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EFFECTS OF ZnO, CuO, Au, AND TiO_2 NANOPARTICLES ON *DAPHNIA MAGNA* AND EARLY LIFE STAGES OF ZEBRAFISH *DANIO RERIO*

The effects of four different kinds of nanoparticles (NPs), namely, CuO, ZnO, TiO_2, and Au, of the sizes ranging from <20 nm to 50 nm on *Daphnia magna*, early life stage of zebrafish, and various enzymes have been investigated. The experimental results showed that the NPs inhibited both the body length and hatching rate of zebrafish larvae; the small nanoparticles exhibited more toxicity. In a 21 day chronic toxicity test, metal ions of higher concentrations significantly reduced the number of *Daphnia magna* offspring. Studies on enzyme activity showed that the NPs reduced the glutathione content and inhibited catalase and superoxide dismutase activities, resulting in shorter body length, lower hatching success, and lower reproduction of zebrafish larvae. Therefore, studies should focus more on the potential toxicity of smaller NPs.

1. INTRODUCTION

In recent years, nanoparticles (NPs) have been widely used in commercial products, thereby creating concerns on their potential to cause adverse effects on the environment and human health. Among these NPs, SiO_2, Al_2O_3, and TiO_2 are of particular concern. Compared with other NPs, the toxicity of copper oxide (CuO NPs), zinc oxide (ZnO NPs), and Au NPs is still poorly understood [1, 2]. TiO_2 and CuO NPs are more toxic than their bulk and ionic counterparts [3]. ZnO, Al_2O_3, and TiO_2 NPs are toxic to *Caenorhabditis elegans*, especially on its reproductive capability [4]. The LC_{50} of Au NPs in *Daphnia magna* was 70 mg/dm^3, and the toxicity of gold NPs on

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Daphnia at high concentrations may be due to the inhibition of nutrient uptake within the gut [5]. However, limited information exists on Au NPs with cetyltriethyl-ammnonium bromide content of <0.1%. Although evidence exists on the toxicity of CuO, ZnO, TiO_2, and Au NPs, different experimental designs with diverse NP sizes, concentrations, and selected aquatic species make it critical to compare the results. Nanomaterial toxicity effects depend on particle composition, size, and shape. The main mechanism of NP toxicity is via oxidative species (OS), which damages proteins and DNA, as well as the catalytic properties, optical properties, and electrical conductivity of cells [6]. Catalase (CAT), glutathione (GSH), and superoxide dismutase (SOD) have been associated with the defense system to oxidative stress [7].

D. magna and *Danio rerio* are commonly used to assess the potential hazard of chemicals. The current European regulatory guideline requires evaluation of chronic effects of active pharmaceutical ingredients on *D. magna*. Based on the lifecycle of *D. magna*, chronic studies require a 21 day exposure period to establish survival and reproductive endpoints. In contrast to the conventional adult fish toxicity testing, alternative methods using zebrafish early life stages are preferred.

Most NP toxicities have been attributed to dissolved metal ions from the particles; however, the great extent of the effects is mainly derived from the inherent particle properties [8]. Limited information exists on the effect of dissolved metal ions from NPs on the survival and reproduction of *D. magna*. In the present study, the effects of four NPs, namely, ZnO, CuO, TiO_2, and Au, with different physicochemical properties, on crustaceans *D. magna* and early life stages of zebrafish *D. rerio* were studied. The activities of three antioxidant enzymes, namely, CAT, SOD, and GSH, associated with the defense system for oxidative stress were also evaluated for biomarker investigation.

2. EXPERIMENTAL

Characterization of nanoparticles. ZnO, CuO, and TiO_2 were purchased from Nanjing Hatai Nano Material Co., Particle sizes were <20 and 30 nm for nano TiO_2, 30 and 50 nm for ZnO NPs, and 40 nm for CuO NPs. The suspensions of CuO, ZnO, and TiO_2 NPs were dispersed in ultrapure water by probe sonication (sonic dismembrator) for 30 min to form homogeneous suspensions. The suspensions were then stirred for 48 h at 200 r/min. Au NPs, with particle size of 20 nm, were purchased from NanoSeedz Ltd., and then serially diluted in sterile ultrapure water. The Au NP solution was characterized by transmission electron microscopy (TEM) and UV-Vis spectroscopy (HITACHI U-3310, Japan). The particle shape of the nanomaterial was visualized by TEM (TEM, JEOL 100CX, USA) operated at 80 kV.

