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Range Findings of Lethal Concentration of Zinc Oxide Nanoparticles (ZnO NPs) to Juvenile Red Tilapia

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HISTORY

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ABSTRACT

Zinc oxide nanoparticles (ZnO NPs) have attracted increasing concerns because of their unique properties and widespread applications in industry, which may adversely impact not only specific ecosystems but also human health. Although numerous studies have investigated the influence of ZnO on fish, there is a paucity of information available regarding the LC₅₀ value of ZnO NPs and effects of ZnO NPs on physical and behavioural of red tilapia Oreochromis sp., which are a vital fish-producing species in Malaysia. In this study, the effects of acute exposure of ZnO NPs (30-50 nm) were conducted in red juvenile tilapia fish (Oreochromis sp.) consisting: i) the assessment of the concentration-response curves and median lethal concentration (LC50) at 96 hours (4 days); and ii) evaluating the toxicity effects of ZnO NPs exposure on red tilapia based on the LC_{50} at 96 hours by observing behavioral and physical changes. 10 juvenile fish in each tank were exposed in the static non-renewable test solution for 96 hour (4 days). The LC50 value of ZnO NPs was 80.326 mg/L after 96 h of exposure. Physical and behavioral alterations were observed, including changes in swimming patterns, buoyancy control, ventilation, aggression, and appearance. Generally, red tilapia at higher concentrations exhibited dense schooling behavior, loss of buoyancy control, lethargy, movement in water, hypo- and hyperventilation, frequent gulping at the surface of the water, and increased aggression towards cannibalism. However, the colour of fish skin from each concentration does not show any changes observed to the fish along the 96-hours period of study.

INTRODUCTION

Nanotechnology is a multidisciplinary and rapidly growing field in science and technology which involves the manufacture, processing, and application of nanometre-scale assemblies of atoms and molecules [1]. Nanomaterials are generally defined as materials with at least one dimension less than 100 nm [2]. They have unique physical and chemical characteristics which deviate vastly from those of individual atoms or molecules and also the same material at a bulk [1,3]. These differences are due to their extremely small sizes and accordingly higher specific surface area and reactivity, which enable them to have novel applications. Heavy metal nanoparticles can be referred to as any metallic chemical element with nano-sized with a relatively high density that is poisonous at low concentrations. They have been assessed in several studies on fish feeding and toxicity because they are abundant in the environment and involved in important physiological tasks in organisms [4,5]. Over the last decade, many nanoscale materials and the field of materials science have advanced exponentially, including zinc oxide particles (ZnO NPs) have been manufactured.

Moreover, metal oxide nanoparticles are one of the most widely used classes of nanomaterials in industrial and domestic applications [6]. ZnO NPs are used in diverse commercial and industrial fields. However, the information from existing studies is not sufficient in evaluating the potential effects of ZnO NPs on fish. Nowadays, our habitats are being destroyed day by day due to increased environmental pollution through various human activities. As eloquently stated by Sabullah et al. [7], heavy metal pollution has increased in Malaysia in these recent years due to mining, natural disasters, and human and industrial activities. Fish are relatively sensitive to these changes in their environment as these metals are increasingly discharged into the aquatic ecosystem due to anthropogenic activities [8]. Pandey et al. [9] also analysed that heavy metals can refer to any metallic chemical element with a relatively high density that is toxic or poisonous at low concentrations.

Heavy metal nanoparticles such as zinc oxide become noticeably lethal when they are most certainly not used by the body and collect in delicate tissues. As stated by Erhirhie et al. [10], acute toxicity is usually from sudden or unexpected exposure to a moderately high concentration of chemicals in a brief time of exposure, subsequently, acute effects symptoms can show up after exposure. In addition, acute toxicity of heavy metals can harm blood composition, finineinfluence the gastrointestinal framework including the liver and other fundamental organs, and also affect other vital organs [11]. The purpose of this research was to ascertain the LC50 value of red juvenile tilapia exposed to ZnO NPs and to investigate the effects of ZnO NPs on juvenile tilapia by examining their physical and behavioral changes through an in vivo toxicity study.

