Effects of the interaction between TiO$_2$ with different percentages of exposed {001} facets and Cu$^{2+}$ on biotoxicity in *Daphnia magna*

Lingling Liu$^1$, Wenhong Fan$^1$, Huiting Lu$^2$ & Wei Xiao$^2$

Anatase TiO$_2$ nanosheets (NSs) with exposed {001} facets have been widely used because of their high activity and particular surface atomic configuration. However, investigations on their biotoxicity are rare. In this study, bioaccumulation of five different TiO$_2$ (with 10%, 61%, 71%, 74% and 78% exposed {001} facets), as well as copper and enzyme activities in *Daphnia magna*, are systematically investigated and rationalized. The results indicated that the addition of Cu$^{2+}$ enhanced agglomeration–sedimentation of TiO$_2$, resulting in the reduction of TiO$_2$ bioaccumulation by 10% to 26%. TiO$_2$ nanoparticles (NPs) increased copper bioaccumulation by 9.8%, whereas the other four TiO$_2$ nanosheets (NSs) decreased it by 43% to 53%, which depended on TiO$_2$ variant adsorption and free Cu$^{2+}$ concentrations in the supernatant. The levels of superoxide dismutase (SOD) enzyme and Na$^+$/K$^+$-ATPase activities suggested that oxidative stress, instead of membrane damage, was the main toxicity in *D. magna*. Meanwhile, the SOD enzyme activities increased with decreasing Cu accumulation and increasing Ti accumulation because of the different functions of Cu and Ti in organisms. This research highlighted the important role of the percentage of exposed {001} facets in nanostructured TiO$_2$ on bioaccumulation and biotoxicity of TiO$_2$ and Cu$^{2+}$ in *Daphnia magna*.

Nanomaterials are widely applied in various fields because of their unique physical and chemical properties. As one of the most commonly used nanomaterials, nano-sized TiO$_2$ are widely used in photocatalysis, cosmetics, paint, medicine, and others. The estimated worldwide productions of nano-sized TiO$_2$ are 2.5 million metric tons per year by 2025, which become a trillion US-dollar business in the future.

The rapid expansion of nano-sized TiO$_2$ increases the risk of aquatic environment exposure, which draws increasing attention. Lovern and Klaper first reported that nano-sized TiO$_2$ was a hazardous material in aquatic organisms and the lethal concentration was only 10 ppm for *Daphnia magna* (D.magna) upon 48 h aqueous exposure. However, the actual concentration of nano-sized TiO$_2$ in natural water is very low (3 ng L$^{-1}$ to 1.6 mg L$^{-1}$), and reaching the lethal concentration is difficult. Due to the special physicochemical characteristics of nano-sized TiO$_2$, the existence of trace nano-sized TiO$_2$ would influence the toxicity of original pollutants in the environment. Nano-sized TiO$_2$ can adsorb other substances in water and influence their biological behaviors and toxicities. The presence of 2 mg L$^{-1}$ nano-sized TiO$_2$ increased the toxicity of the highly toxic marine antifouling compound tributyltin (TBT) up to 20-fold compared with TBT alone in abalone embryos. For heavy metals, it was reported that the presence of TiO$_2$ nanoparticles (NPs) as carriers greatly enhanced the accumulation of Cd and As in carp, and Cu in *D. magna*. On the contrary, Yang’s research showed that nano-sized TiO$_2$ diminished Cd$^{2+}$ bioavailability and toxicity due to Cd$^{2+}$ adsorption by TiO$_2$, which decreased its ambient free ion concentrations.

$^1$Department of Environmental Science and Engineering, School of Chemistry and Environment, Beihang University, Beijing 100191, Beijing, P. R. China. $^2$Department of Environmental Engineering, School of Resource & Environmental Sciences, Wuhan University, Wuhan 430072, Hubei, P. R. China. Correspondence and requests for materials should be addressed to W.F. (email: fanwh@buaa.edu.cn)
The biological toxicity of nano-sized TiO$_2$ is closely related to its physicochemical characteristics, such as size, crystal and surface modifications, and radical formation. It was reported that anatase nano-sized TiO$_2$ was more toxic than rutile, and NPs were more toxic than microparticles for cladocerans, algae, rotifers, and plants. It was reported that biological surface coating of nano-sized TiO$_2$ exerted a negative effect in the molting and development of D. magna. The influence of TiO$_2$ particle size on cadmium toxicity was confirmed, and Cd$^{2+}$ with 30 nm TiO$_2$ NPs presented more serious growth inhibition to algae.

