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Zinc oxide (ZnO) induced toxicity and behavioural changes to oligochaete worm *Tubifex tubifex* (Muller)

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Abstract- Zinc is among the important trace element of the body by participating in several biological processes. But it shows toxicity after a certain concentration. Zinc oxide (ZnO), a hazardous heavy metal, is frequently used in both bulk and nanoparticle format in industrial and household purposes throughout India. In the present study the 24, 48, 72 and 96h LC_{50} values of ZnO was monitored to benthic oligochaete worm *Tubifex tubifex* (Muller) through Finney's probit analysis method. Different abnormal behavioural changes in the worm were also recorded during the exposure period. The 24, 48, 72 and 96h LC_{50} values of Zinc oxide (ZnO) to *Tubifex tubifex* was obtained as 30.88, 24.15, 19.95 and 11.15 mg/l respectively. The mortality rate of *Tubifex tubifex* varied significantly ($p<0.05$) with a dose and time dependent manner (24, 48, 72 and 96 h). The exposed worm showed several erratic behaviors with the gradually increasing concentrations of the metal and with the progress of time of exposure. The safe concentration of this metal was ranged between 0.0001115 -1.11 mg/l. Based on 96h LC_{50} value the Maximum allowable concentration (MAC), no observable effect concentration (NOEC) and the lowest observable effect concentration (LOEC) of Zinc oxide to *Tubifex tubifex* were 1.11, 1.3 and 2.1 mg/l respectively. The present study will be useful in determination of the safe dose of the toxicant before its disposal to the environment.

Keywords*: Zinc, Acute toxicity, LC_{50}, Tubifex tubifex, behavioural response.*

I. INTRODUCTION

In the recent decade, the increasing water pollution causes a great threat to aquatic environment including the aquatic life. The heavy metal pollution causes a serious health problem for the aquatic organisms. Toxicants like heavy metals, pesticides reach the aquatic bodies through industrial and agricultural runoffs. [1]. Increased heavy metal concentration into water bodies can lead to destruction of natural ecosystem by exposing the organisms to high level of metal toxicity [2]. Aquatic organisms like fish, crustaceans and bottom dweller oligochaete worms are most susceptible to this heavy metal toxicity as they cannot escape from the water bodies [3]. Heavy metal toxicity also has a high risk of bioaccumulation and bio magnification [4].

Among different trace metals of body, Zinc (Zn) is an important one. It has important role in structural formation of protein and act as cofactors of several enzymes [5]. It participates in several biological processes. Zinc oxide (ZnO) and Zinc oxide derived nanoparticles are among the most commonly used nano-material in different fields including medical applications, industrial and household products [6]. Despite being a key trace element of body, after a certain concentration it shows toxicity to exposed organisms. Aquatic organisms are more susceptible to its toxicity [7]. The extensive use of ZnO leads to their discharge into the aquatic ecosystem. For these reasons, the risk assessment and toxicity evaluation of Zinc oxide has been needed in aquatic toxicology study. The acute toxicity studies on Silver Carp (*Hypophthalmichthys molitrix*), Grass Carp (*Ctenopharyngodon idella*), rainbow trout, rainbow trout (*Salmo gairdneri*) and some other fish have been reported earlier by previous researchers [8-11].

Tubifex tubifex is a freshwater benthic oligochaete worm used as a test organism for sediment bioassay and for determination of acute toxicity of heavy metals and pesticides [12, 13]. It is a very good bio indicator of pollution as they can survive in areas with heavily polluted organic matter that almost no other species can endure [14]. There are no earlier reports on acute toxicity of ZnO on *Tubifex tubifex*. Therefore, in view of the ecological impact of this pesticide, the present study aimed at determination of LC_{50} of ZnO to the *Tubifex tubifex*, and their behavioural responses during exposure.

The organization of the paper is as follows, Section I contains the introduction, previous related works and objective of the study, Section II contain the methodologies followed during the work including standard protocols and statistical analysis, Section III contain the results obtained from the study, related tables and figures and the discussion of the work, Section IV concludes research work with future directions.

