Effect of titanium dioxide nanoparticles on copper toxicity to *Daphnia magna* in water: Role of organic matter

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A B S T R A C T

Inevitably released into natural water, titanium dioxide nanoparticles (nano-TiO_2) may affect the toxicity of other contaminants. Ubiquitous organic matter (OM) may influence their combined toxicity, which has been rarely reported. This study investigated the effect of nano-TiO_2 on Cu toxicity to *Daphnia magna* and the role of OM (dissolved or particle surface bound) in inducing combined effects. The effect of nano-TiO_2 on heavy metal accumulation depended on the adsorption capacity for heavy metals of nano-TiO_2 and the uptake of nano-TiO_2—metal complexes by organisms. Nano-TiO_2 significantly decreased Cu accumulation in *D. magna*, but the reducing effect of nano-TiO_2 was eliminated in the presence of humic acid (HA, a model OM). In the Cu and HA solution, nano-TiO_2 slightly affected the bioavailability of Cu^{2+} and Cu—HA complexes and thus slightly influenced Cu toxicity. The nanoparticle surface-bound HA reduced the effect of nano-TiO_2 on the speciation of the accumulated Cu; therefore, the combined effects of nano-TiO_2 and Cu on biomarkers similarly weakened. HA-altered Cu speciation may be the main factor responsible for the influence of HA on the combined effects of nano-TiO_2 and Cu. This study provides insights into the combined effects of nano-TiO_2 and heavy metals in natural water.

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1. Introduction

Titanium dioxide nanoparticles (nano-TiO_2) have been widely used in various industries and materials, such as sunscreens, paints, papermaking, wastewater treatment, solar cells, and food additives, because of their unique physical and chemical properties (Robichaud et al., 2009). With the increasing production, nano-TiO_2 is inevitably released into natural water. The predicted nano-TiO_2 concentration in rivers ranges from 3 ng/L to 1.6 μg/L (Gottschalk et al., 2013). Once in an aquatic environment, nano-TiO_2 with unique properties inevitably interacts with other contaminants; thus, the behavior, bioavailability, and toxicity of contaminants to aquatic organisms are modified (Rosenfeldt et al., 2014; Torre et al., 2015; Yang et al., 2014). The behavior and bioavailability of nano-TiO_2 can also be altered by background components in natural water (Pavagadhi et al., 2014; Romanello and de Cortalezzi, 2013; Tong et al., 2013).

Heavy metals are common contaminants in natural water. Heavy metal toxicity is influenced by nano-TiO_2 to varying degrees.

Nano-TiO_2 decreases the concentration of free heavy metal ions through adsorption, as a result, the bioaccumulation and toxicity of heavy metals are reduced (Liu et al., 2015; Rosenfeldt et al., 2014, 2015b; Yang et al., 2012). Nano-TiO_2 can also act as a heavy metal carrier and enhance heavy metal bioavailability when nano-TiO_2—metal complexes penetrate organisms (Fan et al., 2011, 2012b; Hartmann et al., 2012; Miao et al., 2015; Tan et al., 2012; Yang et al., 2014). Nano-TiO_2 may cause differences in heavy metal subcellular distribution (Yang et al., 2014) and synergistically affect heavy metal toxicity (Hartmann et al., 2010). These various findings may be attributed to different exposure conditions and types of nano-TiO_2, heavy metals, and organisms. However, limited information on the comparative analysis of these various results has been published.

Moreover, the influence of background components in natural water on behavior of nanoparticles and heavy metals has been disregarded in most studies. Organic matter (OM), which is ubiquitous in natural water, contains numerous functional groups and has high reactivity. OM plays an important role in metal toxicity in an aquatic environment (Kramer et al., 2004; Lamelas and Slaveykova, 2007; Richards et al., 2001). OM can also alter the surface properties and sorption behavior of nano-TiO_2 (Liu et al., 2008; Yang et al., 2009). OM can significantly influence the

interaction of heavy metals and nano-TiO$_2$ (Chen et al., 2012; Tang et al., 2014). For example, Chen et al. (2012) found that Cd adsorption on humic acid (HA)–TiO$_2$ was higher than that on nano-TiO$_2$. OM can also change the combined bioaccumulation and toxicity of nano-TiO$_2$ and heavy metals. Hu et al. (2011) investigated the combined effects of nano-TiO$_2$ and HA on Cd accumulation in zebrafish; they demonstrated that Cd bioaccumulation in zebrafish in the mixed system of HA and TiO$_2$ nanoparticles was significantly lower than that in the corresponding TiO$_2$ solution but comparable to that in the corresponding HA solution. Rosenfeldt et al. (2015a) reported that Cu toxicity was weakened by nano-TiO$_2$ and further reduced in the presence of OM because of the adsorption and complexation by nano-TiO$_2$ and OM, respectively. After entering natural waters, nano-TiO$_2$ is coated by OM. Nanoparticle surface-bound OM may affect differently from dissolved OM on combined effect of nano-TiO$_2$ and heavy metals (Lin et al., 2012). However, studies have yet to investigate the effect of nanoparticle surface-bound OM on the combined toxicity of nano-TiO$_2$ and heavy metals, and the mechanisms by which OM affect the combined toxicity have yet to be elucidated.

