of white blood cell (WBC) and platelets increased significantly ($p<0.05$) compared to the control. Various pathological alterations such as oedema, epithelial lifting, necrosis, hyperplasia, lamellar disorganization and lamellar fusion were apparent in the gill of ZnO-NPs-exposed fish, while fatty degeneration, increased sinusoidal space, cytoplasmic degeneration, necrosis, and focal fibrosis were observed in the liver compared to the control. Different morphological alterations such as abnormal elliptical shape, irregular boundary with more than one lobopodial projections, shrinkage of erythrocytes, hole-like depression and spherocyte with double projections occurred in the erythrocytes of ZnO-NPs-exposed fish compared to the control. The results of this study revealed that lethal concentration of ZnO-NPs is harmful to Heterobranchus longifilis. Hence, the discharge of ZnO-NPs into water bodies should be regulated to guide against environmental hazards that may arise.

Keywords: Zinc Oxide Nanoparticles, Blood morphology, Haematology, Histology, Lethality, *Heterobranchus longifilis*

INTRODUCTION

Among the environmental factors that cause a great decline in fish population are the emergent nanoparticles like zinc oxide nanoparticles (ZnO-NPs) which are used in the production of most commercial and medical goods. Indiscriminate discharge of waste materials from industries into the aquatic environment may have an adverse effect on the ecosystem which may lead to the extinction of fish species. Fish is regarded as one of the commonest sources of proteins, thus contamination of water bodies where fish resides deserve greater attention. The lethal concentration (96 h LC_{50}) of ZnO-NPs to zebrafish was reported by Zhu *et al*. (2008) to be 4.9 mg/L and LC_{50} of 124.5 mg/l by Xiong *et al*. (2011). Subashkumar and Selvanayagam (2014) reported the LC_{50} value for ZnO-NPs in common carp as 4.897 mg/l. Alkaladi *et al*. (2015) reported 3.1 mg/l of ZnO-NPs as 96 hour

LC_{50} for *Oreochromis niloticus.* The observations of these workers showed that LC_{50} of ZnO-NPs determined for different species were comparatively different, but among these findings on the potential acute toxicity of ZnO-NPs to different types of fish species, there is a dearth of information on the acute toxicity of ZnO-NPs in *H. longifilis*. It is, therefore, necessary to investigate the acute toxicity effect of ZnO-NPs on this fish as it will enable the understanding of the concentration of ZnO-NPs that will cause mortality and the concentration at which it will survive. The small size and the large space of ZnO-NPs which enable it to accumulate and distribute in the tissue may induce histopathological changes (Yang *et al*., 2009). The pathological alterations in organs serve as the basis for abnormalities which may lead to malfunction of the organs and affect the physiology of the fish. Distribution of toxicant to various parts of the body is through the blood, thus making haematological investigation necessary for exposed fish and serves as a biomarker in assessing water quality and fish health condition. Alterations in the levels of erythrocytes and leucocytes are important indicators of organic pollutant contaminations of the environment. Many works have been conducted on haematological effects of nanoparticles of metals in fish. Karthikeyeni *et al*. (2013) and Subramanian *et al*. (2013) reported a reduction in the level of erythrocytes and an increase in the levels of leucocytes in *Oreochromis mosambicus exposed to* iron nanoparticles. Histological parameters also serve as a mirror that reflects other biomarkers of stress and allow the examination of specific target organs that are responsible for vital functions like respiration, accumulation and biotransformation of xenobiotics (Hinton and Lauren, 1990; Fanta *et al*., 2003). The main passage of any toxicant is through the gill, from where it is transported to various parts of the body via the bloodstream accumulation of these ZnO-NPs in the gills and blood may alter the architecture of these tissues that could lead to dysfunctions of organs. The liver is an organ of detoxification, metabolism and excretion of a toxic substance in the body (Hinton and Lauren, 1990). Exposure to toxicants may, therefore, cause histological changes in the liver and histological investigations of the organs of ZnO-NPs-exposed fish may, therefore, produce meaningful results. Federici *et al.* (2007) reported pathological lesions of the internal organs of rainbow trout exposed to 1 mg/l TiO_2-NPs. Hao *et al*. (2009) reported swollen and disrupted gill cells in carp fish exposed to Zn0-NPs. Alkaladi *et al*. (2014) also reported severe vacuolation, necrosis and oedema in the gills of *Oreochromis niloticus* exposed to ZnO-NPs. Amongst this literature on both haematology and histopathology, information on the toxic effect of ZnO-NPs on *Heterobranchus longifilis* is scarce. This work, therefore, investigated the acute toxicity effect of ZnO-NPs on blood cell morphology, haematology and histopathology of the gill and liver of *Heterobranchus longifilis*.

