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Research Article

Enhanced Oxidative Stress and Physiological Damage in *Daphnia magna* by Copper in the Presence of Nano-TiO_2

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This study examines the potential hazard of an individual nanomaterial on the Cu biotoxicity to aquatic organisms. *Daphnia magna* in the absence or presence of nano-TiO_2 was exposed to Cu. Maintaining nano-TiO_2 at a safe concentration cannot eliminate its potential hazard. The biomarkers superoxide dismutase, catalase, and Na^+/K^+-ATPase in *D. magna* were measured. Cu in the presence of nano-TiO_2 induced higher levels of oxidative stress and physiological damage because of the sorption of Cu. Nano-TiO_2 also caused Na^+/K^+-ATPase inhibition possibly by impeding the Na^+/K^+ transfer channel. The correlations among the biomarkers, mortality, and accumulation further showed that the overloading reactive oxygen species generation caused by nano-TiO_2 contributed to deeper oxidative stress and physiological regulation, thereby causing greater toxic injury.

1. Introduction

Nanotechnology has attracted considerable attention in the scientific community since its emergence as a powerful basic and applied science tool [1, 2]. The production of engineered nanomaterials was estimated to reach 2000 tons in 2004 and is expected to increase to 58 000 tons in 2011–2020 [3]. With increasing commercialization of nanoparticles (NPs), concerns about the exposure of humans and the environment to NPs are growing [4]. The unique sizes of NPs result in many special physicochemical properties and may yield extraordinary hazards for human health and the environment [5].

The biological toxicity of NPs is closely related to many physicochemical characteristics such as size, surface area, surface modification, and radical formation. The consideration of these properties in assessing NP toxicity complements the conventional dose- (concentration-) response approach. Nano-TiO_2 is globally important as a sunscreen and pigment, and its physicochemical properties are widely documented [2, 5, 6]. Size is an important factor that determines nano-TiO_2 toxicity because penetration becomes easier with decreasing particle size. Bioavailability toward the sites to be taken up is also increased; thus, more particles can be deposited inside the cell [7]. Nano-TiO_2 particles smaller than 25 nm cause higher algal growth inhibition and greater immobilization of *Daphnia magna* than those bigger than 100 nm [8]. Exposure to 0.22 μm filtered nano-TiO_2 also causes higher mortality in *D. magna* than exposure to unfiltered nano-TiO_2, indicating that toxicity may be directly related to the size of the dispersed NPs [9]. Some ecological studies showed that nano-TiO_2 exposure in aquatic species causes oxidative damage-mediated effects [10, 11]. The exposure of rainbow trout to nano-TiO_2 causes lipid peroxidation, one of the consequences of oxidative stress [10]; changes in antioxidant enzyme activities are also observed in freshwater cladoceran (*Daphnia pulex*) [11]. However, biochemical studies on the effect of nano-TiO_2 on oxidative stress, which has been proposed as an important biochemical biomarker, remain limited to aquatic vertebrate species, and the effect of nano-TiO_2 size fraction has not been investigated. A study on *D. magna* indicated the importance of the colloidal behavior and mode of preparation of nano-TiO_2 to resultant toxicity [12], and the lethal concentration of nano-TiO_2 is only 10 ppm for *D. magna* following 48 h of aqueous exposure [13]. However, little is known about the biological effects under exposure to safer concentrations.

The increasing use of engineered NPs in industrial and household applications may very likely lead to the release of such materials into the environment [5]. At least one study reported enhanced mobility of engineered NPs in simulated groundwater systems [14]. The unique structure and electronic properties of nano-TiO_2 can make it an especially powerful adsorbent [15]. Little is currently known about the fate, transport, and transformation of NPs once they enter the environment [16]. Several studies employing colloidal behavior have investigated the transport of a wide range of engineered NPs through porous media [17]. Colloidal behavior can help predict the behavior of NPs released into the aquatic environment [18]. Zhang et al. [19] reported that nano-TiO_2 can change the uptake of other pollutants and found that carp exposed to cadmium in the presence of nano-TiO_2 accumulated 146% more Cd than controls.

