Acute toxicity of TiO_2, CuO and ZnO nanoparticles in brine shrimp, *Artemia franciscana*

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Abstract

The brine shrimp, *Artemia* spp., is widely used in ecotoxicological research as a biological index. In the present study, aquatic stability and acute toxic effects of TiO_2, CuO and ZnO nanoparticles (NPs) on *Artemia franciscana* were investigated. Acute exposure was conducted in sea water with different concentrations of selected nanoparticles at 24h, 48h, 72h and 96h. The mortality rate of *A. francisca* increased significantly with increasing concentrations and duration of exposure of all NPs. The toxicity pattern of metal oxides to *A. francisscana* was as follows: CuO>TiO_2>ZnO. Our results point to the fact that both TiO_2 and ZnO NPs exhibited moderate toxicity to *Artemia* larvae in 24h as compared with CuO, regardless of their size and concentrations.

Keywords: Acute toxicity, *Artemia franciscana*, Titanium dioxide, Copper oxide, Zinc oxide.

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Introduction

In recent years, the brine shrimp play an important role in aquaculture; approximately 10 million pounds of brine shrimp eggs are harvested each winter and sold as food for tropical fish. (Brix *et al.*, 2003a). *Artemia* is a suitable organism for bioassay and toxicity studies. This species has a key role in the aquatic food chain (Ozkan *et al.*, 2015). Nanoparticles are known for their small size (1–100 nm) and specific physical and chemical properties. These specific properties result in different characteristics (e.g. transparency, UV reflection, physical strength, etc.), which make them very useful material to use in a number of different products. As a result, many industries in the world have increased the production of nanoparticle-containing products specially in this decade (Li *et al.*, 2008).

TiO_2 NPs[1] have been used worldwide in diverse areas including sunblock lotions, cosmetics, paints, food additives, medicines, construction materials and environmental decontamination of air, soil and water (Ozkan *et al.*, 2015). ZnO NPs are widely used as additives in food, sunscreens and cosmetic products and in the manufacture of textiles, paint pigments, semiconductors, catalysts, polishers and water disinfection and chemotherapy (Ates *et al.*, 2013b). CuO NPs have widespread uses in gas sensors, batteries, plastics and metallic coatings, etc. Increasing production, use and application of these nanoparticles will increase the potential of NPs discharge in aquatic ecosystems because of their small size and specific properties which may cause adverse effects to different organisms (Adam *et al.*, 2015).

Nanoparticles can enter in the aquatic ecosystems in a highly dynamic way, which depends on the aquatic environmental conditions. Nanoparticles have been shown to both dissolve (Kasemets *et al.*, 2009, Mortimer *et al.*, 2010) and aggregate (Jo *et al.*, 2012) forms. Different environmental factors (such as dissolved oxygen, ionic strength, pH and natural organic matter, etc.) have been shown to influence the aggregation and dissolution of nanoparticle (Adam *et al.*, 2015). Natural organic matter can reduce aggregation of some nanoparticles by adsorption on to their surface (Zhao *et al.*, 2013).

The main aim of this study were (1) to determine acute toxicities of TiO_2, CuO and ZnO nanoparticles in *A. franciscana*, (2) to characterize NPs over time and (3) to define the effects of NPs based on their morphological characteristics.

Materials and methods

Nanoparticle suspensions preparation

Zinc oxide (ZnO), dioxide titanium (TiO_2) and copper oxide (CuO) nanoparticles were purchased from U.S Research Nanomaterial's Inc., Houston, TX, USA. The physical properties of

[1]. Nanoparticles

these nanoparticles are presented in Table 1.

The stock solutions of the NPs were prepared by suspending appropriate amounts of the NP powders in deionized water at a stock concentration of 20% (w/v) separately. To homogenize the suspension, the contents were vortexes for 20 seconds at 2000 rpm and then exposed to ultrasound for 10 minutes for maximum dispersion. Appropriate volumes of the stock suspension were immediately transferred into the exposure containers which contained *Artemia* in seawater (Ates *et al.*, 2013b).