MATERIALS AND METHODS

Experimental Setup: Juveniles of *Heterobranchus longifilis* (average weight 19.40 ± 3.65 g and average length 12.45 ± 0.13 cm) were obtained from a commercial fish hatchery in Lagos, Lagos State, Nigeria and transported in plastic aquaria containing freshwater from the hatchery to the laboratory. The fish were not fed throughout the day due to stress which may prevent easy digestion and cause mortality. Feeding commenced the following day and they were fed twice daily at 9.00 am and 5.00 pm with commercial feeds (Coppens, 2 mm) at 4 % of initial body weight (USEPA, 1996). The hatchery water was replaced by chlorine free bore-hole water and the fish were kept in a tank of 1000-litre capacity at 24 ± 4 ^{o}C and acclimatized to laboratory conditions for 14 days prior to experiments, the water was kept oxygen saturated with aerators. Unconsumed feed and faecal wastes were removed and renewed every 24 hours to maintain the required toxicant's concentration (FAO, 1986) and to reduce pollution and the risk of disease outbreak and mortality. Feeding was stopped 24 hours before the commencement of the experiment (USEPA, 1996). During the tests, water quality parameters were continuously monitored for temperature, pH, dissolved oxygen (DO), biochemical oxygen demand

(BOD), chemical oxygen demand (COD), total dissolved solids (TDS), turbidity and conductivity according to the procedure of APHA (1995).

Preparation of Nanoparticles Suspension: Commercial zinc oxide nanoparticles with average particle size about 100 nm were purchased from Sigma Aldrich (USA). A stock solution of zinc oxide nanoparticles was prepared by dispersing zinc oxide particles into distilled water and the suspending solution of zinc oxide nanoparticles was magnetically stirred using magnetic stirrer for about two hours to break aggregates or precipitates. The suspension was freshly prepared every day for renewal to maintain the concentration of the toxicant.

Acute Toxicity: The test was carried out in a semi-static condition following the method described in OECD (1992). Based on the result of the range-finding test, a stock solution of 1000 mg per litre of zinc oxide nanoparticles (NPs) was prepared by dispersing the ZnO-NPs in distilled water. The freshly prepared stock solution was then diluted into ten litres of chlorine-free bore-hole water in six different aquaria of different concentrations (0, 60, 80, 100, 120 and 140 mg/l). Ten fish were introduced into each aquarium of 80-litre capacity containing 30 litres of chlorine-free bore-hole water. The first aquarium serves as the control while the remaining five aquaria are with varying concentrations of ZnO-NPs. The experiment was set up in a completely randomized design of six treatment groups replicated thrice with each replicate having ten catfish juveniles (Pathiratne and Athauda, 1998). The fish were observed for 24, 48, 72 and 96-hour intervals and mortality was recorded. The lethal concentration (LC_{50}) at which 50 % mortality of the *Heterobranchus longifilis* occurred was calculated using arithmetic method adopted from Dede and Igbigbi (1997). At the end of the 96-hour experiment, the remaining fish were sacrificed to remove the gill and liver, while the blood was collected for morphological and haematological analyses.

Morphological and Haematological Analyses: Fishes were removed from each test aquaria and sacrificed. Blood was collected from the caudal vein by means of heparinized plastic syringes. The blood samples were collected in a tube containing ethylene diamine tetra acetic acid (EDTA) which acted as an anticoagulant to prevent blood coagulation. The blood samples were analysed for the following blood parameters, red blood cell (RBC) count, haemoglobin concentration (HB), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), white blood cell (WBC) count, platelet, lymphocyte and neutrophil using haematology autoanalyzer (Sysmex KX-21N). The tube of well-mixed EDTA blood was slotted into the sample probe and auto analyzed. Morphological analysis of the blood was carried out using the procedure of Sousa *et al*. (2015).