A previous study revealed that the coexistence of NPs with copper ion (Cu^{2+}) enhances the biotoxicity of Cu^{2+} to daphnids even at low concentrations [20]. In the current study, we measured a range of end points, including biochemical measurements related to physiological functions (e.g., Na^+/K^+-ATPase) and oxidative stress (e.g., superoxide dismutase (SOD) and catalase (CAT)). Na^+/K^+-ATPase is a member of the P-type ATPase family of cation transporters [21], which belongs to a superfamily of ubiquitous pumps involved in the transport of charged substrates across biological membranes [10]. Decreased Na^+/K^+-ATPase activity in cells may impede ion transfer across membranes and cause disorder in the metabolism of substances and energy. SOD activity is sensitive to tissue copper (Cu) because the enzyme requires Cu as a catalytic cofactor. Cu deficiency can also decrease the activities of certain non-Cu-containing enzymes of the oxidant defense system, including CAT [22]. The biotoxicity of Cu^{2+} is reportedly correlated with the interactions between its adsorption and coordination with cosubstrates [23]. Consequently, we used Cu to interfere with nano-TiO_2 and assessed the ecological impact of nano-TiO_2 on aquatic organisms. Therefore, the aforementioned biomarkers were used to reveal the potential risk of the combination of nano-TiO_2 with Cu^{2+}.

2. Experimental

2.1. Preparation of Nano-TiO_2 Suspension. Nano-TiO_2 particles (anatase) were provided by Nanjing High Technology Material Co., Ltd. The N_2-BET-specific surface area was measured using a Nova 2200e BET surface area analyzer (Quantachrome, Boynton Beach, FL) at 114.45 $m^2\,g^{-1}$. Under the aforementioned conditions, the particle size was about 13.5 nm. The stock suspension of nano-TiO_2 (20 mg L^{-1}) was prepared based on the procedure described by Lovern and Klaper [9], in which 2 mg of nano-TiO_2 particles was mixed with 100 mL of deionized H_2O and then placed in a bath sonicator for at least 30 min to break the particles into small, noncoagulating particles. The stock solution was stored at room temperature before usage. The image of nano-TiO_2 particles in water and the adsorption of Cu^{2+} onto the nano-TiO_2 were previously studied [20]. Using a capillary tube, droplets of solution were injected onto the Formvar-coated (Electron Microscopy Sciences, Fort Washington, PA, USA) copper grids. The solution was allowed to dry, and the samples were then placed in the TEM for imaging. The image of nano-TiO_2 particles in water was observed by a JEOL transmission electron microscope (TEM) (JEOL, JEM-2100F), operated at 100 kV electron volts. The image result has been shown in Figure 1.

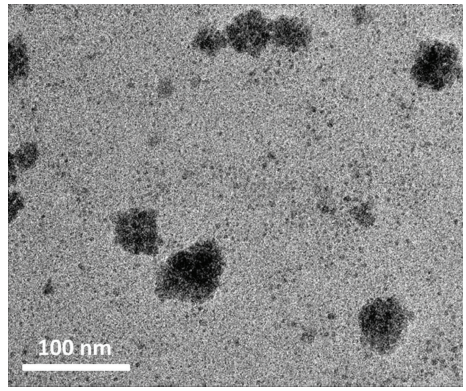

Figure 1: The image of nano-TiO_2 particles distributed in water to form suspensions was observed by TEM.

2.2. Model Organism. Daphnia magna used in this study was cultured at 23°C with a light : dark cycle of 16 : 8 h 2 years after collecting the algae from natural waters near Huo Qi Ying Bridge (116°16′732 E, 39°58′401 N). The green alga *Scenedesmus obliquus* was fed to the daphnids at a concentration of 1×10^5–2×10^5 cells $mL^{-1}\,d^{-1}$. The algae were grown in artificial WC medium [24] and collected by centrifugation at the exponential growth stage. The water used for all exposure experiments was also collected from the Huo Qi Ying Bridge area. The physicochemical parameters of the water were as follows: pH 7.6, Ca^{2+} concentration of 24 mg L^{-1}, total organic carbon concentration of 5.23 mg L^{-1}, and dissolved oxygen concentration of 11.3 mg L^{-1}. The water used in all instances was filtered through a 0.45 μm membrane before use.