This study further investigated nano-TiO$_2$-related factors affecting the heavy metal toxicity and the role of HA (a model of OM) in this process. The nanoparticle surface-bound HA and dissolved HA were applied on the basis of different forms of OM in natural water. As a commonly detected metal in water, Cu was used as a heavy metal model. *Daphnia magna* was selected as test organism. The interaction of HA, Cu, and nano-TiO$_2$ was analyzed to elucidate the mechanism of their combined effects on Cu toxicity. This study provided additional information on the combined effects of nano-TiO$_2$ and heavy metals in natural water.

2. Materials and methods

2.1. Preparation and characterization of nanoparticles and HA

Aeroxide P25 TiO$_2$ with >99.5% purity was provided by Acros Organics (Belgium). HA, from Sigma Aldrich (Germany) was used for the experiments. HA was dissolved in a simplified Elendt M7 medium (SM7) (Samel et al., 1999), containing a mixture of CaCl$_2$ (293.8 mg/L), MgSO$_4$ (123.3 mg/L), K$_2$HPO$_4$ (0.184 mg/L), KH$_2$PO$_4$ (0.143 mg/L), NaNO$_3$ (0.274 mg/L), NaHCO$_3$ (64.8 mg/L), Na$_2$SiO$_3$ (10 mg/L), H$_3$BO$_3$ (0.715 mg/L) and KCl (5.8 mg/L). The resulting HA solution was filtered through 0.45 μm cellulose membranes, and then stored in the dark at 4 °C. TiO$_2$–HA complexes were prepared as described by Yang et al. (2009). In brief, 2 g nano-TiO$_2$ was added to 800 mL HA solution dissolved in ultrapure water (18 mg TOC/L) and shaken at 120 rpm for 5 days. The suspension was centrifuged at 3500 rpm for 30 min. The precipitate was washed twice with ultrapure water, freeze-dried, ground and stored for subsequent experiments. For nanoparticle stock solutions (1 g/L), the nanoparticles were dissolved in ultrapure water, and stored in the dark. The stock solutions without HA sonicated 20 min before use.

The image of nanoparticles in SM7 was obtained by using a transmission electron microscope (TEM, JEM-2100F, Japan). The C and H contents of the whole nanoparticles were determined with an elemental analyzer (Elementar Analysensysteme Gmbh vario, Germany). Surface areas were calculated via BET method (NOVA4200e, USA). The fourier transform infrared spectra of HA, nano-TiO$_2$ and TiO$_2$-HA were obtained from 400 cm^{-1}–4000 cm^{-1} with an infrared microspectrograph (iN10MX, USA). The ultraviolet absorption spectra of the HA solution in the presence or absence of Cu and nanoparticles was obtained by using an ultraviolet spectrophotometer (GBC Cintra 10e, Australia). 3D fluorescence spectra were also obtained with a spectrofluorometer (F-700, Japan). Excitation and emission wavelength respectively ranged from 200 nm to 400 nm and from 220 nm to 550 nm at a scanning speed of 12,000 nm/min.

2.2. Adsorption of Cu^{2+} onto nanoparticles in the presence of HA

The Cu solution (30 μg/L) was prepared by diluting the Cu standard solution (100 μg/mL, National Institute of Metrology) using SM7. The nano-TiO$_2$ or TiO$_2$–HA complexes (2 mg/L) were added to 250 mL of Cu only solution or Cu and HA mixture. Three parallels were used. The mixture was shaken at 150 rpm, and 4 mL samples were collected at different times. The samples were then centrifuged at 12,000 × g for 10 min to settle the nanoparticles (Tan et al., 2012). The total Cu concentration in the supernatant was determined through inductively coupled plasma mass spectrometry (VGPQ2 Turbo). The amount of Cu adsorbed on the nanoparticles was calculated by mass difference between the initial samples and the supernatant in equilibrium.

2.3. Test organism

D. magna was cultured in a climatic chamber at 22 °C with 16 h light: 8 h dark cycle and fed daily with the green alga *Chlamydomonas reinhardtii*. The culture medium for *D. magna* was collected from the Jingmi River (39°58′5.58″ N, 116°16′53.3″ E) with a pH of 8.50. The concentrations of Ca, Zn, and Cu were 34 mg/L, 6 μg/L and 0.7 μg/L, respectively, and Cd was not detectable. The TOC content was approximately 3 mg/L. The culture medium was uncontaminated, and might slightly influence the sensitivity of organisms. The medium was filtered to remove larger particles before use. The medium was changed every other day.