MATERIALS AND METHODS

Zinc oxide nanoparticles (ZnO NPs)

This study was designed to evaluate the toxicity effects of ZnO NPs on red tilapia. 500 g of ZnO NPs (10-30 nm, powder) with a purity of 99% were purchased from US Skyspring Nanomaterials Inc. (Houston, USA). The powder appeared white to light yellow in colour.

Ethics statement

This toxicity study was carried out carefully under animal utilisation protocol (UPM/IACUC/AUP-R065/2018) with the approval of the Institutional Animal Care and Use Committee (IACUC) from Universiti Putra Malaysia (UPM). All the methods were made to minimise the pain of the fish.

Fish handling for acclimatisation purposes

Red tilapia possesses several physical traits that are laterally compressed for their body and have long dorsal fins, although the dorsal fin area is heavily spined. Red tilapia was chosen as the organism of study because it is the third most fish cultured in Malaysia (reference), widely available in store and appeals to the masses due to its red colour. Juvenile red tilapia, Oreochromis sp. was purchased from Puchong Hatchery, Universiti Putra Malaysia (UPM) located in Puchong, Selangor. 300 juvenile red tilapia were used in the experiment. Initially, the weight and length of each fish were measured and calculated, with the mean (n = 250) recorded as 8 g \pm 0.5 g and 7.6 ± 0.2 cm (3 inches), respectively. To avoid fish stress that could result in mortality during weight and length measurement, which involved hands-on manipulation, we decided to proceed quickly and in a gentle manner, solely to minimize stress. After that, the fish were acclimatised for 2 weeks.

All fish were held in the laboratory for 14 days (48 h settling-in + 12 days acclimatisation) before testing. During the acclimatisation period, the fish were held in a polytank containing 100 L of water. For ethical considerations and to prevent fish mortality due to stress, the water must be adequate and have sufficient quality for experimental purposes for at least 12 days immediately before testing, and must be under the

following conditions, which were the photoperiod was kept 12 h light:12 h dark. Temperature, dissolved oxygen (DO), and pH were maintained within acceptable ranges during acclimatisation. Detailed information on the water parameters is presented in **Table 1**.

Thus, to ensure that the water quality was maintained at all times, the physicochemical parameters of tank water were measured daily using a YSI 556 Multiprobe System (MPS). For fish feeding, a commercial feed named Starfeed, containing 35% crude protein, 12% moisture, 5% crude fibre, and 4% crude fat, was fed to the fish twice a day, constituting approximately 5% of their average mean weight. Surplus food and faeces were removed from the tank using siphoning techniques to prevent waste accumulation and maintain water quality.

 Table 1. During the acclimatisation period, the following water parameters were checked on a daily basis.

Type of water parameters					
pH	7.2				
Temperature	$28^{\circ}C \pm 1$				
Dissolved oxygen (DO)	7.8				
Total dissolved solid (TDS)	0.98				
Turbidity (NTUs)	1.05				

During the final 48 hours of acclimatisation before the toxicity test, no fish mortality was observed. If mortality exceeded 5%, the fish were deemed too stressed to be used for the test. In the final 24 hours of acclimatisation, fish were not fed to remove faeces and prevent water quality deterioration in the test tank. Healthy fish, determined by their normal physical behaviour, were used for the toxicity studies.

Preparation for acute toxicity test

25 tanks were used for the preliminary acute toxicity study after the acclimatization period. 10 juvenile red tilapias were used in each tank in this study. Each test tank measured 45 cm \times 29.5 cm \times 30 cm and contained 20 L of water. Each test concentration had three replicates, but only one replicate was used as the control. All replicate and control tanks were filled with 10 fish per tank. The total number of juvenile red tilapia in all tanks at the following concentrations and the control was 250. Layout for treatment and replicates are presented in **Table 2**.