2.3. Exposure of D. magna to Cu in the Absence or Presence of Nano-TiO_2. *Daphnia magna* (21–25 d) was exposed to different concentrations of dissolved Cu^{2+} (as copper nitrate) in the absence or presence of nano-TiO_2 particles for 3 d. The concentrations of Cu^{2+} used in the study were 10, 20, 30, 40, 50, 70, and 100 μg L^{-1}. Thirty *D. magna* of the same age in 200 mL to 300 mL of water were used in each exposure treatment, with two replicates for each treatment. *Daphnia magna* was not fed during its exposure period. The numbers of dead individuals were noted each day, and the mortality rate was calculated at the end of the exposure. A control test without Cu^{2+} contaminant was also conducted under the same conditions.

2.4. Determination of SOD, CAT, and Na^+/K^+-ATPase in D. magna. Twenty exposed daphnids were weighed after

removing the water on their body surfaces. Tissues of *D. magna* were homogenized by ultrasonication in 0.5 mL of sucrose buffer (0.25 M sucrose and 0.1 M Tris-HCl, pH 8.6) and centrifuged at 16 000 × g for 20 min. The supernatant fluid was diluted to 1.5 mL using a homogenate, and 1 mL of supernatant fluid was used to determine SOD, CAT, and Na^+/K^+-ATPase using commercially available kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's protocol. SOD activity was based on the inhibition by SOD of NADPH oxidation by molecular oxygen in the presence of EDTA, manganese chloride, and 2-mercaptoethanol [25]. CAT activity was calculated and expressed as nmol H_2O_2 consumed $s^{-1}\,g^{-1}$ protein^{-1} [26]. Na^+/K^+-ATPase was assessed based on the amount of inorganic phosphate liberated from the hydrolysis of the substrate ATP [27].

3. Results and Discussion

3.1. Induction of Oxidative Stress by Cu in the Absence or Presence of Nano-TiO_2. The main potential mechanism of NP toxicity is believed to be via oxidative stress with reactive oxygen species (ROS), which damages lipids, carbohydrates, proteins, and DNA. To interpret the differences between the toxicity of Cu only and Cu^{2+} adsorbed onto nano-TiO_2 (Cu + nano-TiO_2), SOD and CAT activities in *D. magna* were detected during the exposure (Figure 2). Cell toxicity is principally induced by oxidative stress [28]; thus, the SOD and CAT activities in *D. magna* were investigated because they are antioxidant biomarkers for metal pollution. The SOD and CAT activities decreased to different extents. These activities were significantly ($P < 0.01$) induced in the groups with and without nano-TiO_2 compared with the control. Figure 2(a) shows that in the presence of nano-TiO_2, the highest induction (208.2% of the control) was reached at 10 $\mu g\,L^{-1}$, whereas in the absence of nano-TiO_2, the highest induction (203.3% of the control) was at 20 $\mu g\,L^{-1}$. The induction then decreased proportionally with increased Cu^{2+} concentration. The SOD activities had no significant difference between the two groups ($P > 0.05$, one-way ANOVA). The activity of CAT, a part of the SOD-CAT system that defends against oxygen toxicity [29], differed from that of SOD. Figure 2(b) showed that the CAT activities in the Cu^{2+}-exposed *D. magna* were significantly higher than those in the Cu^{2+}/nano-TiO_2-exposed *D. magna* ($P < 0.01$, one-way ANOVA). In the presence of nano-TiO_2, the highest induction (368.9% of the control) was reached at 10 $\mu g\,L^{-1}$, whereas in the absence of nano-TiO_2, the highest induction (504.81% of the control) was at 20 $\mu g\ L^{-1}$. The induction then decreased proportionally with increased Cu^{2+} concentration. The presence of nano-TiO_2 reduced the CAT activity in *D. magna*, and the largest observed drop was 55.7%. The antioxidant enzyme activities increased at the nano-TiO_2 concentration of 5 $mg\,L^{-1}$ [11]. However, at Cu concentrations greater than 40 $\mu g\,L^{-1}$ and at a safe nano-TiO_2 concentration of 2 $mg\,L^{-1}$, the activity of the entire antioxidant-system (SOD + CAT) considerably decreased, indicating decreased antioxidant capacity in *D. magna* and suggesting that Cu + nano-TiO_2 was more dangerous than Cu alone in aquatic environments.