Metal analysis. Ionic copper (Cu) and zinc (Zn) in the nanomaterial samples were quantitatively determined using an atomic absorption spectrophotometer (PE-AA 800,

USA). The NP suspensions were centrifuged at 10 000*g* for 20 min, and then filtered with 0.22 μm membrane.

Daphnid. The *D. magn*a Straus were originally derived from Belgium. The daphnids were cultured in a glass beaker at the density of 40 adults in 2 dm^3 of Elendt M7 medium (ISO10706,2000) at 20 °C, with a natural photoperiod (16:8 h light/dark). The culture medium was renewed and the offspring was removed two times weekly. Cultured daphnids were fed daily with approximately 0.2 mg carbon of micro-alga *Chlorella vulgaris* (Institute of Hydrobiology, Chinese Academy of Sciences) per daphnid.

Neonates less than 24 h old, derived from the second to fifth brood, were exposed to NP suspensions. A preliminary test and four definite trials were conducted for each nanomaterial. Twenty daphnids per concentration were exposed in each experiment. After 24 h and 48 h of exposure, the immobile daphnids were counted. Immobile daphnids were considered as those that cannot swim within 15 s after gentle agitation. The tested concentrations for each endpoint were 0.5, 1, 2, 4, and 8 mg/dm^3 for ZnO NPs (30 nm) and CuO NPs; 1, 2, 4, 8, and 10 mg/dm^3 for ZnO NPs (50 nm); and 0.05, 0.1, 0.2, 0.4, and 0.8 mg/dm^3 for Au NP (20 nm).

The chronic test was carried out in accordance with OECD guidelines (OECD, 1998) for the 21-d toxicity test. *D. magna* studies were conducted by exposing young female daphnia (< 24 h of age) to the test substance in Elendt M7 over a 21 day period. Ten animals were held individually in glass beakers in each test concentration and in a control series. At the end of the test, the total number of offspring produced at each exposure concentration and the controls was determined. The tested concentrations for each endpoint were 0.1, 0.2, 0.4, 0.6, and 0.8 mg/dm^3 for ZnO NPs (30 nm, 50 nm); 0.2, 0.4, 0.8, 1.0 and 1.6 mg/dm^3 for CuO NPs (40 nm).

Toxicity on the early life stages of zebrafish D. rerio. Adult zebrafish were fed separately at constant temperature (26 °C) and photoperiod (16 h light: 8 h dark). Males and females, in the ratio of 1:1, were placed in a breeding box separately with a plastic partition before the onset of darkness the day before the test. On the following day, mating, spawning, and fertilization took place within 30 min after the plastic partition was removed. The collected eggs were transferred to a binocular using a pipette. Fertilized eggs in the four to eight cell stages were placed in 24-well plates; each well contained 2 cm^3 of test media and 1 embryo. After 96 h of exposure, hatching and malformations of the embryos were evaluated. The tested concentrations for each endpoint were 5, 10, 15, 25, and 25 mg/dm^3 for ZnO NPs (30 nm, 50 nm) and CuO NP; 100, 500, 800, and 1000 mg/dm^3 for TiO_2 NPs (< 20 nm, 30 nm), and 0.25, 0.5, 1, 2, and 4 mg/dm^3 for Au NP (20 nm).

Antioxidant enzymes. CAT, GSH, and SOD were proposed as the markers involved in the antioxidant system of defense in various aquatic species such as bivalves

[9]. SOD, CAT, and GSH kits were purchased from NanJing JianCheng Bioengineering Institute. The juvenoids and zebrafish embryos were homogenized in normal saline to determine activities of the enzymes. The homogenate was centrifuged at 3000*g* for 15 min at 4 °C to precipitate large particles; the supernatants were used directly as enzyme sources. Enzymatic measurements were conducted with a Shimadzu UV-1700 spectrophotometer (Shimadzu, Japan). Hydrogen peroxide enzyme decomposed into hydrogen peroxide and suspended rapidly with the addition of ammonium molybdate. The remaining hydrogen peroxide and ammonium molybdate produced a light yellow complex. CAT activity was determined at 405 nm because of the produced light yellow consumption. GSH activity was determined at 420 nm because the dithio-bisnitrobenzoic acid reacted with the mercapto compound and produced a yellow compound. SOD activity was measured by the inhibition of cytochrome c reduction. The increased absorbance corresponding to the reduction of cytochrome c by O_2^- was measured at 550 nm. The protein concentrations were obtained using the Coomassie brilliant blue method.