Table 2. Layout for treatment and replicates for the acute toxicity test.

Type of concentration (mg/L)		Replicate (R)	
Control (0)		R1	
10	R1	R2	R3
20	R1	R2	R3
40	R1	R2	R3
60	R1	R2	R3
80	R1	R2	R3
100	R1	R2	R3
120	R1	R2	R3
150	R1	R2	R3

The stock solution of the ZnO NPs was freshly prepared. The powder of ZnO NPs was dispersed and sonicated with ultrapure water. The prepared stock solutions were used to make different concentrations for the exposure in the test tank. There were 8 concentrations were made in this exposure study. For zinc oxide (ZnO) nanoparticles, the concentrations of zinc oxide were ranging from 0, 1, 10, 20, 40, 80, 100, 120, 150 mg/L. A preliminary test was conducted to establish the substance concentration for the definitive test in this study. Triplicate tests for every 8 concentrations were done for this preliminary study including one control. During the acute toxicity study (96 h LC_{50}), water parameters were checked twice per day in each test tank which was including DO, pH, and temperature, total dissolved solid (TDS) and turbidity by using a YSI meter. Gentle aeration of test vessels was used in static systems during the exposure period.

The physical and behavioral changes of the fish were observed in each tank. There was no feeding during the acute toxicity study period within 96 h. All test tanks were inspected every 12 h to evaluate the acute toxicity of ZnO NPs. The evaluation included the assessment of mortality, morphological alterations, and abnormal behavior in accordance with the IACUC guidelines. Any fish exhibiting these endpoints, as outlined in **Table 3**, were immediately removed and euthanized using tricaine methanesulfonate (MS-222) at a concentration of > 250 mg/L. All dead fish were buried in the soil behind the laboratory.

 Table 3.
 Endpoints that were used to assess acute toxicity on juvenile red tilapia.

Criteria of endpoints that were used to trigger the decision to remove an animal from the experiment, or to terminate the sample from the experiment besides mortality

i.	Totally loss of buoyancy control (floating or sinking at the bottom
	with nearly no movement)
ii.	Over-aggressive in water or frequently jumping out of water
:::	Vany weals, latheness, and with near immediative in water

iii. Very weak, lethargy, and with near immobility in water

iv. Over-gasping or over-gulping (totally suffocate due to toxicant)

Note: All the criteria of endpoints listed above have been discussed and agreed upon by the IACUC committee

The toxicity of ZnO NPs was measured using the LC50 method and analysed via probit analysis using the IBM SPSS software (version 25, Chicago, IL, USA). Following the acquisition of LC₅₀ data resulting from the 96 h of acute toxicity test, it is imperative to compare the obtained LC₅₀ values with the toxic classification criteria established by the United States Environmental Protection Agency (US) [12], shown in **Table 4**.

Table 4. Toxicity category for aquatic organisms, according to their 96-h LC50. All data of the acute toxicity study are adopted from U.S. EPA [12].

Category for toxicity	Concentration of acute toxicity (mg/L)		
Very highly toxic	< 0.1		
Highly toxic	0.1 - 1		
Moderately toxic	> 1 - 10		
Slightly toxic	> 10 - 100		
Practically non-toxic	> 100		
Note: Ecotoxicity categories for Terrestrial and Aquatic Organisms based on U.S EPA [12].			

RESULT AND DISCUSSION

Behavioural Changes of Fish

Based on the OECD revised draft on Fish, Acute Toxicity Test [13] in Table 5, there are 8 aspects (labelled A - H) that encompass physical and behavioural changes in the fish.

The degree of physical and behavioural changes within the concentration range are shown in **Table 5**. There are four stages for classifying this level of change which are normal, mild, intermediate, and terminal/severe. No physical alterations were noted in the fish, suggesting that the hue of the fish skin did not undergo any modifications at any concentration throughout the 96-hour period of examination.