Test organisms

Brine shrimp cysts were purchased from Bandar Imam (Iranian Fishery Research Organization, Bandar Emam) and were certified to be *A. franciscana*. Cysts were stored in the dark until used for testing. Cysts were hatched in seawater (salinity 28 g L^{-1}) at 27ºC under vigorous aeration (Brix *et al.*, 2003b).

Water quality parameters

Water quality parameters (pH, dissolved oxygen, EC, TDS and salinity) were measured in each test chamber and water temperature was recorded in the physical system at the initiation of the test and every day thereafter. Water temperature was measured using a digital thermometer. Test solution pH was measured using a ColeParmer Model 5398-00 digital pH meter. Dissolved oxygen, salinity, EC and TDS was measured using a sensefon378 digital model.

Exposure setup

Acute toxicity was conducted according to the Organization for Economic Cooperation and Development testing guidelines (OECD 202) with 20 organisms in each test (OECD, 2004). A total of 60 *Artemia* larvae were exposed to different concentrations of the NPs (In each beaker we used 20 *Artemia* larvae) for 24h, 48h, 72h and 96h. For ZnO NPs concentrations were 100, 120, 140, 160, 180 and 200 mgL^{-1}, for CuO the concentrations were 1, 3, 5, 7, 9 and 10 mgL^{-1} and for TiO_2 NPs the test concentrations were 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mgL^{-1}. A control group was also set up without the test compound, using only the filtered sea water. Exposures were carried out in triplicate groups in 1.0 L beakers in 500 mL of filtered seawater.

Slight aeration was provided through the bottom of the beaker to prevent settling of NPs from the suspension. For all test groups the light regime of 16:8 h light: dark was maintained. No food was provided during the course of the exposure. Details of the experimental conditions are summarized in Table 2.

Artemia larvae were exposed to each NP solution for 24, 48, 72 and 96 h. At the end of each time healthy *Artemia* larvae were counted and then we could calculate the dead larvae. This step was repeated for 24, 48, 72 and 96 h exposure tests.

Table1 : Size distribution and other characteristics of nanoparticles.

Nano Particles	APS	SSA	Purity	Color
CuO	40 nm	~20 m^2 g^{-1}	99%	Black
ZnO	10-30 nm	20-60m^2 g^{-1}	+99%	Milky white
TiO_2	20 nm	10 - 45 m^2-g^{-1}	+99%	White

SSA: Specific Surface Area, APS: Average Particle Size.

Table 2: Physico-chemical properties of the test.

Characteristics/Parameter	Range	Mean
Room temperature	29 – 31.2 (˚C)	30.0
Water temperature	28 – 28.2 (˚C)	28.1
Dissolved oxygen	6.7 – 6.9 (mg L^{-1})	6.8
TDS	24.8 – 25.8 (g L^{-1})	25.3
Salinity	24.8 – 25.5 (%)	25.1
EC	38800 - 39200 (mho cm^{-1})	39000
pH	7.8 – 8.1	7.9

After the acute toxicity tests, morphological changes and mortality rate of the *Artemia* exposed to NPs were recorded under a phase contrast microscope (Nikon Eclipse 80i). Every exposed group was compared to the control group and the potential anomalies were recorded. Completely motionless *Artemia* were counted as dead, and the percentages of mortality compared to the control were calculated.

The LC_{50} value and the related 95% confidence limits were calculated using the Probit Method (Zhu *et al.*, 2008; Strigul *et al.*, 2009) Significant differences between controls and treated samples were determined using the Bonferroni nonparametric post hoc tests, where $p<0.05$ was considered to be significantly different.