Recent research on nanostructured TiO$_2$ focused on tailoring its shape, size, and exposed facets for enhancing its performance in photocatalysis, solar energy conversion, photochromic devices, and sensors, which was highlighted by the anomalous physicochemical properties of anatase TiO$_2$ nanomaterials enhancing its performance in photocatalysis, solar energy conversion, photochromic devices, and surface of anatase TiO$_2$ is much more reactive than the thermodynamically more stable $\{101\}$ surface for the dissociative adsorption of H$_2$O molecules than the thermodynamically more stable $\{101\}$ facets. The high-energy $\{001\}$ facets of anatase TiO$_2$ have been proved to produce extra-active centers not only for small organic molecules adsorption, but also for the adsorption of heavy metal ions such as Cd$^{2+}$ and Pb$^{2+}$, and their photoactivity exceeds that of P25 by a factor of more than nine times. However, the effects of TiO$_2$ NSs with different exposed $\{001\}$ facets on heavy metal accumulation and toxicity remain unexplored.

In this research, TiO$_2$ NPs and NSs with different percentages of exposed $\{001\}$ facets were prepared and characterized. Bioaccumulation of TiO$_2$ and Cu$^{2+}$ was investigated under different exposure conditions with D. magna as test organism. The changes in metabolic enzymes, such as superoxide dismutase (SOD) and Na$^+$/K$^+$-ATPase, were also discussed. The results of the present study provide a strong evidence for the environmental risks of TiO$_2$ NPs and NSs.

### Results and Discussion

**Characterization of prepared TiO$_2$.** TiO$_2$ samples with different percentages of exposed $\{001\}$ facets were synthesized by changing $R_F$ and their physical properties are shown in Table 1. The percentage of the exposed $\{001\}$ facet of TiO$_2$ was calculated using the reported method according to crystal structure. All the prepared TiO$_2$ was anatase phase, according to X-ray diffraction results (not shown here). The BET surface areas of these TiO$_2$ samples decreased from 156 m$^2$/g to 97 m$^2$/g with increasing $\{001\}$ facet percentage from 0% to 78%. At the same time, the porosity of TiO$_2$ increased from 55.0% to 67.5%, the pore volumes increased from 0.33 cm$^3$/g to 0.56 cm$^3$/g, and the average pore size from 1.4 nm to 2.0 nm. The existing nanopores (or porosity) were from the aggregation of TiO$_2$ NPs and NSs. TiO$_2$ NPs (NP10) and NSs (NS78) were characterized by TEM, as shown in Fig. 1. The morphology of TiO$_2$ NSs with 78% $\{001\}$ facets (Fig. 1b) was different from that of TiO$_2$ NPs with 10% $\{001\}$ facets (Fig. 1a). According to Table 1, the shape of TiO$_2$ changed from NPs to NSs with increasing $R_F$. TiO$_2$ with 61%, 71%, 74%, and 78% $\{001\}$ facets were faceted nanosheets, whereas those with 10% $\{001\}$ facets were nanoparticles. The morphologies of TiO$_2$ NS61, NS71, and NS74 were similar to that of NS78 (TEM pictures not shown).

### Adsorption of Cu$^{2+}$ on TiO$_2$. The adsorption of Cu$^{2+}$ on TiO$_2$ was evaluated from the decrease of Cu$^{2+}$ concentrations in the supernatant. Figure 2 shows rapid adsorption of Cu$^{2+}$ on TiO$_2$ with the adsorption equilibrium reaching within the first 60 min. The decrease on Cu$^{2+}$ concentrations ranges from 50% to 70%, dependent on the percentage of exposed $\{001\}$ facets in the samples. The TiO$_2$ NSs with higher exposed $\{001\}$ facets could adsorb more Cu$^{2+}$ in water than the TiO$_2$ NPs. This finding is related to the surface properties of $\{001\}$ facets. The high-energy $\{001\}$ facets of anatase TiO$_2$ have more surface defects such as unsaturated Ti atoms and abundant oxygen holes that are more effective for the dissociative adsorption of H$_2$O molecules than the thermodynamically more stable $\{101\}$ facets. As a result, a large number of OH groups are generated on $\{001\}$ facets. Adsorbates tend to be adsorbed at steps, defects, and domain boundaries because the surface atoms at these sites have fewer coordination numbers. Consequently, the neutral and unoccupied surface sites of TiO$_2$ with $\{001\}$ facet are Ti-(OH)(OH$_2$) in water. (These surface OH groups participate in.) These surface OH groups have been proved to produce extra-active centers not only for small organic molecules adsorption, but also