Histopathological Assay: Following the methods of Pearse (1985) and Woods and Ellis (1994), gill and liver samples were fixed immediately in 10% neutralized buffered formalin. After 72 hours the samples were washed in running tap water for 12 hours followed by dehydration through a series of ethanol. The samples were cleared in xylene and infiltrated through increasing concentrations of paraffin wax to xylene in a 60 ^{0}C oven. Once thoroughly infiltrated, they were embedded in paraffin wax blocks. Each block was sectioned at 15 ųm using a microtome (Leica). These sections were stretched using hot water bath (60 ^{o}C) and mounted on albumenized slides. Slides were dried (30 ^{o}C) in an oven overnight for histological staining. The sections were stained with Haematoxylin–Eosin (HE), dehydrated in serial alcohol and covered with coverslips using DPX and left to dry. The sections were then examined by a light microscope and photographed using a digital camera (Takashima, and Hibiya, 1995). The histopathological alterations in the gills and liver were semi-quantitatively determined through degree of tissue change (DTC) based on the severity of the lesions as described by Poleksić and Mitrović-Tutundžić (1994). The severity of

damages was classified into three stages: Stage I (slight alteration); Stage II (moderate alteration) and Stage III (severe alteration) (Camargo and Martinez, 2007).

Statistical Analysis: The data obtained from the experiment were analysed using analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) (Duncan, 1955) and expressed as mean ± SE. Statistical significance for all the tests was set at $p<0.05$. All statistical analyses were performed using Microsoft Excel 2003 and IBM SPSS statistics 20.0.

RESULTS

Physico-Chemical Parameters of Water: The results obtained from the study revealed a significant increase ($p<0.05$) in the values of TDS, BOD, COD, conductivity and turbidity as the concentration of ZnO-NPs increased. However, the values of DO decreased in the groups exposed to ZnO-NPs compared to control. The values of temperature and pH had no significant difference ($p>0.05$) when compared with the control (Table 1).

Mortality Rate (LC_{50}): The lowest percentage mortality (10 %) was observed in the lowest concentration (60.00 mg/l) of ZnO-NPs, while the highest percentage mortality (100 %) was observed in the group exposed to the highest concentration (140 mg/l) of ZnO-NPs after 96 hours (Table 2). The percentage mortality of *H. longifilis* recorded for each concentration of ZnO-NPs was directly proportional to the increase in ZnO-NPs concentration. However, the ZnO-NPs-exposed groups recorded mortality compared to the control group without a record of mortality. The lethal concentration (LC_{50}) value was estimated to be 100.00 mg/L (Table 3).

Haematology: The haematological parameters such as RBC, HB, PCV, MCV, MCH and MCHC of *H. longifilis* exposed to ZnO-NPs for 96 hours decreased significantly ($p<0.05$) as the concentrations of ZnO-NPs increased compared to the control. However, the values of leucocytes and platelets in the ZnO-NPs–exposed fish were significantly higher ($p<0.05$) when compared to the control, but had no significant increase ($p>0.05$) in the lymphocyte and neutrophil of ZnO-NPs–exposed fish compared to the control (Table 4).

Histopathology of Gills: Varying histopathological alterations were revealed in the gills of *H. longifilis* exposed to different concentrations of ZnO-NPs for 96 h (Figure 1a – e). The gill of the control fish showed normal cellular architecture such as normal structure of primary and secondary lamellae (Figure 1a), while the gills of fish exposed to ZnO-NPs revealed varying degrees of histopathological lesions (Figure 1b – e). The commonest alterations depicted were epithelial hyperplasia, epithelial lifting, lamellar fusion, disruption of cartilaginous core, lamellar disorganization and curling of secondary lamellae which are considered to be at stage I of severity. The most frequent lesions under stage II of severity were also aneurysm, epithelial oedema, cellular degeneration and rupture of epithelial cell. However, in 60, 80 and 100 mg/l of ZnO-exposed groups, epithelial hyperplasia and lamellar degeneration were observed (Figure 1b, c and d). Aneurysm and oedema were recorded in 80, 100 and 120 mg/l of ZnO-NPs-exposure groups (Figure 1c, d and e) and lamellar fusion was observed only in the gill of fish exposed to 100 mg/l of ZnO-NPs (Figure 1d). Gills of fish exposed to the highest concentration showed severe lesions such as disruption of cartilaginous core, epithelial lifting, cellular degeneration, rupture of epithelial cell and epithelial necrosis (Figure 1e). The different alterations revealed in 96 h of exposure to ZnO-NPs resulted in a mean degree of tissue change (DTC) of 24.50 at a concentration of 60 mg/l; mean DTC of 33.40 at a concentration of 80 mg/l; mean DTC of 39.60 at a concentration of 10 mg/l and 40.50 at a concentration of 120 mg/l. The overall mean DTC of 34.50 was recorded indicating moderate damage after 96 h of exposure to ZnO-NPs, while the mean DTC values in the control group remained within the range for normal functioning of gills.