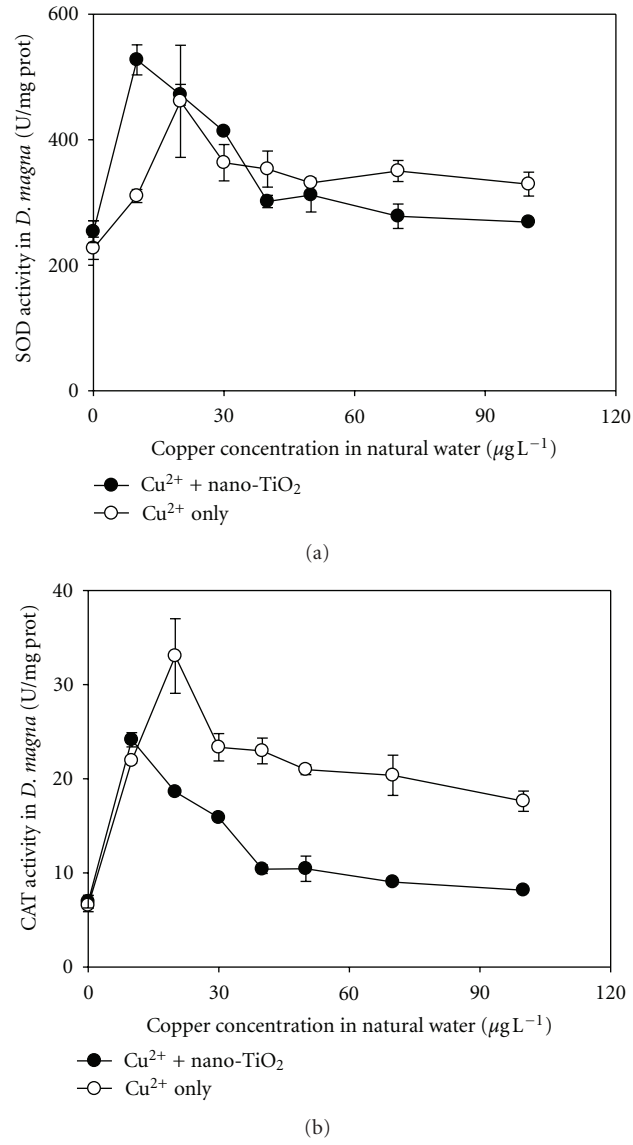

Figure 2: Induction of oxidative stress in *D. magna* after 3 d of exposure to Cu^{2+} at different concentrations in the absence and presence of 2 $mg\,L^{-1}$ nano-TiO_2. Mean ± standard deviation ($n = 2$). (a) SOD: superoxide dismutase. (b) CAT: catalase.

3.2. Inhibition of Na^+/K^+-ATPase by Cu in the Absence or Presence of Nano-TiO_2. Figure 3 shows that compared with the group without nano-TiO_2, the group with nano-TiO_2 exhibited a statistically significant decrease in Na^+/K^+-ATPase activities, with a reduction range between 21.3% and 45.3% ($P < 0.01$, one-way ANOVA). Na^+/K^+-ATPase indicates the ability of ion transfer in the cell membrane channel. Na^+/K^+-ATPase enzyme is present at high concentrations in salt-transporting tissues such as intestines and gills, where it maintains the ionic and electrical gradients necessary for transepithelial salt movements [30]. Santore et al. [31] had