| No.  | $R_F$ | Percentage of $\{001\}$ | Phase | CS (nm) | $S_{BET}$ (m$^2$/g) | APS (nm) | PV (cm$^3$/g) | Porosity (%) |
|------|-------|------------------------|-------|---------|---------------------|---------|--------------|--------------|
| NP10 | 0     | 10                     | A     | 8.9     | 156                 | 7.4     | 0.33         | 55.0         |
| NS61 | 0.67  | 61                     | A     | 12.5    | 128                 | 8.8     | 0.35         | 56.5         |
| NS71 | 1     | 71                     | A     | 13.6    | 114                 | 16.0    | 0.52         | 65.8         |
| NS74 | 1.33  | 74                     | A     | 15.1    | 108                 | 19.0    | 0.53         | 66.3         |
| NS78 | 2.67  | 78                     | A     | 17.9    | 97                  | 20.0    | 0.56         | 67.5         |

Table 1. Effects of $R_F$ on physical properties of TiO$_2$. A, CS, APS, and PV represent anatase, crystalline size, average pore size and pore volume, respectively.
metal ion adsorption on TiO$_2$.

The exchange reaction of the metal cations with the -OH on the surface was presumed as presented in the following equation: TiOH$^3+$ + Cu$^{2+}$ → TiOCu$^{4+}$ + H$^+$. Therefore, the adsorption capacity of Cu$^{2+}$ on TiO$_2$ depends on the amount of OH groups on the TiO$_2$ {001} surface. Hence, it is obvious that TiO$_2$ NSs with 78% exposed {001} facets could adsorb the most Cu$^{2+}$ in water.

**Accumulation of TiO$_2$ in *D. magna***. Ti accumulation in *D. magna* was determined after exposure to different TiO$_2$ samples at the 1 mg L$^{-1}$ concentration with and without Cu$^{2+}$. As shown in Fig. 3, Ti accumulation in *D. magna* in the presence of Cu$^{2+}$ was lower than that without Cu$^{2+}$, suggesting that Cu$^{2+}$ inhibited the ingestion of TiO$_2$ by *D. magna*. When TiO$_2$ and Cu$^{2+}$ coexisted, Ti accumulation in *D. magna* exposed to the NP10 sample decreased by 26.4%. However, Ti accumulation in *D. magna* exposed to the other four NS samples (NS61, NS71, NS74, and NS78) decreased by 10%. Moreover, the accumulated Ti in *D. magna* increased slightly with increasing percentage of {001} facet of TiO$_2$ NSs from 3692 μg g$^{-1}$ (in NS 61) to 5088 μg g$^{-1}$ (in NS 78) dry weight in the absence of Cu$^{2+}$, except for the NS71 sample. Thus, the coexistence of Cu$^{2+}$ and TiO$_2$ NSs has a negative effect on bioaccumulation of TiO$_2$ in organisms.

Free nanomaterials tend to aggregate in aquatic environments because of their large specific surface area. In addition, the less-mobile aggregated nanomaterials can easily combine with filter feeders and sediment-dwelling animals. The aggregation is influenced by factors such as primary size, pH and ionic strength in aquatic environment. According to the results of dynamic light scattering, these nano-sized TiO$_2$ aggregated in water were from 1.363 μm to 1.572 μm in size in water with the absence of Cu$^{2+}$, which further grew to about 2.1 μm in size when Cu$^{2+}$ was adsorbed onto the TiO$_2$ surface. The addition of Cu$^{2+}$ facilitates the aggregation of TiO$_2$, in agreement of the reported increases the aggregation level when BPA was added into nano-TiO$_2$ dispersions. Dudev also demonstrated that the
The hydrodynamic diameter of anatase TiO₂ nanoparticles (ANTNPs) increased in the presence of Ca²⁺, resulting in the aggregation of ANTNPs²⁶. When the aggregated particle size exceeds a certain limit, the settlement behavior would become the key factor. The agglomeration–sedimentation processes resulted in the decreased concentrations of the NPs in the supernatant and then diminished the bioavailability of NPs²⁷. The aggregation of nano-sized TiO₂ has an important function in the environmental effects of NPs because the size and shape of NPs will determine the magnitude of any potentially toxic effect. In this experiment, Cu²⁺ enhanced the aggregation of TiO₂ and formed the bigger aggregate in water, retarding the effective uptake of these particles by D. magna. Therefore, the existence of Cu²⁺ predictably weakened the bioaccumulation of nano-TiO₂.