II. MATERIALS AND METHODS

Test animal

The live specimens of benthic oligochaete worm *Tubifex tubifex* (Mueller), Phylum: Annelida, Class: Clitellata, Order: Oligochaeta and Family: Naididae, with an average body size of 10.5 ± 0.5 mmwere acquired from local fish market and allowed to acclimate in laboratory conditions for 72 hours prior to the experiment. The test organisms were kept in natural and pollution free water (temperature 29.0 ± 0.4 °C, pH 7.2 ± 0.3, free CO_2 13.6 ± 0.5 mg/l, dissolved oxygen 5.7 ± 0.3 mg/l, total alkalinity 182 ± 8.1 mg/l as $CaCO_3$, hardness 113 ± 6.8 mg/l as $CaCO_3$) to determine natural mortality. Physicochemical parameters of water used during experiment were measured as per standard procedure [15]. The photoperiod was kept as per average day and night around 12 hours. Only the healthy organisms were chosen for experiment.

Test chemical

The heavy metal for the experiment, Zinc oxide (ZnO) was purchased from SRL (51508).Stock solution of the test chemical and its dilutions were made following the standard method [15].

Acute toxicity bioassay

The acute toxicity test was conducted in a static system to determine the 24, 48, 72 and 96 h LC_{50} of ZnO following the standard guideline [16, 17]. Initially a rough test was conducted to determine the range of the concentrations where mortality occurs. The test organisms were exposed to ten different nominal concentrations (5, 15, 30, 45, 60, 75, 90, 100, 120 and 140 mg/l), along with one control (without test chemical), keeping 10 test organisms in each test concentration in a 250 ml glass beaker having 200 ml of water. No gathering stress was observed during experimentation. Mortality and behavioural changes of test organism were recorded at 24, 48, 72, and 96 h. The dead organisms were removed immediately after their death to avoid any organic decomposition. The investigation was repeated thrice for good statistical significance, following the standard recommendations [18]. Feeding was stopped 24h prior to the exposure of toxicant and worms were not fed during the experiment period following the earlier methods [19, 20, 21]. No mortality was observed in the control group of *Tubifex tubifex*. The behavioural changes (wrinkling effect, clumping tendency, mucus secretion, hyperactive movement and body fragmentation) in the worms were also noted immediately after the exposure to toxicants until the end of experiment as per standard protocol [22, 23].

Statistical Analysis

All the statistical analysis were done using Graphpad prism and MS-Excel 2016. Finney's probit analysis method [24] was used to calculate the 24, 48, 72 and 96h LC_{50} values, which were also subsequently verified following the methods of Miller and Tainter, 1944 [25] and Behrens-Karber method with the help of Klassen formula [26]. Safe concentration of profenofos was calculated following the methods and guidelines of Burdick [27], International Joint Commission (IJC) [28], National Academy of Sciences (NAS/NAE) [29] and Company Wide Quality Control (CWQC) [30] based on the 96h LC_{50} values. Maximum allowable concentration (MAC), no observable effect concentration (NOEC) and the lowest observable effect concentration (LOEC) were also determined.

III. RESULTS & DISCUSSION

The results of percent mortality in different test groups after 96h of exposure to ZnO are shown in Table 1. No mortality was observed in control worms throughout 96h. With the increasing concentration of the toxicant, the mortality of the test organism increases. The Lethal concentrations as well as LC_{50} values for 96h along with its 95% lower and upper confidence limits (ML and MU), slope and intercept of ZnO as calculated by Finney's probit analysis are shown in Tables 2. The 24, 48, 72 and 96 h LC_{50} values of ZnO to *Tubifex tubifex* were 30.88, 24.15, 19.95 and 11.15 mg/l respectively. The mortality rate of *Tubifex tubifex* varied significantly ($p<0.05$) with increasing exposure times (24, 48 and 72 and 96h) and doses (2 way ANOVA followed by Tukey test; Table 1, Figure 1). This observation indicated that mortality is dose and time dependent (Figure 2). The LC_{50} values decreases with the increase in exposure period of the toxicant. It indicates that toxicity of this metal increases with the increase in exposure time.