Statistical analysis. One-way analysis of variance (ANOVA) with a Mann –Whitney post hoc test were used to test the differences between the control and concentrations of various nanomaterials ($p < 0.05$). The software package SPSS 13.0 was used for statistical analyses (SPSS Inc., Chicago, IL).

3. RESULTS AND DISCUSSION

3.1. TRANSMISSION ELECTRON MICROSCOPY (TEM)

Figure 1 shows the TEM images of ZnO, CuO, Au, and TiO_2 NPs of various sizes. Particle size has important control over other physical and chemical properties such as zeta potential and metal binding [10]. ZnO (30 nm) and TiO_2 (20 nm) NPs were smaller than ZnO (50 nm) and TiO_2 (30 nm) ones which may be the reason why the toxicity of ZnO (30 nm) and TiO_2 (20 nm) was higher than that of ZnO (50 nm) and TiO_2 (30 nm). Figure 2 presents the absorption spectrum of the Au NPs with an absorption peak at 852 nm.

3.2. EFFECTS ON EARLY LIFE STAGES OF ZEBRAFISH D. RERIO

Among the endpoints evaluated in zebrafish exposed to NPs, larval body length proved to be the most sensitive and repeatable biomarker [11]. Spine malformations and pericardial oedema were observed in the early life stages of the zebrafish *D. rerio* (Fig. 3). Such malformations are a common response of zebrafish to NPs. Incardona et al. [12] found that pericardial edema was caused by the inhibition of an essential component of the sarcomere in the cardiomyocytes and the edema was proceeded by

spine deformities, which may be the reason why these two malformations often occur in NPs. Compared with the control, the four tested NPs reduced the larval growth; however, CuO and Au NPs significantly reduced the body length (Fig. 4). These small changes of growth (lower than 5%) are already related physiologically.

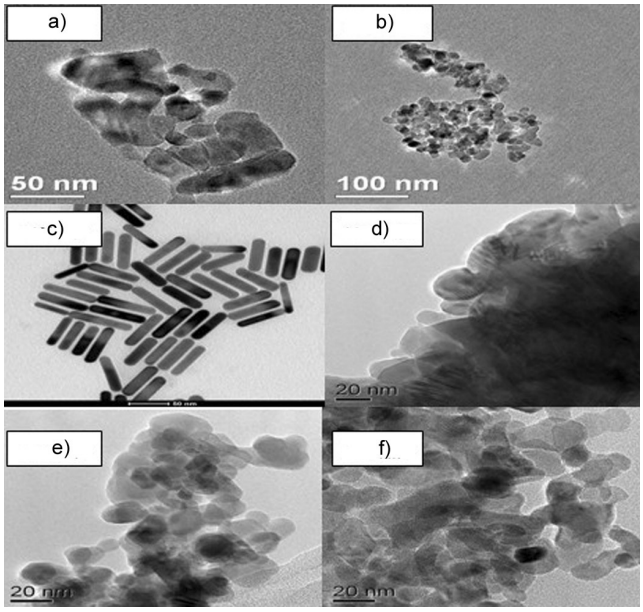

Fig. 1. TEM images of the NPs of various sizes: a) ZnO (30 nm), b) ZnO (50 nm) c) Au (20 nm), d) CuO (50 nm), e) TiO_2 (30 nm), f) TiO_2 (< 20 nm)