Results

No *Artemia* larvae died during toxicity tests in the control group. During all acute toxicity tests, the measured pH of the test dispersions remained within the range of 7.8 and 8.1 and did not vary by more than 1.0 unit in any given test. The water temperature ranged from 28.0ºC to 28.2ºC during all acute tests. The oxygen content of the test dispersions in all acute toxicity tests was between 6.7– 6.9 mgL^{-1}. Thus, all tests met validity criteria set by the OECD guidelines. The physico-chemical characteristics of the test water are presented in Table 2.

The toxicity of NPs to *Artemia* larvae increased with increasing NP concentration and duration of exposure. The concentrations that killed 50 % (LC_{50}) of *Artemia* larvae varied with the NPs as shown in Table 3. The results of the 69 acute toxicity tests (66 cases and 3 controls) performed with TiO_2, CuO and ZnO, expressed as LC_{10}, LC_{50} and LC_{90} values, are summarized in Tables 4 and 5. Hence, the tests met the biological validity criterion as required in the OECD guideline 202.

Table 3: Mortality (percent) of the *Artemia franciscana* exposed to NPs.

Nano particles	Concentration (mgL^{-1})	Time			
		24 h	48 h	72 h	96 h
ZnO	Control	0.00	0.00	0.00	0.00
	100	0.00	0.00	11.67	15.00
	120	0.00	3.33	18.33	23.33
	140	1.67	16.67	25.00	31.67
	160	5.00	20.00	33.33	43.33
	180	8.33	23.33	43.33	53.33
	200	11.67	26.67	50.00	63.33
CuO	Control	0.00	0.00	0.00	0.00
	1	3.33	8.33	13.33	20.00
	3	8.33	16.67	26.67	33.33
	5	16.67	23.33	38.33	46.67
	7	33.33	26.67	48.33	60.00
	9	41.67	53.33	61.67	70.00
	10	50.00	63.33	71.67	83.33
TiO_2	Control	0.00	0.00	0.00	0.00
	10	1.67	8.33	16.67	28.33
	20	6.67	11.67	23.33	36.67
	30	10.00	18.33	28.33	46.67
	40	13.33	21.67	33.33	51.67
	50	16.67	30.00	40.00	56.67
	60	23.33	38.33	46.67	61.67
	70	28.33	41.67	55.00	68.33
	80	38.33	50.00	61.67	80.00
	90	45.00	53.33	68.33	85.00
	100	46.67	60.00	71.67	91.67

Table 4: Lethal concentrations (LC_{50}) of NPs on *Artemia franciscana*.

Nano particles	Concentrations (mgL^{-1})			
	24 h	48 h	72 h	96 h
CuO	11.42	8.51	6.21	4.32
ZnO	293.10	247.13	201.21	173.20
TiO_2	115.55	86.11	57.31	30.54

Table 5: The toxicity (LC_{10}, LC_{50}, LC_{90} (mgL^{-1}) at 24h, 48h, 72h, 96h) of NPs on *Artemia ranciscana*.

Nano particles	Time	LC_{10}	LC_{50}	LC_{90}
CuO	24h	2.83	11.42	46.15
	48h	1.69	8.51	42.87
	72h	1.01	6.21	37.99
	96h	0.72	4.32	25.85
ZnO	24h	189.19	293.10	454.07
	48h	139.38	247.13	438.16
	72h	96.86	201.21	417.97
	96h	91.05	173.20	329.48
TiO_2	24h	30.27	115.55	448.28
	48h	16.92	86.11	438.20
	72h	9.33	57.31	352.00
	96h	5.48	30.54	170.21