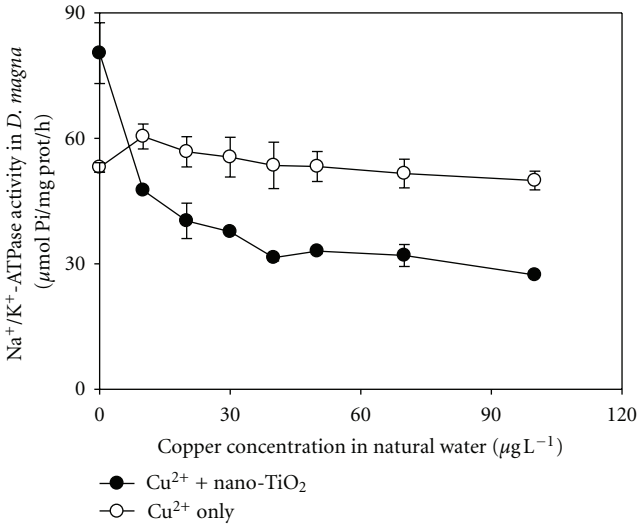

FIGURE 3: Inhibition of Na^+/K^+-ATPase activity in *D. magna* after 3 d of exposure to Cu^{2+} at different concentrations in the absence and presence of 2 mg L^{-1} nano-TiO_2. Mean ± standard deviation ($n = 2$).

proposed that Cu^{2+} accumulation in the gills of freshwater fish inhibits Na^+ influx and reduces Na^+/K^+-ATPase activity. Exposure to Cu^{2+} leads to concentration-related losses of plasma ions [32], particularly sodium and chloride, and damaged gill structure and function [33]. Given that Na^+/K^+-ATPase activity tends to compensate for effects at the gill by showing normal activity when branchial Na^+/K^+-ATPase activity is low, Na^+/K^+-ATPase inhibition did not result in plasma Na^+ or K^+ depletion. Therefore, Cu + nano-TiO_2 has an inhibitive effect on the antioxidant enzyme (Figure 3).

3.3. Mechanism of Enhanced Biotoxicity of Cu^{2+} by Nano-TiO_2. Table 1 shows the accumulated Cu in *D. magna* after 3 d of exposure to Cu^{2+} at different concentrations in the absence or presence of 2 mg L^{-1} nano-TiO_2. The mortality of *D. magna* is shown in Table 2.

In invertebrate species, SOD and CAT are considered to play greater antioxidant roles [34]. Figure 4(a) shows that the SOD activity and accumulated Cu had a definite positive correlation ($P < 0.05$, one-way ANOVA). The relationship between SOD activity and mortality in *D. magna* also had a significant correlation ($P < 0.05$, one-way ANOVA) (Figure 4(b)). In the groups with and without nano-TiO_2, the CAT activity decreased significantly ($P < 0.01$, one-way ANOVA) with increased accumulated Cu (Figure 4(c)). The mortality also decreased significantly ($P < 0.01$, one-way ANOVA) with increased CAT activity (Figure 4(d)). Normally, Cu participates in the formation of ROS. In the presence of superoxide ($O_2^{\bullet -}$), Cu^{2+} can be reduced to Cu^+, which is capable of catalyzing the formation of hydroxyl radical ($OH^\bullet$) from hydrogen peroxide (H_2O_2) [33]. The hydroxyl radical is the most powerful oxidizing radical and is capable of reacting with practically every biological molecule and destroying the antioxidant defense system [35]. The relationship between the oxidative stresses at the organismal (e.g., uptake) and biochemical levels in daphnids indicated that in the SOD-CAT system, free Cu ions and Cu + nano-TiO_2 may produce the same level of $O_2^{\bullet -}$ because the induced SOD and CAT activities had a definite positive correlation with accumulated copper. Therefore, Cu + nano-TiO_2 induced Cu biotoxicity by oxidative stress (Table 2). Cu can generate ROS (e.g., $O_2^{\bullet -}$, H_2O_2, and $OH^\bullet$) only in digestive tissues [36]. Thus, Cu + nano-TiO_2 must enter digestive tissues to generate ROS according to Figure 4. Cu + nano-TiO_2 generated the devastating ROS, which destroyed the antioxidant defense system. Ultimately, the overload of ROS damaged the daphnid as the induced SOD-CAT system capability dropped to a very low level.