Accumulation of copper in D. magna. Cu accumulation in D. magna at different exposure conditions was investigated in this study, as shown in Fig. 4a. Compared with the control experimental run (treated only with Cu²⁺), the existence of TiO₂ also influenced the bioaccumulation of Cu²⁺ in D. magna. When D. magna was exposed to water with a mixture of Cu²⁺ and NP10, Cu²⁺ accumulation was enhanced by 9.8%. However, Cu²⁺ accumulation in D. magna was reduced by 43% to 53% when Cu²⁺ coexisted with TiO₂ NSs. Generally, the forms of Cu²⁺ ingested by D. magna were free Cu²⁺ and adsorbed Cu²⁺ on TiO₂. When copper coexisted with TiO₂, TiO₂ could adsorb Cu²⁺. Thus its free ion concentration decreased in the ambient environment, which diminished a portion of Cu²⁺ internalization and bioavailability. In contrast, Cu²⁺ accumulation was enhanced when D. magna swallowed Cu²⁺-adsorbed TiO₂. The factor that dominates in Cu accumulation depends on the unique physicochemical characteristics of TiO₂ and exposure condition.

The observed increase in copper accumulation with the presence of TiO₂ NP10 is similar to previous report regarding the P25²⁸. The explanation for reduced Cu accumulation with the presence of TiO₂ NSs is as follows. Firstly, the Cu²⁺-adsorption capacities of TiO₂ NSs were larger than those of TiO₂ NPs, leading to the decrease in Cu²⁺ concentration. Yang studied Cd²⁺ toxicity caused by TiO₂ NPs, and the results are similar to those of the present study. They suggested that nano-sized TiO₂ could reduce free Cd²⁺ concentration in the media, which further lowers its bioavailability and toxicity to green alga Chlamydomonas reinhardtii²⁹. As shown in Fig. 4b, a relative positive relationship exists between Cu accumulation in D. magna and Cu ion concentration in the media. The decrease of free Cu ion concentration was the main factor for the decrease of copper accumulation. Secondly, when Cu²⁺ coexisted with nano-sized TiO₂, nano-sized TiO₂ adsorbed Cu²⁺ and formed big aggregates in water. The large agglomeration–sedimentation of nano-sized TiO₂ reduced Ti accumulation in D. magna and weakened the role of Cu as carrier. Thirdly, TiO₂ NSs themselves may be toxic because of their insolubility in the gut and could alter Cu²⁺ toxicity in an antagonistic, synergistic, or additive way.

SOD enzyme and Na+/K⁺-ATPase activity in D. magna. The SOD enzyme activities in D. magna were investigated because they are antioxidant biomarkers for oxidative stress. As shown in Fig. 5a, when D. magna was exposed only to different nano-sized TiO₂, the SOD enzyme activity decreased from 55.5% to 86.6% compared with the control experiment. SOD enzyme activities increased with increasing percentage of [001] facet of TiO₂ NSs, although the NS78 sample had the largest Ti accumulation. When D. magna was exposed to different TiO₂ and Cu²⁺, SOD activity decreased by 31.0% to 64.7% compared with the control experiment (only Cu²⁺). The decrease in SOD activities indicated that both TiO₂ and Cu²⁺ induced a certain degree of oxidative stress and SOD enzyme inactivation³⁰. The nanotoxicity...
theories were generated by the reactive oxygen species (ROS) and oxidative stress effects. Nanoparticle stress resulting in ROS generation has already been reported by the Dalai group and could be related to TiO₂ NP cytotoxicity potential. When *D. magna* was exposed to two foreign materials, SOD activities in the organisms were further deactivated. In addition, SOD activities in the exposed group were evidently lower than Cu²⁺ only, implying that Cu and nano-sized TiO₂ together are more dangerous than Cu alone in aquatic environments.