As shown in Table 5. no behavioural changes were observed from the control (0 mg/L) to 20 mg/L. However, at 40 mg/L, the fish started to gulp at the surface of the water, but this was only a mild condition. At 60 mg/L, several types of mild behavioural changes were found in fish, including dense schooling, lethargy, hyperventilation, hypoventilation, and gulping at the water surface. However, the behavioural changes started to become intermediate at 80 mg/L. The behavioural changes become terminal/severe when the fish are exposed to 100 mg/L ZnO NPs at certain aspects, such as dense schooling, hyperventilation, lethargy in water, hypoventilation, hyperventilation, and gulping at the surface of water.

At 150 mg/L, all aspects exposed by fish were at the terminal/severe level, and only at this concentration was found that, the fish started to become aggressive and tend to fight each other, contributing to the mortality of fish. The behaviour response of fish tends to get severe (dead symptom), especially at a higher concentration most of the fish tested were total loss of buoyancy control (floating or sinking at the bottom with nearly no movement), very weak with near immobility in water and over-gasping or over-gulping (totally suffocate due to toxicant) rightly before died. These were the criteria of endpoints that were used and agreed upon by the IACUC committee.

Similar behavioural studies were also reported by Keerthika et al. [14]; Keerthika et al. [15], where *Labeo rohita* was used in a toxicity study and exhibited behavioral response due to a higher concentration of iron oxide nanoparticles in the water. Also, Chen et al. [16] recorded the stress behaviour exposed on larval zebrafish (*Danio rerio*) when introduced to a high concentration of titanium dioxide. Fish behaviour responses to toxicants presents in the aquatic environment provide a promising tool for toxicology studies [17], as it reveals the consequences of toxicants at the lower level and can lead to higher ecosystem-level adverse outcomes [14].

Behaviour is a series of actions that are carried out through the central and peripheral nervous systems, and is the cumulative result of genetic, biochemical, and physiological processes that are essential to life, such as feeding, reproduction, and predator avoidance [18]. Adaptation to changing environments allows fish to adjust external and internal stimuli to cope with sudden changes in the environment [19,14]. Physical and behavioural changes are ideal ways to study an organism's response to stress due to environmental pollutants and serve as a connection between physiological and ecological processes. **Table 5.** The degree of physical and behavioural changes that have been observed in during 96 h acute toxicity study of ZnO NPs to juvenile red tilapia.

Con	centration	0	10	20	40	60	80	100	120	150	
(mg	/L)										
aspe	ct										
Α	Dense schooling	-	-	_	-	+	$^{++}$	++	+++	+++	
	behaviour										
В	Loss of buoyancy	_	_	_	_	_	+	+	++	+++	
	control										
С	Lethargy/	_	_	_	_	+	$^{++}$	++	+++	+++	
	weak										
	movement in										
	water										
D	Hyperventil-ation	-	-	-	-	+	++	++	+++	+++	
Е	Hypoventil-ation	_	_	_	_	+	+	++	+++	+++	
F	Gulping at	_	_	_	+	++	++	++	+++	+++	
	surface of water										
G	Aggression and/	_	_	_	_	_	_	_	_	+++	
	or cannibalism										
Н	Appearance	The	colo	ur of	fish	skin	does	not sh	ow any	change	es

observed to Fish shin toes not show any enanges observed to the fish along the 96-hours period of study. **Note:** Clinical Sign for Fish Behavior Assessment based on OECD, (2018). – normal, + mild, ++ intermediate, +++ terminal/sevre

Acute toxicity

In this experiment, no mortality was recorded from 0 (control) to 20 mg/l. the lowest concentration that could cause fish mortality was 40 mg/L, which was observed at 96 h. Complete mortality was observed at 150 mg/L, with mortality starting at 24 h. there was a significant correlation between ZnO NPs concentration and the mortality rate of *Oreochromis* sp. Besides, Figure 1 shows that the mortality rate of *Oreochromis* sp. during the toxicity test increased with increasing concentration of ZnO NPs in the water. the trend of this graph confirms that the ZnO NPs have concentration-dependent toxicity on the juveniles of *Oreochromis* sp. in water. The LC₅₀ value (50% of mortality rate) of ZnO NPs occurred at a log concentration of 1.905, which corresponds to 80.326 mg/L.