This 96h LC_{50} value was also assessed and estimated following the methods of Miller and Tainter, 1944 (11.22 mg/l) and Behrens-Karber's method (17.75 mg/l) (Table 3). The three methods are comparable and in good concordance.
The safe concentrations calculated for *Tubifex tubifex* exposed to ZnO are shown in Table 4. The safe concentration of ZnO to *Tubifex tubifex* ranged between 0.0001115 -1.11 mg/l may be considered before their disposal to the natural environment.

Based on computed LC_{50} 96h on *Tubifex tubifex*, contaminants MAC value is 1.11 mg/l for ZnO in 27±0.5°C. In addition, contaminants LOEC were calculated to be 2.1

mg/l for ZnO. NOEC was determined to be 1.3 mg/l for ZnO (Table 5).

Different abnormalities in behavior were observed in the exposed worms in comparison to control. But no behavioral changes were recorded in control. Control worms were active and alert to the slightest disturbance with their well synchronized clumping tendency. The effects of pesticide were observed as hyperactive movement (HM), clumping tendency (CT), mucus secretion (MS) and wrinkling activity (WE) are shown in Table 6. The clumping tendency of *Tubifex tubifex* was decreased with the increasing concentration of the test chemical and exposure time in comparison to control. The hyperactive movement of the worms increases with the increasing concentration of ZnO but decreases with exposure period. Mucous secretion and wrinkling effects of the worm were increased at 45 mg/l and above. The clumping tendency was gradually decreased with the increasing concentration of the toxicants and was absent at 75 mg/l and above irrespective of exposure times. But the rate of mucous secretion was very high at these concentrations.

Discussion

In the current study, Zn toxicity was indicated by *Tubifex tubifex* mortality. For any toxicological study, determination of the LC_{50} value is the initial step [31]. The present study illuminates the acute toxicity of ZnO on the benthic worm *Tubifex tubifex*. The result of the 96h LC_{50} value of ZnO was determined to be 11.15 mg/l using Finney's probit analysis method. This value is supported by the values obtained from Behrens–Karber's method (17.75 mg/l) and Miller and Tainter method (11.22 mg/l). Similar results were obtained by previous researchers during acute toxicity study of Zn. The present 96h LC_{50} value (11.15 mg/l) of Zn to *Tubifex tubifex* is higher than the values obtained by earlier researchers in *Oncorhynchus mykiss* (0.95 and 3.79 mg/l) [32, 33], *Salmo gairdneri* (0.55 mg/l, 0.24 mg/l and 4 mg/l) [34, 35, 36], *Hypophthalmichthys molitrix*(1.23 mg/l) and *Ctenopharyngodon idella* (4.6 mg/l) [8]. But it was slightly lower than the value found in *Oncorhynchus mykiss* (12.88 mg/l) [9], *Oreochromis niloticus* (63.98 mg/l) [31], *Labeo rohita* (65 mg/l) [37] and *Channa punctatus* (48.68 mg/l) [38]. This variation in toxicity of Zinc was probably due to the variation in physic-chemical properties of water [39], size, weight, age group and the habitat of the test organisms [40]. These kinetic variables play a vital role in the variation in LC_{50} values.

Behavioural changes signify an integrated response of test species to toxicant stress. Behaviour provides a unique perception among the body physiology and ecology of an organism and its environment. Similar kind of behavioural reactions were also observed in *Branchiura sowerbyi* exposed to alpha-cypermethrin and Triazophos [17, 23, 41]. With the increasing concentration of the toxicant and exposure time, the clumping tendency of the worm decreases. High amount of mucus secretion was recorded with the increase in exposure time. These abnormal ethological behaviours indicates towards the avoidance reaction of the worms towards toxicant. Previous researchers suggested that the metal effects the respiration of the worm by blocking the gas exchange through formation of mucus-metal complex over the body surface [42].