Table 1: Water quality parameters during exposure of *Heterobranchus longifilis* to lethal concentrations of ZnO-NPs for 96 hours

CONC. (mg/l)	TDS (mg/l)	DO (mg/L)	BOD (mg/L)	COD (mg/L)	Conductivity (µ S/cm)	Turbidity	Temp (°C)	pH
0	1.50 ± 1.13^{a}	8.23 ± 0.58^{b}	5.55 ± 0.05^{a}	31.3 ± 1.27^{a}	55.02 ± 0.04^{a}	1.44 ± 1.14^{a}	25.0 ± 0.58^{a}	7.5 ± 0.58^{a}
60	9.00 ± 1.15^{b}	2.26 ± 0.13^{a}	13.22 ± 0.17^{b}	54.4 ± 1.14^{b}	79.18 ± 0.12^{b}	11.0 ± 1.13^{b}	25.0 ± 0.58^{a}	7.0 ± 0.05^{a}
80	10.10 ± 0.58^{b}	1.89 ± 0.12^{a}	21.67 ± 1.16^{c}	125.8 ± 0.58^{c}	98.12 ± 0.14^{c}	15.1 ± 0.58^{c}	25.0 ± 0.58^{a}	6.5 ± 1.04^{a}
100	15.70 ± 0.06^{c}	1.66 ± 0.25^{a}	35.36 ± 1.25^{d}	286.4 ± 0.23^{d}	115.03 ± 0.11^{d}	26.3 ± 0.02^{d}	25.0 ± 0.58^{a}	6.0 ± 0.42^{a}
120	17.40 ± 0.27^{d}	1.04 ± 0.21^{a}	65.89 ± 1.26^{e}	457.6 ± 0.34^{e}	245.06 ± 2.15^{e}	37.3 ± 1.13^{e}	25.0 ± 0.58^{a}	6.0 ± 0.12^{a}

Mean (± SE, n=3) with the same superscript in the same column are not significantly different (P>0.05, TDS = Total dissolved solids; DO = Dissolved Oxygen; Temp. = Temperature; BOD = Biochemical Oxygen Demand; COD = Chemical Oxygen Demand. Standard for Water Quality: DO = 4-6 mg/l; BOD = 4-6 mg/l; COD = 30 mg/l; pH = 6.5-9.0; Temp. = 20-33 ^{0} C; Turbidity. = 4 mg/l (NESREA, 2011) and (WHO, 2004)

Table 2: Mortality rate of *H. longifilis* exposed to lethal concentrations of ZnO-NPs for 96 hours

Concentration (mg/l)	Number of fish	Mortality (%)
0.0 (control)	10	0
60.0	10	10
80.0	10	30
100.0	10	40
120.0	10	50
140.0	10	100

Table 3: Lethal concentration (LC_{50}) value of *Heterobranchus longifilis* exposed to ZnO-NPs after 96 hours

Concentration (mg/l)	Concentration difference	Number of alive fish	Number of dead fish	Mean number of dead fish	Mean death × Concentration difference
0.00	0	10	0	0.00 ± 0.00	0
60.00	60	9	1	0.7 ± 0.05	42
80.00	20	7	3	2.3 ± 0.08	46
100.00	20	6	4	3.3 ± 0.06	66
120.00	20	5	5	4.0 ± 0.08	80
140.00	20	0	10	8.3 ± 1.23	166
Total					400

Values on the fifth column are means ± SE of three replicates, LC_{50} = 100 mg/l

Table 4: Haematological parameters of *Heterobranchus longifilis* exposed to different concentrations of zinc oxide nano-particles for 96 hours