To discuss further the mechanism of the enhanced biotoxicity of Cu + nano-TiO_2, the relationship between Cu accumulation and Na^+/K^+-ATPase was analyzed. As shown in Figure 5(a), the presence of nano-TiO_2 led to decreased Na^+/K^+-ATPase level. Cu^{2+} and Cu^{2+}+nano-TiO_2 exposure was differentiated by the inhibition of Na^+/K^+-ATPase in *D. magna*. Therefore, we speculated that the function of this enzyme was inhibited due to the damage caused by Cu [33] and nano-TiO_2 [10]. The reason was that nano-TiO_2 is a powerful adsorbent and may compete with the active binding sites for essential elements of organisms such as K^+ and Ca^{2+}. Therefore, the Na^+/K^+-ATPase activity was affected. Cu^{2+} can cause the inhibition of Na^+/K^+-ATPase enzyme activity by interfering with the binding of Cu^{2+} and protein-thiol. The binding site of Cu^{2+} has special interactions. However, Federici et al. [10] found that Na^+, K^+, and Ca^{2+} in fish tissues were generally unaffected, and exposure to nano-TiO_2 caused a statistically significant decrease in intestinal Na^+/K^+-ATPase activity. Therefore, we speculated that nano-TiO_2 may have impeded the Na^+/K^+ transfer channel due to its small particle size [37], which increased the biotoxicity of physiological effects compared with the system without nano-TiO_2, including the inhibition of ion transfer across the membrane and disturbance of the metabolism of substances and energy. Consequently, the inhibition worsened.

Considering both exposure systems together, the relationship between mortality and Na^+/K^+-ATPase in *D. magna* had no significant correlation (Figure 5(b)). By contrast, considering the two systems independently, the mortality and Na^+/K^+-ATPase activity in each system had a positive correlation, that is, the mortality of *D. magna* decreased significantly with increased Na^+/K^+-ATPase level. The observed levels of Na^+/K^+-ATPase were explained by the toxicity of Cu ions in *D. magna*. The function of Na^+/K^+-ATPase is considered to respond to physiological function in aquatic organisms; thus, the observed decline in Na^+/K^+-ATPase indicated physiological effect inhibition. At the same mortality level, Na^+/K^+-ATPase inhibition by Cu + nano-TiO_2 was lower than that by Cu only. Given that ROS generation was uncontrollable because of the breakdown of antioxidant action, we believed that the protective response was inactivated and overtaken by inflammation and cytotoxicity. Therefore, these defects or aberrations can determine disease susceptibility during the exposure, and worsened Na^+/K^+-ATPase inhibition by Cu + nano-TiO_2. Finally, the high