Table 6. Toxicity category for aquatic organisms, according to their 96-h LC50. All data of the acute toxicity study are adopted from U.S. EPA [12].

Category for toxicity	Concentration of acute toxicity (mg/l)
Slightly toxic	> 10 - 100
Note: Ecotoxicity categories for Ter	restrial and Aquatic Organisms based on U.S EPA [12].



Fig. 1. Graph showing the mortality dose-response curve of ZnO NPs (96 h) for *Oreochromis* sp. based on parameter estimates from the Probit analysis. The LC_{50} value for ZnO NPs determined by probit analysis at different concentrations (0, 10, 20, 40, 60, 80, 120, 150 mg/L) at the end of 96 h.

Based on the result in Fig. 1, acute concentrations value of ZnO NPs can be classified as slightly toxic as it takes place between >10 - 100 mg/L according to the U.S. EPA [12] (Table 6). To sum up, extensive aquatic toxicity data from this study could be used to determine the ecological risks to aquatic ecosystems, and it adds new knowledge that could be adapted to aquaculture, which is about the safety level of ZnO NPs that will be incorporated in feeding for red tilapia.

One of the main differences between this study and the previous study about acute toxicity is the value of the LC_{50} of ZnO NPs according to test species. In line with some previous studies, Suganthi et al. [20] stated that 50% of Oreochromis mossambicus (Nile tilapia) mortality was observed between 100-110 mg/L. Furthermore, a recent study by Taherian et al. [21] found 25.50 mg/L as LC_{50} of ZnO NPs on Oncorhynchus mykiss (rainbow trout) for 96 h. Other than that, the LC_{50} of ZnO NPs of freshwater fish, Labeo Rohita observed was 31.15 mg/L [22] at a 95% confidence interval. Moreover, LC_{50} of ZnO of Rutilus rutilus caspius (Caspian roach) was found by occurred at a concentration of 48 mg/L [23].

However, in another aspect, the size of ZnO can influence the value of LC50 of ZnO, a comparison that prevailed by Mohamed et al. [24], due to the assessment of ZnO toxicity between NPs and BPs, both LC50 of ZnO (NPs & BPs) results were obtained for Oreochromis sp. was 5.6 mg/L and 84 mg/L respectively after 96 h and it is similar to Abdel-Khalek [25], also reported Zn (BPs & NPs) toxicity on Nile tilapia (Oreochromis niloticus) were 1.36 g/L and 0.18 g/L, respectively. Meanwhile, another previous assessment of LC50 study based on the type of toxicant by Rashidian et al. [26], stated that the outcome of LC50 value varied accordingly to commercial and green synthesized ZnO NPs which has recoded 59.95 and 78.9 mg/L respectively. The inconsistencies in the previous LC50 of ZnO NPs outcomes were due to various reason, as elucidated by Morgaleva et al. [27], three main aspects cause the level of acute toxicity different in each organism which are the type of test animal, aggregation, and concentration of nanoparticles. Also, Vajargah et al. [28] indicated that species, size, genus, and living environment of test organisms can influence the value of acute toxicity of pollutants. In short, it explains to us that every type of acute toxicity assay is highly possible to produce various results due to these factors.

CONCLUSION

The acute toxicity study of ZnO NPs revealed the behavior response of fish due to the higher concentration of ZnO NPs. The LC50 value of ZnO NPs was obtained at 80.326 mg/L for 96-hour exposure, which is practically considered slightly toxic based on the U.S EPA [12].

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