The controls showed no mortality in 24h, 48h, 72h and 96 h. As pointed out above, the exposures were conducted in the absence of feeding. The experimental mortalities for the controls clearly demonstrate that deprivation from food did not induce any lethal effects on *Artemia* larvae even up to 96 h. In the treatments, the mortalities increased with increasing NPs concentration and time ($p<0.05$). In 24 h, the average mortality ranged from 0% (100, 120 mgL^{-1}) to 11.6% (200 mgL^{-1}) for ZnO NPs, from 33% (1 mgL^{-1}) to 50% (10 mgL^{-1}) for CuO NPs. and from 1.6% (10 mgL^{-1}) to 46.6% (100 mgL^{-1}) for TiO_2 NPs. The lethal effects recorded for 96 h exposure were more prominent. The average mortality was about 15% in 120 mg mL^{-1} suspensions of the ZnO NPs and increased to 63.3% in 200 mgL^{-1} suspensions (Fig. 1). For TiO_2 NPs the average mortality was about 28.3% in 10 mg mL^{-1} suspensions and increased to 91.6% in 100 mgL^{-1} suspensions (Fig. 2). Likewise, the CuO NPs was 20% in 1mgL^{-1} suspensions and increased to 83% in 10 mgL^{-1} suspensions (Fig. 3). Our images confirmed the accumulation of the NPs inside the gut and other parts of *Artemia*. (Figs. 4 and 5).

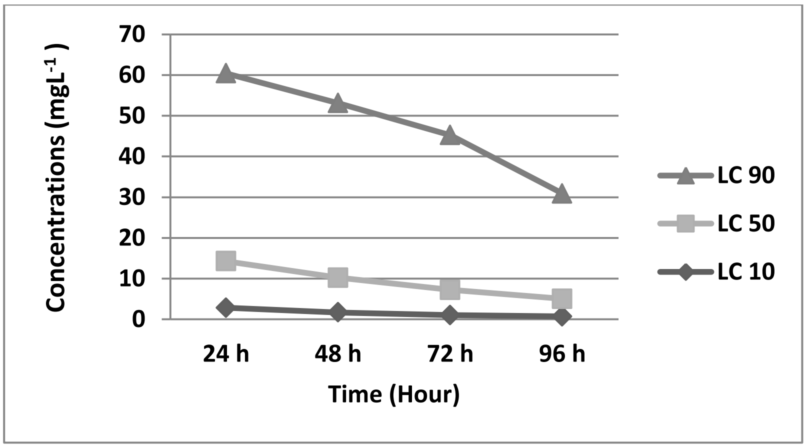

Figure 1: Acute toxicity of ZnO NPs Concentration (mgL^{-1}) in *Artemia franciscana*.

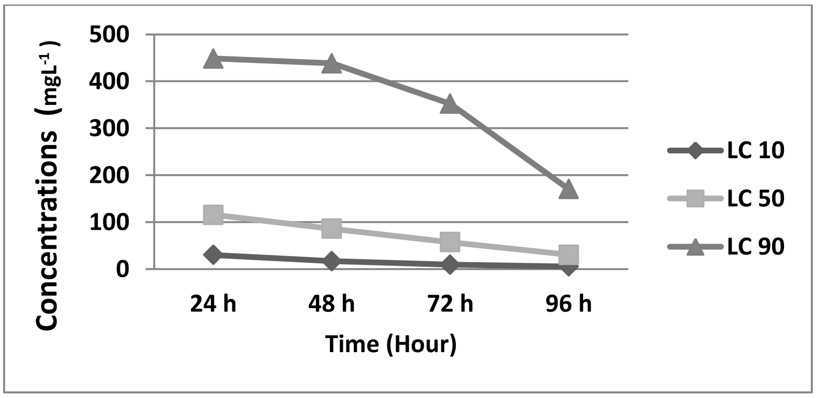

Figure 2: Acute toxicity of TiO_2 NPs Concentration (mgL^{-1}) in *Artemia franciscana*.