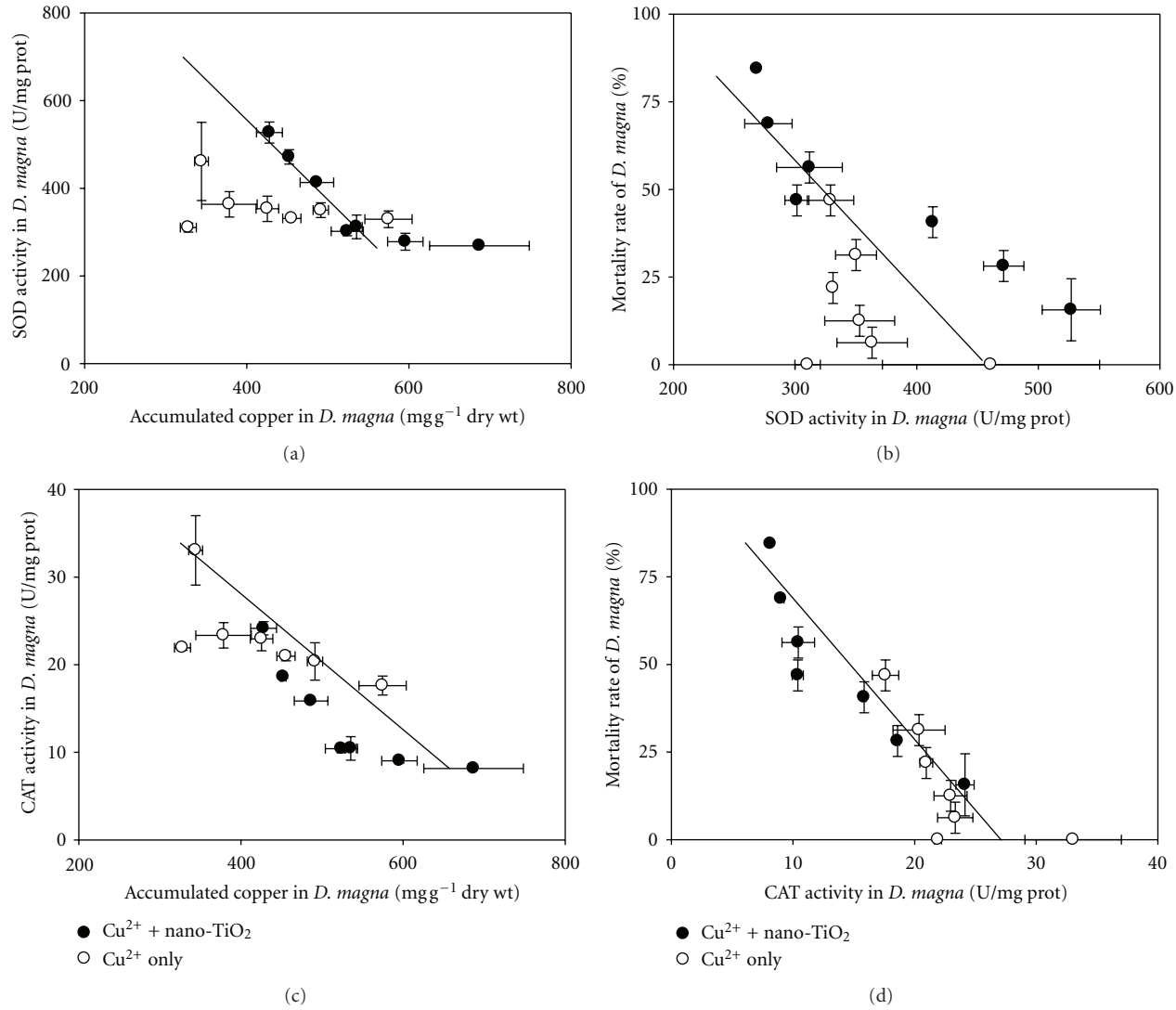

Figure 4: Relationships between (a) SOD activity and accumulated copper, (b) mortality and SOD activity, (c) CAT activity and accumulated copper, and (d) mortality and CAT activity in *D. magna* after 3 d of exposure to Cu^{2+} at different concentrations in the absence and presence of 2 mg L^{-1} nano-TiO_2. Mean ± standard deviation ($n = 2$).

Table 1: Accumulated copper in *D. magna* after 3 d of exposure to Cu^{2+} at different concentrations in the absence and presence of 2 mg L^{-1} nano-TiO_2.

		Exposure groups with copper (μg L^{-1})							
Exposure system		Control	10	20	30	40	50	70	100
Cu	Accumulated copper (μg g^{-1})	229.48 ±35.02	327.94 ±9.94	344.23 ±8.49	378.59 ±34.21	425.38 ±13.95	455.56 ±11.18	491.42 ±9.44	574.84 ±29.16
Cu + nano-TiO_2	Accumulated copper (μg g^{-1})	134.63 ±14.74	428.03 ±15.91	452.32 ±3.53	486.57 ±20.69	523.97 ±19.86	535.44 ±6.96	595.67 ±21.86	687.05 ±61.48

Data are presented as the mean ± standard deviation ($n = 2$).

Table 2: Mortality of *D. magna* after 3 d of exposure to Cu^{2+} at different concentrations in the absence and presence of 2 mg L^{-1} nano-TiO_2.