The result of the present acute toxicity study showed that *Tubifex tubifex*was sensitive towards Zn toxicity. The obtained 96h LC_{50} value indicates that Zinc can affect the aquatic organisms and disrupt the natural aquatic food chain even at low concentration. The ethological changes due to Zinc toxicity may be used as a bio indicator for heavy metal toxicity studies.

Table 1: Mean values (±SD) of % mortality of *Tubifex tubifex* exposed to different concentrations of ZnO at different times of exposure (24, 48, 72 and 96h). The mortality increases significantly with increasing concentration and time of exposure. Mean values within columns indicated by different superscript letters (a-j) and mean values within rows indicated by different superscript letters (m-p) are significantly different (2 way ANOVA followed by Tukey test).

Concentration (mg/l)	**% Mortality**			
	24h	**48h**	**72h**	**96h**
0 (Control)	0 ± 0^{am}	0 ± 0^{am}	0 ± 0^{am}	0 ± 0^{am}
5	10 ± 2.5^{bm}	20 ± 4^{bn}	30 ± 5^{bo}	40 ± 3^{bp}
15	30 ± 2^{cm}	30 ± 3.5^{cm}	40 ± 3.5^{cn}	50 ± 3.5^{co}
30	40 ± 3.5^{dm}	50 ± 4.5^{dn}	60 ± 5^{do}	60 ± 4^{do}
45	50 ± 2^{em}	60 ± 2^{en}	70 ± 4.5^{eo}	80 ± 3.5^{ep}
60	70 ± 4^{fm}	70 ± 2.5^{fm}	70 ± 2.5^{em}	80 ± 4^{en}
75	70 ± 3.5^{gm}	70 ± 3^{fm}	80 ± 3^{fn}	90 ± 4.5^{fo}
90	80 ± 5^{hm}	90 ± 4^{gn}	90 ± 4^{gn}	100 ± 0^{go}
100	90 ± 3^{im}	90 ± 2.5^{gm}	100 ± 0^{hn}	
120	90 ± 3.5^{im}	100 ± 0^{hn}		
140	100 ± 0^{j}			

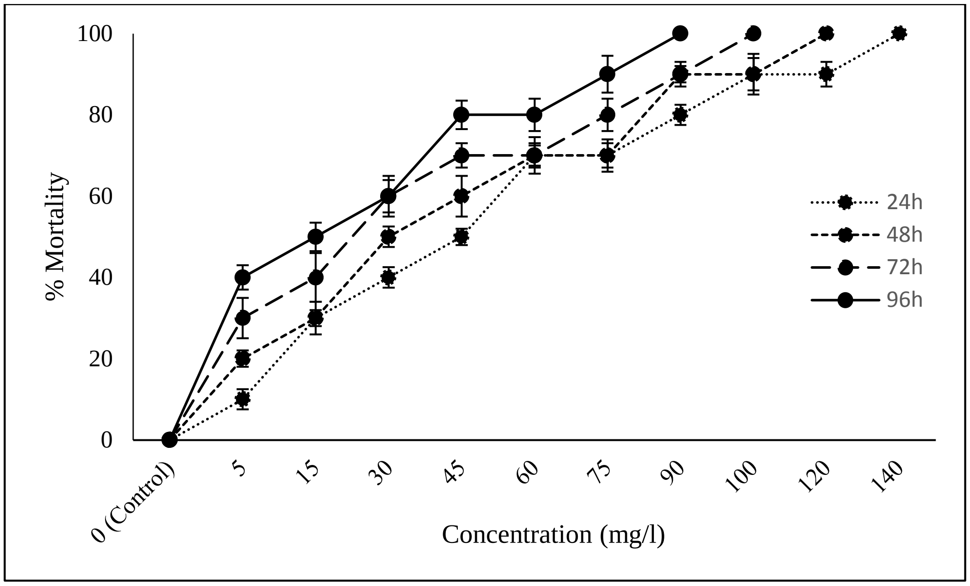

Figure 1: % Mortality of *Tubifex tubifex* against different concentrations of ZnO during 96h of exposure. ($p<0.05$).