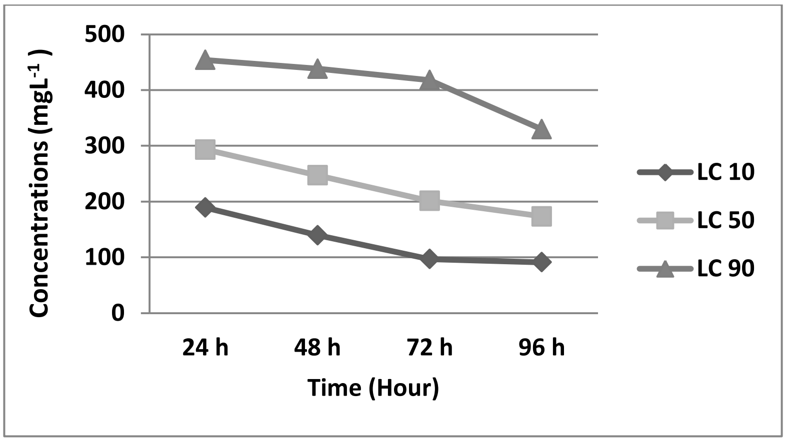

Figure 3: Acute toxicity of CuO NPs Concentration (mgL^{-1}) in *Artemia franciscana*.

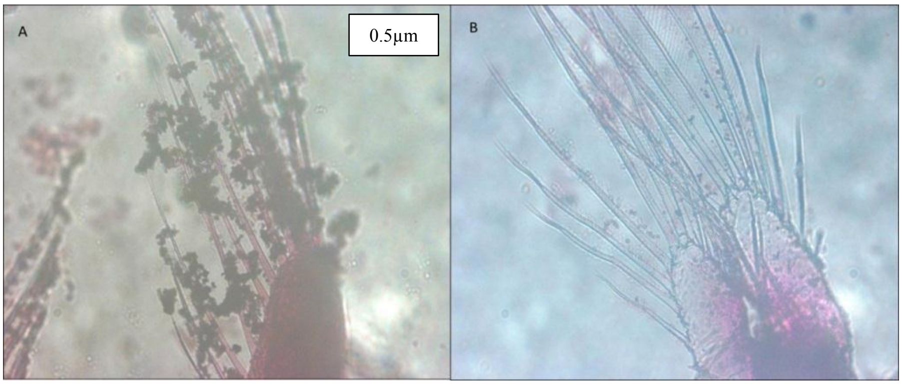

Figure 4: (A) Tail section of the brain shrimp *Artemia fransicana* in control group; (B) NPs stick to tail of brain shrimp *Artemia fransicana* (Scale 1mm: 0.5 µm).

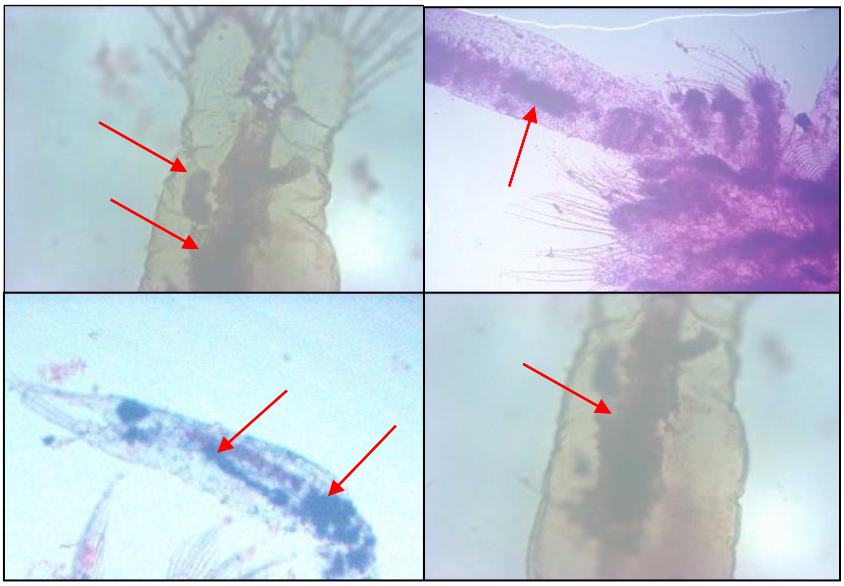

Figure 5: NPs in *Artemia* gut mark with arrows.