Na⁺/K⁺-ATPase indicates the ability of ion transfer in the cell membrane channel. Figure 5b shows the activities of Na⁺/K⁺-ATPase enzyme in *D. magna* under different exposure conditions. Compared with the control group, the Na⁺/K⁺-ATP activities exhibited no significant difference after being exposed only to different TiO₂. The result is similar to that in C.S. Ramsden’s report, demonstrating that no changes in Na⁺/K⁺-ATPase activity were observed in the brain, gill, or liver tissues of the zebrafish after exposure to TiO₂ NPs or bulk. 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. No significant changes in K⁺, Na⁺ and Ca²⁺ concentration were observed in exposure TiO₂-only conditions, which resulted in the absence of any treatment-related change in Na⁺/K⁺-ATPase activities. When TiO₂ co-existed with Cu²⁺, the Na⁺/K⁺-ATPase activities in *D. magna* were slightly lower than the treatment with TiO₂ only. The addition of Cu²⁺ changed the ionic strength of the solution, and Cu²⁺ accumulation in the body inhibited Na⁺ influx and reduced Na⁺/K⁺-ATPase activity in organisms. These results suggest that membrane damage is not the main toxicity to *D. magna* under this research.

**Mechanism of TiO₂ NSs effects on Cu²⁺ biotoxicity.** In the coexistence system, Cu²⁺ affected the stabilities of TiO₂ NS suspensions and their ingestion by organisms. The addition of TiO₂ NSs changed...
Cu²⁺ uptake and biotoxicity in *D. magna*. As suggested above, the oxidative stress damage instead of membrane damage is the major toxicity. To further investigate the main mechanisms of oxidative stress toxicity, the relationship between superoxide dismutase (SOD) activity and Cu/Ti accumulation was considered when *D. magna* was exposed to Cu²⁺ and different TiO₂ samples. According to Fig. 6, a linear relationship between SOD activity and Cu/Ti accumulation in *D. magna* (*P* < 0.01, one-way ANOVA) appears. SOD activities decreased with increasing copper accumulation and decreasing Ti accumulation in *D. magna*. These results are related to the physiological effect of Cu and Ti on organisms. Generally, Cu²⁺ is a hazardous substance to *D. magna* and could produce strong oxidative damage. Cu²⁺-induced cellular toxicity can be explained by the participation of Cu²⁺ in the formation of ROS. Cu²⁺ can be reduced to Cu⁺ in the presence of superoxide (O₂⁻•), and Cu⁺ is capable of catalyzing the formation of hydroxyl radical (OH•) from hydrogen peroxide (H₂O₂)³⁵. OH• is a strong oxidizing radical that can practically react with every biological molecule and destroy the antioxidant defense system. SOD enzyme activities in *D. magna* were deactivated with the accumulation of Cu. On the contrary, Ti is the ninth most abundant element in the earth’s crust, and has a certain stimulating and promoting effect on the growth of plants³⁶. Its beneficial effects on plants have been known since the 1930s³⁷. One mechanism of Ti action is that Ti⁴⁺/Ti³⁺ participates in the metabolism reaction involved in electron transfer in the redox system³⁸. Ti species also involve in activating enzyme activities, such as peroxidase, catalase and nitrate reductase activities in plant tissues³⁹. For these reasons, Ti in daphnids possibly maintains higher SOD enzyme activities to help in the scavenging of generated ROS. However, this supposition needs further studies.

In summary, it was found that bioaccumulation and biotoxicity of nanostructured TiO₂ in *D. magna* was dependent on the percentage of exposed {001} facets. With the co-existence of nanostructured TiO₂ and Cu²⁺, the percentage of exposed {001} facets influenced on the interaction between TiO₂ and Cu²⁺ and therefore played an important role on Cu²⁺ bioaccumulation and biotoxicity in *D. magna*. Firstly, Ti bioaccumulation in *D. magna* increased slightly with increasing percentage of {001} facets, and the addition of Cu²⁺ reduced Ti bioaccumulation in organisms due to the aggregation of TiO₂ induced by adsorbed Cu²⁺. Secondly, TiO₂ NPs enhanced copper accumulation, whereas the other four TiO₂ NSs...
reduced it. Such difference is probably relevant to the different Cu\(^{2+}\) adsorption capacities of TiO\(_2\) with different percentage of exposed \{001\} facets. Thirdly, five types of TiO\(_2\) and Cu ingested by \textit{D. magna} produced relatively strong oxidative stress and inhibited SOD enzyme activity, but the membrane damage was not the main toxicity. Moreover, the SOD activities decreased with increasing copper accumulation because of its oxidative toxicity, whereas SOD increased with increasing Ti accumulation in \textit{D. magna} probably because of Ti's positive physiological effect. In sum, it was confirmed that the co-existence of copper and TiO\(_2\) is more dangerous than copper alone in aquatic environments. The mechanism of TiO\(_2\) NSs on copper biotoxicity requires further exploration.