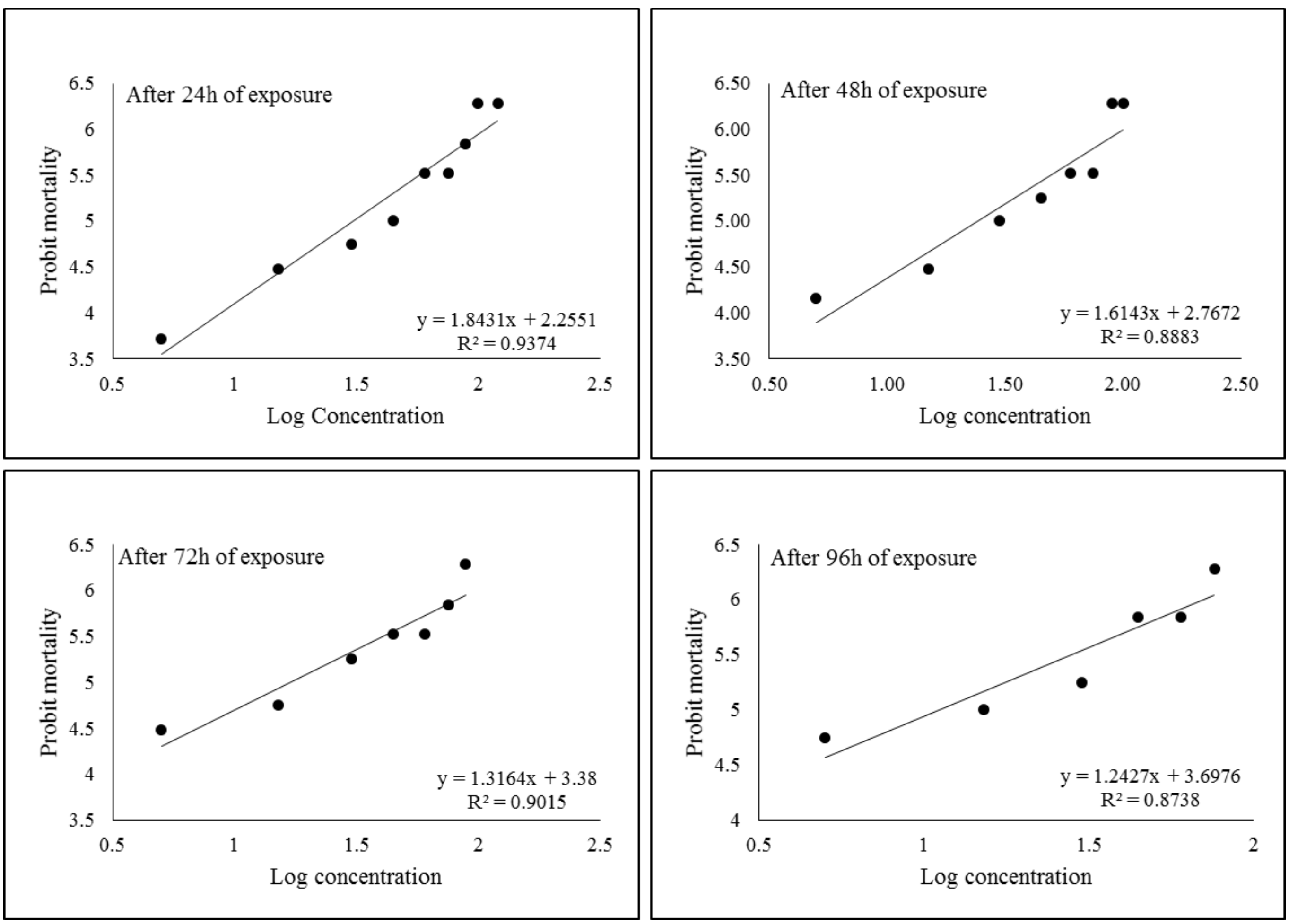

Figure 2: Probit mortality of *Tubifex tubifex* against different log concentrations of ZnO after 24, 48, 72 and 96hof exposure. ($p<0.05$).