Conc. (mg/l)	RBC ($x10^6$/µl)	HB (g/dl)	PCV (%)	MCV (fL)	MCH (pg)	MCHC (g/dl)	WBC ($x10^3$/µl)	PLT ($x10^3$/µl)	LYMP (%)	NEUT (%)
0.00	2.90 ± 0.01^{e}	11.50 ± 0.02^{c}	34.20 ± 0.06^{d}	131.2 ± 0.01^{d}	43.30 ± 0.02^{c}	33.20 ± 0.01^{c}	205.7 ± 0.01^{a}	8.00 ± 0.58^{a}	98.3 ± 0.06^{a}	1.50 ± 0.06^{a}
60.00	2.80 ± 0.00^{d}	11.20 ± 0.12^{c}	33.90± 0.06^{c}	124 ± 0.12^{d}	40.50 ± 0.12^{b}	33.90 ± 0.06^{d}	210.2 ± 006^{b}	10.00 ± 0.58^{b}	98.2 ± 0.06^{a}	1.70 ± 0.06^{a}
80.00	2.50 ± 0.00^{c}	10.50 ± 0.58^{b}	31.50 ± 0.12^{b}	122.8 ± 0.06^{c}	39.90 ± 0.06^{a}	32.30 ± 0.06^{b}	216.8 ± 0.06^{c}	10.00 ± 0.58^{b}	98.1 ± 0.06^{a}	1.80 ± 0.06^{a}
100.00	2.40 ± 0.00^{b}	9.60 ± 0.58^{a}	31.10 ± 0.12^{b}	120.0 ± 0.58^{b}	39.30 ± 0.12^{a}	32.70 ± 0.06^{b}	223.2 ± 0.06^{d}	13.00 ± 0.58^{c}	98.5 ± 0.06^{a}	1.80 ± 0.06^{a}
120.00	2.39 ± 0.00^{a}	9.40 ± 0.58^{a}	29.20 ± 0.06^{a}	119.9 ± 0.06^{a}	38.60 ± 0.06^{a}	30.90 ± 0.06^{a}	223.7 ± 0.06^{d}	14.00 ± 0.58^{d}	98.2 ± 0.06^{a}	1.90 ± 0.06^{a}

Means (mean ± SE, n=3) with the same superscript in the same column are not significantly (P > 0.05) different. RBC = Red blood cells; HB = Haemoglobin; PCV = Packed cell volume; MCV = Mean corpuscular volume; MCH = Mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; WBC = White blood cells; PLT = Platelet; LYMP = Lymphocytes and NEUT = Neutrophil

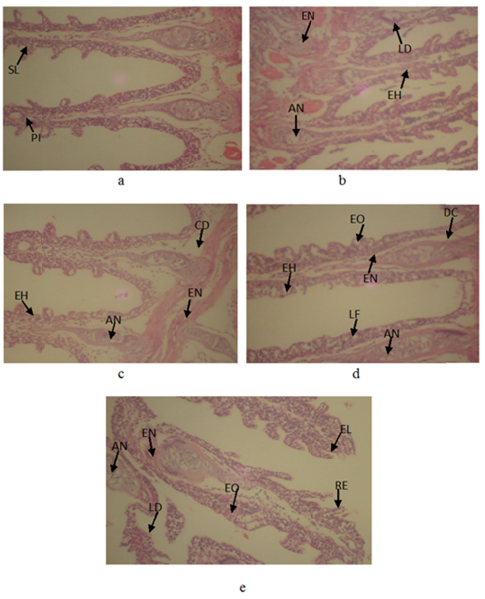

Figure 1 a-e: Histopathological alterations in the gill of *Heterobranchus longifilis* exposed to ZnO-NPs for 96 h (H&E, X 100). a) Gill of *Heterobranchus longifilis* in the control experiment shows normal architecture with normal primary lamellae (PL) and secondary lamellae (SL); (b) epithelia necrosis (EN), aneurysm (AN), epithelial hyperplasia (EH) and lamellar disorganization (LD) in the gill of fish exposed to 60 mg/L of ZnO- NPs; (c) Cellular degeneration (CD) and epithelial hyperplasia (EH) in the gill of fish exposed to 80 mg/L of ZnO-NPs; (d) Epithelia oedema (EO), Disruption of cartilaginous core (DC), Lamellar fusion (LF) and epithelial hyperplasia (EH) in the gill of fish exposed to 100 mg/L of ZnO-NPs; (e) Epithelia lifting (EL), Lamellar disorganization (LD), Epithelial oedema (EO), Rupture of epithelial cells (RE), aneurysm (AN) and epithelial necrosis (EN) in the gills of fish exposed to 120 mg/L of ZnO-NPs