**Methods**

**Preparation of TiO\(_2\) NSs and NPs.** TiO\(_2\) NSs samples were prepared through solvothermal method using Ti(O\(_x\)C\(_4\)H\(_{2}\)\(_x\))\(_4\) and HF solution as precursors\(^{14,15}\). Briefly, 25 mL of Ti(O\(_x\)C\(_4\)H\(_{2}\)\(_x\))\(_4\) and 3 mL of HF solution (with a concentration of 40 wt.%) were mixed in a dried 100 mL Teflon-lined autoclave, then heated and kept at 180°C for 24 h. The nominal atomic ratio of F to Ti (\(R_F\)) was 1. After the solvothermal reaction, the white precipitates were collected after thorough rinse in ethanol and distilled water thrice, and drying in an oven at 80°C for 6 h. Four TiO\(_2\) NSs samples with different percentages of exposed \{001\} facets were prepared by changing \(R_F\) (0.67, 1.00, 1.33 and 2.67). Based on the geometric configurations derived from TEM images, the four prepared TiO\(_2\) NSs samples appear 61%, 71%, 74%, and 78% exposed \{001\} facets, which were labeled as NS61, NS71, NS74, and NS78, respectively. TiO\(_2\) NPs with 10% exposed \{001\} facets were hydrothermally prepared in pure water without HF and labeled as NP10. The preparation details of TiO\(_2\) NSs and NPs are summarized in Table 1. Finally, TiO\(_2\) stock suspensions (1 g L\(^{-1}\)) were prepared by dispersing TiO\(_2\) NSs or NPs in Milli-Q water using ultrasonic treatment for 30 min (50 W L\(^{-1}\), 40 kHz). The stock solution was stored at room temperature before utilization.
Characterization. Transmission electron microscopy (TEM) analysis was conducted using a JEM-2100F electron microscope (JEOL, Japan) with an accelerating of 200 kV voltage. X-ray diffraction (XRD) (type HZG41B-PC) was used to characterize the crystalline phase and crystallite size of the TiO\textsubscript{2} samples. Brunauer–Emmett–Teller (BET) specific surface area (S_{BET}) of the powders was analyzed via nitrogen adsorption in a Micromeritics ASAP 2020 nitrogen adsorption apparatus (USA). All the as-prepared samples were degassed at 180°C prior to nitrogen adsorption measurements. The BET surface area was determined by a multipoint BET method using adsorption data in the relative pressure (P/P\textsubscript{0}) range of 0.05 to 0.3. A desorption isotherm was used to determine the pore size distribution via the Barrett–Joyner–Halenda (BJH) method, assuming a cylindrical pore modal.

Adsorption of Cu\textsuperscript{2+} on TiO\textsubscript{2}. To study the sorption of Cu\textsuperscript{2+} on nano-sized TiO\textsubscript{2} with different percentages of \{001\} facets, 1 mg/L nano-sized TiO\textsubscript{2} suspensions were prepared using the TiO\textsubscript{2} stocks in SM7 medium respectively. Add Cu\textsuperscript{2+} solution with a known concentration into TiO\textsubscript{2} suspension and mix rapidly. Tow replicates were set for each treatment. At 5, 30, 60, 240 and 360 min, 5 mL of the mixture was drawn out. The samples were then centrifuged for 5 min at 12,000 rpm using a versatile compact centrifuge (Himac CF 16RX, Hitachi, Tokyo, Japan) to separate particles from the solution. Cu\textsuperscript{2+} concentration in the supernatant was determined through ICP-MS (VG PQ2 TURBO). The adsorption amount of Cu\textsuperscript{2+} on TiO\textsubscript{2} was determined by calculating the mass difference between before and after adsorptions.