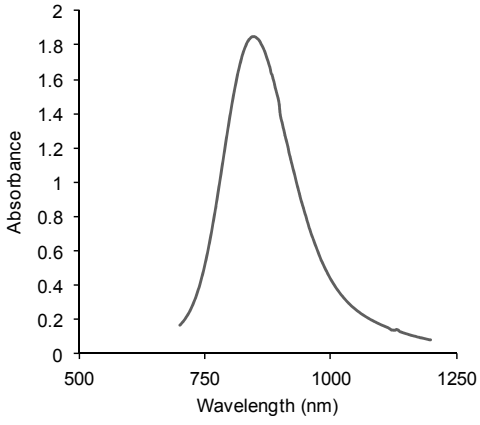

Fig. 2. Absorption spectrum of Au NP

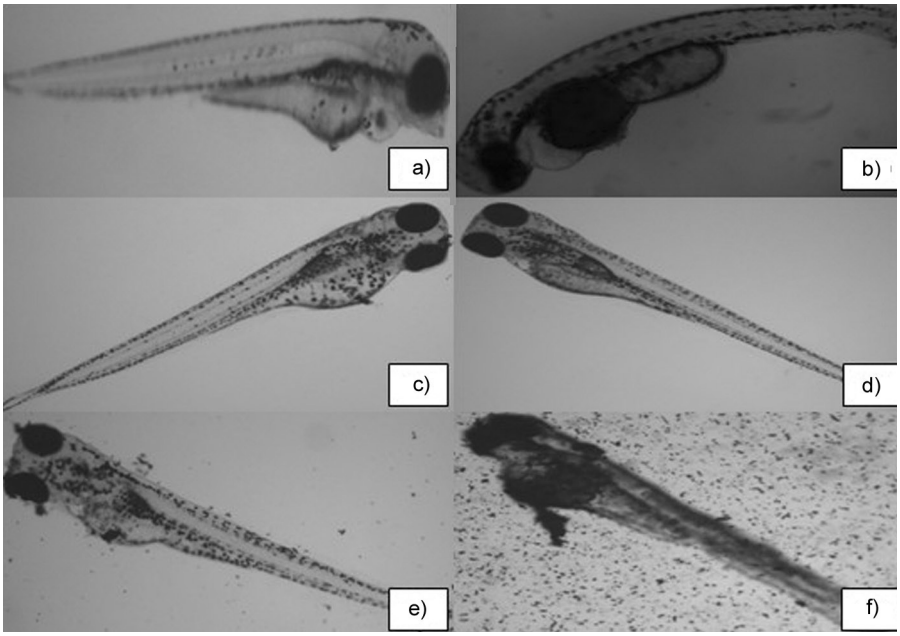

Fig. 3. Various types of larval malformations after 96 h post fertilization:
a) ZnO (30 nm), 10 ppm, b) Au (20 nm), 2 ppm, c) CuO 50 ppm, d) control,
e) TiO_2 (30 nm), 500 ppm, f) TiO_2 (<20 nm), 1000 ppm

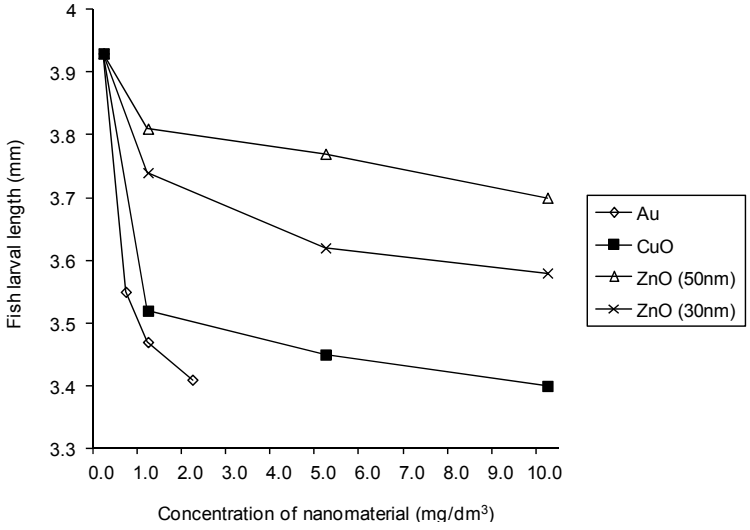

Fig. 4. Fish larval length of the NPs 96 h post fertilization

The effects of the four tested NPs on the larvae were observed after hatching. The hatching success of the zebrafish embryos at 96 h post fertilization is shown in Fig. 4.

All embryos hatched successfully in the control group. The hatching rates of the embryos exposed to ZnO NPs (30 nm, 50 nm) and CuO NPs decreased with the increasing concentrations of 1 mg/dm^3 to 25 mg/dm^3. In addition, no embryo hatched after exposure to ZnO NPs (30 nm, 50 nm) over the concentration range of 50 mg/dm^3 to 100 mg/dm^3. At 1000 mg/dm^3, both TiO_2 NPs at 20 nm and 30 nm reduced the hatching success to 5% and 20%, respectively. At 2.5 mg/dm^3, Au NPs reduced the hatching success to 20% (Fig. 5).