Discussion

Our results point to the fact that both TiO_2 and ZnO NPs exhibited moderate toxicity to Artemia larvae in 24h as compared with CuO regardless of their size and concentration.

The toxicity pattern of metal oxides to *Artemia francisscana* was (Cu>TiO_2>ZnO). In the present study CuO NPs was found to have a 24h LC_{50} of 11.42 mgL^{-1}, which was over 26 times higher than that of ZnO NPs and 10 times higher than TiO_2 NPs.

The publications on the marine crustaceans are mostly related to the genus *Artemia*, and more specifically to the anostracan *Artemia salina* (Ates *et al.*, 2013a) and *A. franciscana* (Minetto *et al.*, 2014). However, the overall results are quite representative, because they come from immobilization bioassays, biomarker measures and bioaccumulation evaluation, performed with both adults and nauplii and diversifying the exposure scenario. In both the publications, the authors could verify the toxic effects of the $nTiO_2$ in the overall concentration range of 0.5–100 mg L^{-1} (Ates *et al.*, 2013a; Minetto *et al.*, 2014).

Ozkan *et al*. (2015) assessed the toxicity effects of Tio_2 and $AgTiO_2$ NPs in *A. salina* and studied morphological changes in this species. In this study, aquatic stability and toxic effects of TiO_2 and $AgTiO_2$ nanoparticles (NPs) were investigated in *A. salina* nauplii. *n*$AgTiO_2$ was found to be more toxic to nauplii compared to *n*TiO_2. The mortality rate in nauplii increased significantly with increasing concentrations and duration of exposure. TiO_2 elimination ranged between 27.8 % and 96.5 % at 50 and 1 mgL^{-1} TiO_2 exposure to nauplii, respectively (Ozkan *et al.*, 2015).

Ates *et al*. (2013b), studied comparative evaluation of the impacts of Zn and ZnO nanoparticles on brine shrimp (*A. salina*) larvae and the suspensions of the NPs did not exhibit any significant acute toxicity within 24 h, mortalities increased remarkably in 96 h and escalated with increasing concentration of NP suspension to 42% for Zn NPs (40–60 nm) ($LC_{50\ \sim 100}$ mgL^{-1}) and to about 34% for ZnO NPs (10–30nm) (LC_{50}>100 mgL^{-1}). The suspensions of Zn NPs were more toxic to *Artemia* than those of ZnO NPs under comparable regimes (Ates *et al.*, 2013b).

The LC_{50} 96 h Of TiO_2 in Ozkan *et al.*(2015) research was 18.77 mgL^{-1} (Ozkan *et al.*, 2015). Our results show higher LC_{50} 96 h (30.54 mgL^{-1}), but lower LC_{50} 96 h concentration compared with the results of Ates *et al*. (2013a) who reported LC_{50} 96 h of above 100 mgL^{-1}. The difference in toxicity thresholds may be related to differences in particle size, preparation methods, or test designs and inconsistent test conditions such as pH, photoperiod and dissolved oxygen (DO). *Artemia* were unable to eliminate the ingested particles, which was thought to be due to the formation of massive particles in the guts and other parts of body, and had adverse effects on *Artemia* such as on movement, swimming speed, feeding and etc. Based on the experimental results in this paper, it can be concluded that:

- The NPs used in this research (CuO, ZnO and TiO_2) may have acute dose-dependent eco-toxicological effects on *A. franciscana*.
- NPs with different compositions exhibited different toxicities in *A. franciscana*. The CuO was observed to be most toxic among the tested compounds, while ZnO had least toxicity.
- The studied NPs can be ranked in the following order according to the *A. franciscana* acute toxicity: CuO>TiO_2>ZnO.
- Toxicity of NPs may be the result of the effects from NPs itself, dissolution products, and NPs agglomerates that develop during the experiment.
- The results of this study indicated that the potential eco-toxicity and environmental health effects of NPs should be given due attention.

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