Model organisms. The D. magna used in this study was kept in the laboratory for two years, and were cultured at 23°C with a light:dark cycle of 16:8 h. The daphnids were cultured in natural water collected from Huo Qi Ying Bridge (116°16'732 E, 39°58'401 N). The water used was filtered through a 1.2 μm membrane before use. The green alga Chlamydomonas reinhardtii was fed to D. magna at a density of 1 × 10\textsuperscript{5} to 2 × 10\textsuperscript{5} cells mL\textsuperscript{-1} per day, and the water was replenished every two days. The alga was grown in artificial WC medium\textsuperscript{36} and was collected at its exponential growth stage by centrifugation.

Acute exposure of D. magna to TiO\textsubscript{2} with or without Cu\textsuperscript{2+}. The water used for the exposure experiments was synthetic water, which was simplified Elendt M7 medium (SM7, containing only CaCl\textsubscript{2}, MgSO\textsubscript{4}, K\textsubscript{2}HPO\textsubscript{4}, KH\textsubscript{2}PO\textsubscript{4}, NaHCO\textsubscript{3}, NaNO\textsubscript{3}, Na\textsubscript{2}SiO\textsubscript{3}, H\textsubscript{3}BO\textsubscript{3}, and KCl and without disodium ethylenediaminetetraacetic acid, trace metals, or vitamins). The stocks of five different TiO\textsubscript{2} were added to 25, 105, and 250 mL SM7, with TiO\textsubscript{2} concentration fixed at 1 mg/L. Two groups of samples were set: one contained TiO\textsubscript{2} and another contained TiO\textsubscript{2} and Cu\textsuperscript{2+} fixed at 50 μg/L. The control group comprised 500 mL SM7 and Cu\textsuperscript{2+}. Each treatment had three replicates, which contained 50 14-day old D. magna (1 individual/10 mL). The D. magna were not fed during the exposure time. All the samples were treated under the same conditions. All glassware and exposure chambers were previously acid washed and thoroughly rinsed with distilled water.

Determination of Ti and Cu bioaccumulation in D. magna. At the end of exposure, ten D. magna were taken out and rinsed with pure water for three times. They were then placed in a drying oven at 80°C. These dried D. magna were digested in 68% HNO\textsubscript{3} (Aristar grade) and (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}-H\textsubscript{2}SO\textsubscript{4} (98%, Aristar grade) solution at 110°C\textsuperscript{41}. The digestion solution was transferred into a volumetric flask with 2% HNO\textsubscript{3} and diluted for Ti and Cu analysis through ICP-MS. TiO\textsubscript{2} and Cu accumulation was calculated based on the dry weight of D. magna (μg/g dry wt).

Determination of SOD and Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activities in D. magna. The other twenty exposed D. magna were weighed after wiping off the water from their surfaces. Tissues of D. magna were homogenized in 0.5 mL sucrose buffer (0.25 M sucrose and 0.1 M Tris-HCl, pH 8.6) by ultrasonication, after which they were centrifuged at a speed of 16000 × g for 20 min. The supernatant fluid was diluted to 1.5 mL using a homogenate. One milliliter of supernatant fluid was used to determine SOD enzyme and Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activities using commercially available kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer’s protocol.

SOD is a kind of catalytic enzyme that can convert superoxide into oxygen and hydrogen peroxide to protect cells. SOD activity is assayed using a spectrophotometric method based on inhibition of a superoxide-driven NADH oxidation, which consists of a purely chemical reaction sequence which involves EDTA, Mn(II), mercaptoethanol, and molecular oxygen\textsuperscript{42}. Na\textsuperscript{+}/K\textsuperscript{+}-ATPase can keep a high concentration of K\textsuperscript{+} inside the cell and Na\textsuperscript{+} outside the cell to maintain the balance of osmotic pressure. Na\textsuperscript{+}/K\textsuperscript{+}-ATPase is assessed based on the amount of inorganic phosphate liberated from hydrolysis of the substrate ATP\textsuperscript{43}.

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**Author Contributions**

W.H.F. and L.L.L. designed the experiments; W.X. and H.T.L. prepared materials; W.H.F. and L.L.L. performed the experiments and analyzed the data; W.H.F., L.L.L., H.T.L. and W.X. wrote the paper.

**Additional Information**

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