Table 2: LC50 values (with 95% confidence limits, regression equation, R^2 and r values) of ZnO to the *Tubifex tubifex* at different times of exposure (24, 48, 72 and 96h)

Hours	Lethal Concentration Values (mg/l)						95% Fiducial Limits of LC_{50}		Probit Regression Equation Y=ax+b	R^2 Value	r value
	LC_1	LC_{10}	LC_{30}	**LC_{50}**	LC_{70}	LC_{90}	Lower	Upper			
24h	1.60	6.05	15.86	**30.88**	60.15	157.49	21.78	43.78	Y=1.84x + 2.25	0.94	0.96
48h	0.76	3.61	11.10	**24.15**	52.54	161.43	15.98	36.48	Y=1.61x + 2.76	0.88	0.94
72h	0.26	1.73	6.66	**16.95**	43.14	166.14	10.04	28.63	Y=1.32x + 3.37	0.90	0.94
96h	0.14	1.01	4.17	**11.15**	29.82	123.41	6.10	20.37	Y=1.24x + 3.69	0.87	0.93

Table 3: 96h LC_{50} value according to the method of Miller and Tainter and Behrens-Karber's method

96h LC_{50} value of ZnO according to	
Miller and Tainter method	11.22 mg/l
Behrens–Karber's method	17.75 mg/l

Table 4: Safe concentration range of ZnO based on the 96h LC_{50} value

Chemical	96h LC50	Method	Safe Value (mg/l)
ZnO	11.15 mg/l	Burdick	1.11
		CWQC	0.01115
		NAS/NAE	1.11-0.0001115
		IJC	0.55

Table 5: Maximum allowable concentration (MAC), the lowest observable effect concentration (LOEC) and no observable effect concentration (NOEC) values of ZnO to *Tubifex tubifex*

Maximum allowable concentration (MAC)	1.11 mg/l
Lowest observable effect concentration (LOEC)	2.1 mg/l
No observable effect concentration (NOEC)	1.3 mg/l

Table 6: Behavioural responses of *Tubifex tubifex* (CT= Clumping Tendency, HM=Hyperactive Movement, MS= Mucous Secretion, –: absent, +: mild, ++: moderate, +++: high) exposed to different concentrations of ZnO at different times of exposure.

Behavioural responses of *Tubifex tubifex* at different times of exposure																
Dose (mg/l)	**Dose (mg/l)**				**Dose (mg/l)**				**Dose (mg/l)**				**Dose (mg/l)**			
	24h	48h	72h	96h	24h	48h	72h	96h	24h	48h	72h	96h	24h	48h	72h	96h
5	-	-	-	-	+++	+++	++	+	-	-	+	+	-	-	-	-
15	+	+	-	-	++	++	+	+	-	-	+	+	+	-	-	-
30	++	+	+	-	++	+	+	+	-	+	+	++	+	+	+	-
45	++	++	+	+	+	+	-	-	+	++	++	++	++	+	+	+
75	+++	++	++	+	+	-	-	-	++	++	++	+++	++	++	++	++
90	+++	++	++	+	-	-	-	-	++	++	++	+++	+++	+++	++	++

IV. CONCLUSIONS

The median lethal concentration of Zinc oxide to *Tubifex tubifex* was estimated in terms of LC_{50} values. The 24h, 48h, 72h and 96h LC_{50} value of Zinc oxide to *Tubifex tubifex* were 30.88, 24.15, 19.95 and 11.15 mg/l respectively. It shows that *Tubifex tubifex* is very much sensitive toward toxicity of Zinc even presence in a minute quantities. Thus, it can be used as an excellent model organism to observe the potential hazards of acute exposure of the heavy metals to aquatic organisms. This study also shows the importance of behavioural parameter in assessing the hazards of the non-degradable heavy metals to aquatic organisms. This approach may help in the approximation of potential health hazards to non-target aquatic organisms upon exposure to Zinc.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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