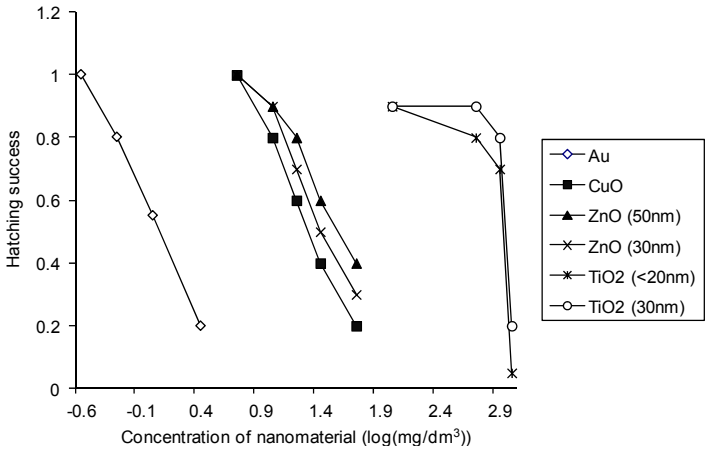

Fig. 5. Hatching success rate of zebrafish (survival of larvae) exposed to the NPs at 4 days post fertilization (mean±SE)

3.3. ACUTE TOXICITY IN D. MAGNA

The median effective concentrations (EC_{50}) and 95% confidence limits of the test substance at 48 hour were calculated using appropriate statistical methods. The calculated EC_{50} of ZnO NPs (30 nm; 50 nm) in daphnids were 1.78 and 6.73 mg/dm^3, that of Au NPs was 0.32 mg/dm^3, and that of CuO was 5.66 mg/dm^3. In contrast to the 50 nm ZnO NPs, 30 nm ZnO NPs provoked significantly lower EC_{50} effects on daphnids because small particles were more likely to enter the lymphatic system or blood system by penetrating through the intestinal barrier, thereby increasing the inflammation [13]. Recently, Jiang et al. reported that the basic cell signaling functions, including cell death, were interrupted by NPs [14]. This observation indicates that studies should focus more on the potential toxicity of NPs with smaller particles.

The effects of the four tested NPs on the reproduction of *D. magna* are shown in Table. 2. All daphnias in the control were alive, and the mean number of live offspring produced per surviving parent animal at the end of the test was 70. One possible factor affecting the reproduction of *D. magna* is the dissolution of metals from the tested NPs [15]. The number of offspring was significantly reduced with the increase in met-

al ion concentration, as shown in Table 2. However, Kim found that TiO_2 NP exposure did not inhibit the propagation of *D. magna* in the chronic bioassay [16].

Table 1

Characteristics of the particles used in the study

NPs	Purity [%]	Diameter [nm]
Nano-ZnO	>99.5	30
Nano-ZnO		50
Nano-CuO		40
Nano-TiO_2		<20
Nano-TiO_2		30
Nano-Au		20

Compared with the control, exposure to 30 nm ZnO NPs at 0.6 mg/dm^3 increased concentration of Zn ions to 367 μg/dm^3, significantly inhibiting the offspring per daphnia. Dead adults were observed at 0.4 and 0.8 mg/dm^3 of ZnO (50 nm) treatments, with the number of 4 and 5, respectively. When the copper ion concentration was increased to 130 μg/dm^3, the number of offspring was significantly reduced to 43.

Table 2

Offspring of *D. magna* exposed to several concentrations of NPs

Concentration of nanomaterial [mg/dm^3]		Concentration of dissolved metal ions [mg/dm^3]	Average number of offspring
Control		0	70
ZnO (30 nm)	0.1	0.064	64
	0.2	0.197	59
	0.4	0.281	50
	0.6	0.367	40
ZnO (50 nm)	0.1	0.142	66
	0.2	0.228	60
	0.4	0.296	53
	0.6	0.342	46
CuO (40 nm)	0.2	0.038	66
	0.4	0.050	59
	0.8	0.088	56
	1.6	0.130	43

3.4. ANTIOXIDANT ENZYME ACTIVITIES

Figure 6a shows that the GSH activity of ZnO NPs at concentration of 25 mg/dm^3 was significantly lower than the enzyme activity at 5 mg/dm^3 and 10 mg/dm^3. The

GSH activity of CuO, Au and TiO_2 decreased with the increase in NP concentration. Figure 6.b showed that the CAT activity of CuO, ZnO, Au and TiO_2 NPs at high concentrations were significantly lower than at low concentration. And the CAT activity