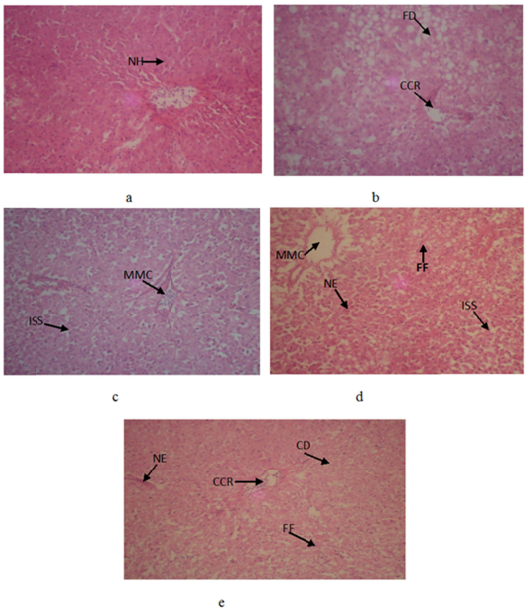

Figure 2 a-e: Histopathological changes in the liver of *Heterobranchus longifilis* exposed to ZnO-NPs for 96 h (H&E, X 100). (a)Liver of *Heterobranchus longifilis* in the control experiment showing normal hepatocyte; (b) fatty degeneration (FD) and central vein congested with red blood (CCR) in the liver of fish exposed to 60 mg/l of ZnO NPs; (c) melanomacrophase hepatocytes (MMC), and increased sinusoidal space (ISS) in the liver of fish exposed to 80 mg/l of ZnO NPs; (d) focal fibrosis (FF), necrosis (NE), melanomacrophase hepatocytes (MMC) and nuclear alterations (NA) in the liver of fish exposed to100 mg/l of ZnO NPs ; (e) cytoplasmic degeneration (CD), central vein congested with red blood (CCR), focal fibrosis (FF) and necrosis (NE) in the liver of fish exposed to 120 mg/l of ZnO NPs

The severity of damage in the gills of ZnO-NPs-exposed fish increased as the concentration of ZnO-NPs increased (Table 5).

Histopathology of Liver: The histopathological effects of ZnO-NPs on the liver of *Heterobranchus longifilis* exposed to varying concentrations of ZnO-NPs for 96 hours (Figure 2a – e). The liver of control fish showed normal hepatocytes with sinusoids (Figure 2a), there were moderate lesions such as fatty degeneration in the liver of fish exposed to 60 mg/l of ZnO-NPs (Figure 2b) and mild congestion of blood in the central vein of the liver in fish exposed to 60 and 120 mg/l ZnO-NPs (Figure 2b and e). Melanomacrophase of hepatocytes and increased sinusoidal space were evident at 80 and 100 mg/l ZnO-NPs (Figure 2c and d). Severe alteration like focal fibrosis and necrosis were shown at 100 and 120 mg/l (Figure 2d and e) while cytoplasmic degeneration alone was exhibited in the liver of fish exposed to 120 mg/l ZnO-NPs (Figure 2e). At the end of 96 h of exposure, the damages depicted resulted to DTC value of 0.10 in the control group; DTC of 36.60 at 60 mg/l; DTC of

Table 5: Degree of tissue change in the gills and liver of *Heterobranchus longifilis* exposed to ZnO-NPs for 96 hours

Organ	Concentration (mg/l)				
	Control (0.00)	60.00	80.00	100.00	120.00
Gill	0.10 ± 0.00	24.50 ± 0.06	33.40 ± 0.06	39.60 ± 0.23	40.50 ± 0.58
Liver	0.00 ± 0.00	36.60 ± 0.06	58.60 ± 0.17	58.90 ± 0.12	68.60 ± 0.58

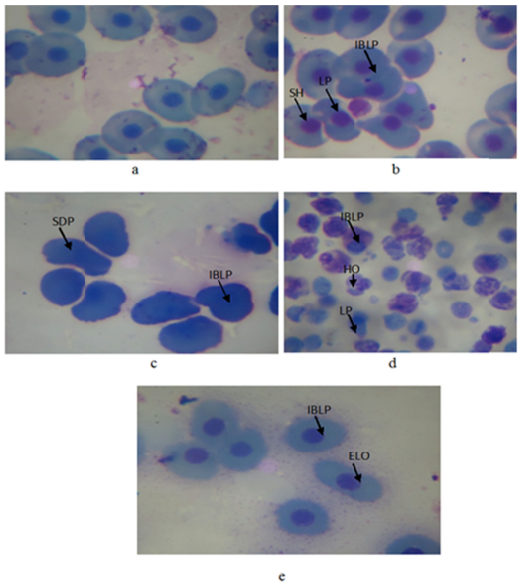

Figure 3 a-e: Morphological changes of blood cells in *Heterobranchus longifilis* exposed to ZnO-NPs for 96 h (H&E, 100 ×) (A)Blood cell of *Heterobranchus longifilis* in the control experiment shows normal erythrocyte shape; (B) irregular boundary with more than one lobopodial projections (IBLP), shrinkage (SH) and Lobopodial projection (LP) in the blood cell of fish exposed to 60 mg/L of Zinc oxide NPs; (C) Spherocyte with double projections (SDP) and Irregular boundary with more than one lobopodial projections (IBLP) in the blood cell of fish exposed to 80 mg/L of Zinc oxide NPs; (D) Hole- like depression (HO), Lobopodial projection (LP) and Irregular boundary with more than one lobopodial projections (IBLP) in the blood cell of fish exposed to 100 mg/L of Zinc oxide NPs; (E) Elongated cell with lobopodial projection (ELO) and Irregular boundary with more than one lobopodial projections (IBLP) in the blood cell of fish exposed to 120 mg/L of Zinc oxide NPs