		Exposure groups with copper (μg L^{-1})							
Exposure system		Control	10	20	30	40	50	70	100
Cu	Mortality rate (%)	0	0	0	6.25	12.5	21.88	31.25	46.88
Cu + nano-TiO_2	Mortality rate (%)	0	15.63	28.13	40.63	46.88	56.25	68.75	84.38

Data are presented as the mean ± standard deviation ($n = 2$).

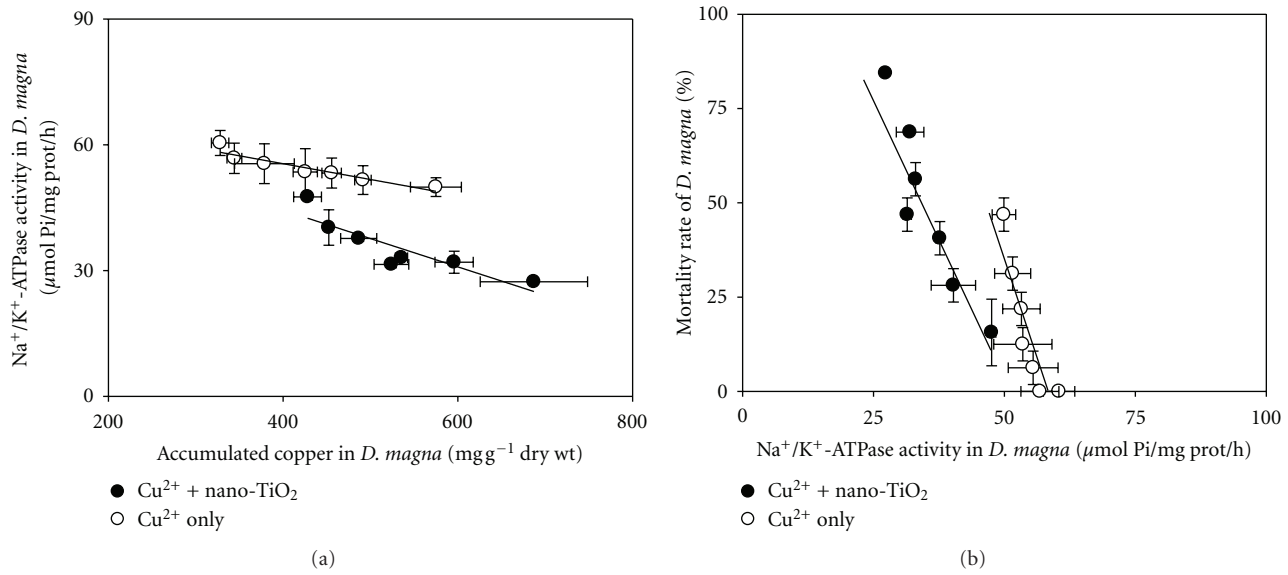

Figure 5: Relationships of Na^+/K^+-ATPase activity with accumulated copper (a) and mortality (b) in *D. magna* after 3 d of exposure to Cu^{2+} at different concentrations in the absence and presence of 2 mg L^{-1} nano-TiO_2. Mean ± standard deviation ($n = 2$).

ROS concentration fatally induced damage to cell structures, lipids, and proteins, and the aquatic organisms died from digesting the mixture of toxicants.

4. Conclusions

Even at low and safe levels, nano-TiO_2 can enhance oxidative stress by ROS generation due to its high adsorbability. Cu + nano-TiO_2 generated ROS and the antioxidant defense system was damaged due to the inhibition by Cu or Cu + nano-TiO_2. Nano-TiO_2 was able to impede the Na^+/K^+ transfer channel because of its particle size, thus causing Na^+/K^+-ATPase inhibition. The increased ROS generation caused by Cu + nano-TiO_2 led to higher toxicity. These ROS led to higher inhibition of Na^+/K^+-ATPase and physiological functions were damaged. These results indicated that the sorption of NPs played an important role in their toxicity to aquatic organisms. Our study provided one of the first detailed overviews on oxidative stress and the physiological effects of Cu + nano-TiO_2 in *D. magna* and further elucidated nanosafety by revealing the correlation among the antioxidation system, Na^+/K^+-ATPase, mortality, and bioaccumulation.

Acknowledgments

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