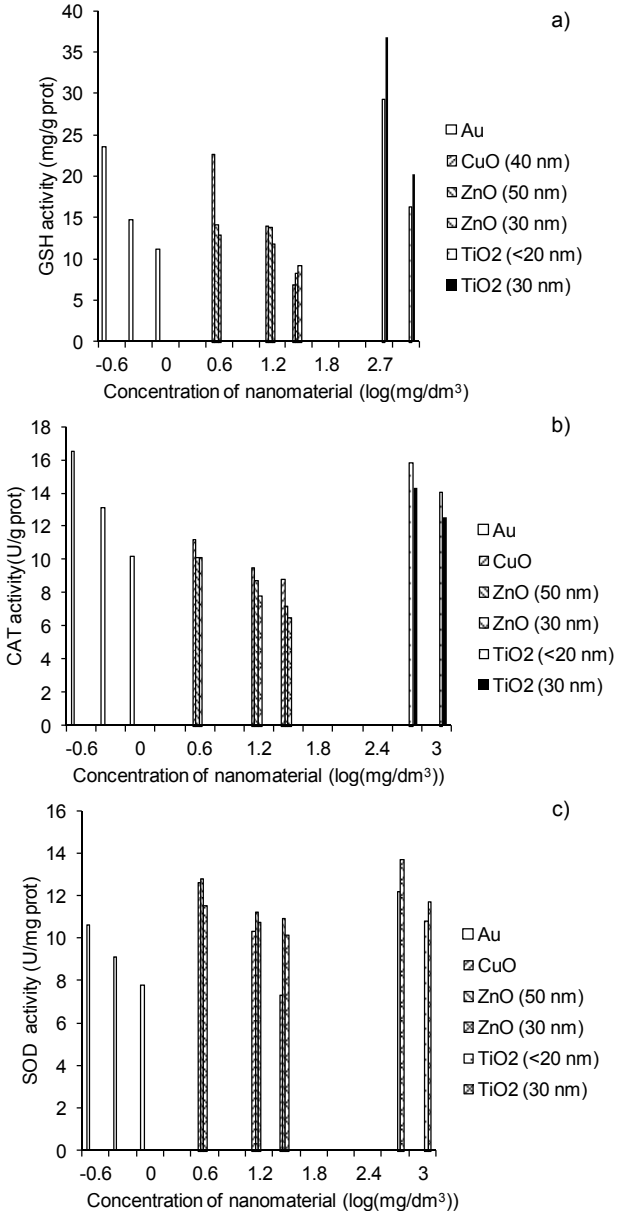

Fig. 6. GSH (a), CAT (b), and SOD (c) activities
of juvenile zebrafish exposed to the NPs after 4 days

of NPs decreased with the increase in NP concentration. Figure 6c shows that the enzyme activities of CuO NPs at concentrations of 15, 25 mg/dm^3 were significantly lower than at 5 mg/dm^3. The SOD activity of CuO, ZnO, Au and TiO_2 decreased with the increase in NP concentration. It might be the continued growth in ROS began to attack protein of SOD enzyme and made it inactivate. It showed that the antioxidant defense system functions began to fail. The induction of SOD, CAT, and GSH activities may be related to the defense mechanisms. The GSH content, as well as the CAT and SOD activities decreased with the increase in NP concentration (Fig. 6). ZnO, CuO, TiO_2, and Au NPs reduced the GSH content and inhibited the CAT and SOD activities, which caused embryo oxidative damage and changes in physiology in the zebrafish, including hatching failure, shorter body length, and lower reproduction. Gomes et al. showed that the digestive gland is susceptible to CuO NPs in relation to oxidative stress, and is also the main tissue for their accumulation [17]. Heinlaan et al. showed that metal oxide particles do not necessarily have to enter the cells to cause toxicity [18]. The intimate contact between the cell (crustacean gut environment) and the particle is more important because this may cause changes in the vicinity of the organism–particle contact area and generate extracellular ROS that may damage cell membranes.

5. CONCLUSIONS

- ZnO, CuO, TiO_2, and Au NPs are toxic to the daphnids of *D. magna* and early life stages of zebrafish. In particular, 30 nm ZnO and 20 nm TiO_2 NPs induced more toxicity than 50 nm ZnO and 30 nm TiO_2 NPs because smaller particles were more likely to enter the cell.
- Reproductive toxicity can be adequately explained by the dissolution of the metal ion. The number of offspring was significantly reduced with the increase in metal ion concentration.
- The NPs reduced the GSH content and inhibited the CAT and SOD activities, causing oxidative damage and physiology changes to zebrafish embryo and *D. magna* daphnids.

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