58.60 at 80 mg/l; DTC of 58.90 at 100 mg/l and DTC of 68.60 at 120 mg/l with the mean DTC of 55.68 indicating a severe damage (Table 3). The severity of the damage in the liver of fish exposed to ZnO-NPs increased from slight to severe as the concentration of the contaminant increased.

Red Blood Cell Morphology: Figure 3a-e reveals the morphology of the erythrocyte of *H. longifilis* exposed to varying concentrations of ZnO-NPs for 96 hours. The erythrocyte of the fish shows an elliptical shape with the nucleus centrally located in the control group. Irregular boundary with more than one lobopodial projection was seen in erythrocytes of all exposed fish (60, 80, 100 and 120 mg/l) (Figure 3b, c, d and e), the erythrocyte also shows single lobopodial projection at 60 mg/l and 100 mg/l concentrations (Figure 3b and d). Shrinkage of erythrocyte occurred only at the lowest concentration 60 mg/l (Figure 3b). Spherocyte with double projections in the blood cell was also observed at 80 mg/l concentration (Figure 3c). The erythrocytes showed hole-like depression at 100 mg/l concentration (Figure 3d), while elongation of cell with lobopodial projection occurred at 120 mg/l (Figure 3e).

DISCUSSION

The physicochemical parameters of water to which *H. longifilis* was exposed greatly deviated from the recommended limits for fish survival in its habitat (WHO, 2004; NESREA, 2011). This could be due to the contamination of the water by the toxic effect of ZnO-NPs and may adversely affects the metabolic and physiological activities of the fish. The high LC_{50} value of 100 mg/l of ZnO-NPs obtained for *H. longifilis* implies that *H. longifilis* is more tolerable to ZnO-NPs contaminants than other fish species. This could be attributed to the possession of air-breathing organs by *H. longifilis* which enables it to tap atmospheric oxygen when in stress condition. The 96 hour LC_{50} of 100 mg/l of ZnO-NPs recorded for *H. longifilis* in this study was in contrast to the 96

hour LC_{50} of 4.897 mg/l of ZnO-NPs reported in common carp by Subashkumar and Selvanayagam (2014) and 96 hour LC_{50} of 4.92 mg/l of ZnO-NPs in zebrafish by Xiong *et al.* (2011). The higher doses of ZnO-NPs which resulted to decrease in RBC, HB and PCV values in the blood could be due to the inability of the fish to cope with metabolic stress induced by the toxic effect of ZnO-NPs. This may result in incapability of *H. longifilis* to further increase RBC and HB production to meet up with the increased oxygen demand. This could cause damage to the RBC thereby causing anaemia in fish. The decrease in the values of erythrocyte counts or in the packed cell volume is an indication of the destruction of RBC due to the toxic effect of ZnO-NPs. This may also cause anaemia at high concentration or erythropoiesis at a lower concentration which may eventually lead to physiological dysfunctions (Omoregie *et al.,* 1990). The decrease in the packed cell volume of *H. longifilis* exposed to ZnO-NPs may be an indication of haemodilution. This finding was also in accord with the report of Kori-Siakpere and Ubogu (2008) who reported a decrease in packed cell volume of *Heteroclarias* exposed to zinc. Reduction in the values of haemoglobin and packed cell volume in *H. longifilis* could also be as a result of lysing of erythrocytes indicating severe damage of the RBC as a result of ZnO-NPs effect. Dose-dependent decrease in RBCs of *H. longifilis* exposed to ZnO-NPs altered the morphology of red blood cells that lead to haemolysis as reported by Kori-Siakpere and Ubogu (2008) in *Heteroclarias* in response to ZnO-NPs. A significant decrease ($p<0.05$) in RBC, PCV and HB could be as a result of liver damage which inhibits the production of RBCs (Alkaladi *et al*., 2015). The significant decrease ($p<0.05$) observed in the MCV values of *H. longifilis* exposed to ZnO-NPs could be as a result of spleen contraction after stress, which released an erythropoietic agent that could be responsible for reduction in cellular blood iron, resulting in reduced oxygen carrying capacity of blood and eventually stimulating erythropoiesis (Kori-Siakpere and Ubogu, 2008). The increased levels of WBC could be as a result of an immunological reaction to produce antibody and helped the fish to cope with the stress induced by the toxicant. This finding is similar to the report by Sampath *et al*. (1993). Production of reactive oxygen species (ROS) helps to recruit WBCs and could be a response to attack the contaminant (ZnO-NPs) which serve as the foreign body that helps to give strength to the affected cells. Increase in the levels of WBC is, therefore, a defensive response to ROS effect. Production of ROS also induces the mobilization of platelets to the site of injury for cell repair. Increase in the values of platelets could, therefore, be a response to providing healing or repair to damaged gills and liver. Decrease in the level of the immune system of ZnO-NPs-exposed-fish as a result of its toxic effect could be attributed to the increased WBCs count; this is considered as a defensive mechanism that triggered the immune system. Also, an increase in the levels of WBC as the concentration of ZnO-NPs increased could be that different concentrations exerted a varying degree of stress. Mortality of some of the fish during acute experiment could be attributed to increasing rate of respiratory function of the gill; by increasing the ventilation rate in response to the reduction in oxygen uptake caused by the histopathological alterations, thereby increasing the rate of water flow through the gill which may have adverse effect on the function of the gill (Reebs, 2009). The various histopathological lesions recorded in the gills of *H. longifilis* exposed to lethal concentrations of ZnO-NPs for 96 hours could be as a result of the accumulation of ZnO-NPs in the gills, being the first target organ of pollutants or due to the large surface area between the external and internal fish environment. Gills also carry out the functions of gas exchange and ion osmoregulation, thus making them sensitive to adverse environmental conditions. Hyperplasia of the gill which occurred at varying concentrations of ZnO-NPs was in agreement with the report of Hao *et al*. (2009), who recorded hyperplasia-like thickening of the primary lamellae in the filaments of zebrafish exposed to titanium nanoparticles (TiO_2-NPs). Presence of lamellar fusion, epithelial lifting, oedema, aneurysm and necrosis in this study was similar to the reports of Xiong *et al.* (2011)

and Subashkumar and Selvanagam (2014). This result also conformed to those of Federici *et al*. (2007) and Li *et al*. (2009) who exposed zebrafish and Medaka to ZnO-NPs, respectively. All these morphological changes were reported to occur due to serious hyperplasia. Hyperplasia of epithelial cells, epithelial lifting and lamellar fusion will increase the space of contact of toxicants with the vascular system of the gill resulting in impairment of respiration as well as fish health (Subashkumar and Selvanayagam, 2014). Lamellar disorganization and rupture of epithelial cells recorded in this study could make it difficult for the recognition of different cell types which may result into fusion of secondary lamellar, thereby reducing the surface area for gaseous exchange and adversely affect the physiology of the fish (Susithra *et al*., 2007). Aneurysm could be due to the collapse of pillar cells in secondary lamellae or rupture of blood vessels which release a large quantity of blood that resulted in lamellar disorganization. Necrosis, oedema, lamellar fusion and hyperplasia of secondary lamellar were also reported by Kaoud *et al*. (2011) as visible damages in tissues affected by a pollutant. Manahan (1991) reported the occurrence of necrosis as a consequence of enzymatic inhibition, damages in the cellular membrane integrity and disturbances in the synthesis of proteins and carbohydrate metabolism. The hepatocyte lesions observed in liver tissues agree with the report of Subashkumar and Selvanayagam (2014). The hepatic alterations were associated with a degenerative necrotic condition. Degeneration of cytoplasm, recorded could probably be as a result of hypermetabolic activity of liver or direct toxicities of ZnO-NPs, and this is in conformity with the report of Subashkumar and Selvanayagam (2014). Congestion of blood reported in this work could be attributed to counter alteration, irritation and stimulation of vasodilation (Li *et al*., 2009). Necrosis recorded in the liver of exposed fish could be as a result of oxidative stress generated by ZnO-NPs toxicity. The multifunctional roles, metabolism of xenobiotics, and detoxification of contaminants by liver could also be attributed to its damage. Fatty degeneration recorded in the liver of exposed fish could be as a result of metabolic disorder due to ZnO-NPs effect.

Conclusion: The results of this study revealed that exposure of *H. longifilis* to lethal concentrations of ZnO-NPs caused varying morphological, haematological and histopathological alterations in a concentration-dependent manner. These alterations may compromise the health status of the fish and threaten its survival and may even increase the health risks of fish consumers. There is, therefore, the need to regulate the discharge of nanoparticles into the environment to prevent environmental hazards.

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