2.4. Toxicity test

The Cu, nano-TiO$_2$, and HA concentration for the toxicity test were set at 30 μg/L, 2 mg/L and 1 mg TOC/L, respectively. SM7 (pH = 8.0) was used as test medium. The toxicity test for *D. magna* included the eight following groups: (a) nano-TiO$_2$ suspension, (b) TiO$_2$–HA complexes suspension, (c) Cu only solution, (d) mixture of Cu and HA, (e) mixture of Cu and nano-TiO$_2$, (f) mixture of Cu and TiO$_2$–HA complexes, (g) mixture of Cu, HA and nano-TiO$_2$, (h) mixture of Cu, HA and TiO$_2$–HA complexes. The HA and Cu solution was allowed to reach equilibrium within 12 h before the nanoparticles were added. Three parallels were used for each group. *D. magna* exposure was performed in 500 mL beakers containing 500 mL of test solution for 48 h. Fifty healthy *D. magna* (14 ± 2 days) with similar sizes were added to each beaker. The experimental toxicity conditions were the same as the culture conditions for *D. magna*, as described in Section 2.3, except river water was replaced with SM7. *D. magna* were not fed during exposure.

2.5. Ti and Cu bioaccumulation in D. magna

Ten surviving *D. magna* were removed and washed with ultrapure water after 48 h exposure. *D. magna* were dried at 80 °C to a constant weight and digested in 68% HNO$_3$ and concentrated sulfuric acid–ammonium sulfate solution (Tan et al., 2012). Cu and Ti concentrations in the digest were determined by the 797 VA computrace system (Metrohm, Switzerland) and an inductively coupled plasma optical emission spectrometer (Optima 5300DV, USA), respectively. The recuperation experiments were performed to evaluate reliability of the digestion method. The average recovery rate of Ti and Cu was approximately 85% and 98%, respectively. Bioaccumulation was expressed on basis of the dry weight of *D. magna*.

2.6. Determination of metallothionein and enzyme activity

Twenty surviving individuals were weighed after removing water from surface and disrupted with an ultrasonic cell crusher in 1 mL sucrose buffer (0.25 mol/L sucrose, 0.1 mol/L Tris–HCl, pH = 8.6) in an ice bath. The homogenate was then centrifuged (0 °C, 16,000 × g) for 20 min. The supernatant was used to determine metallothionein (MT), superoxide dismutase (SOD) and Na^+/K^+-ATPase activities. MT was determined using the modified silver saturation method (Scheuhammer and Cherian, 1991), and its detailed procedures were available from our previous study (Fan et al., 2012a). The SOD and Na^+/K^+-ATPase activities were determined by using the test kits (Nanjing Jiancheng Bioengineering Institute, China) according to the instruction manual. The SOD activity was based on the inhibitory effect of SOD on the NADPH oxidation by molecular oxygen (Paoletti and Mocali, 1990). Its activity unit (U) was defined as the SOD amount per mg tissue protein when the inhibition rate reached 50% in 1 mL reaction solution. The Na^+/K^+-ATPase activity was calculated on the basis of the amount of inorganic phosphate liberated from ATP hydrolysis (Lanzetta et al., 1979). The activity unit (U) was defined as the amount of inorganic phosphate produced through ATP decomposition catalyzed by the ATP enzyme per mg tissue protein per hour.

2.7. Statistical analysis

Results were expressed as mean value ± standard deviation (n = 3). Variance homogeneity was assessed. One-way analysis of variance (ANOVA) was conducted to evaluate the significance of different treatments. Least significant difference post-hoc test was performed for pairwise comparison in cases with significant effects of the treatment. Statistical significance was accepted if p value was <0.05. Statistical analyses were conducted using IBM® SPSS® Statistics 20.0.

3. Results

3.1. Material characterization

The image of the nanoparticles in SM7 was obtained through TEM (Fig. 1). The morphological characteristics of TiO_2–HA complexes were different from those of nano-TiO_2. The C content of TiO_2–HA complexes was significantly higher than that in nano-TiO_2 (Table 1). This finding indicated that HA was successfully coated nano-TiO_2. The surface area of TiO_2–HA complex was less than that of nano-TiO_2 because of microporous blockage. The zeta potential increased and the average particle size decreased after HA coated nano-TiO_2, because HA enhanced the dispersibility and stability of nano-TiO_2 in water. The infrared spectra of HA, nano-TiO_2 and TiO_2–HA complexes are presented in Fig. S1 (Supporting Information). The peaks from 400 cm^{-1} to 800 cm^{-1} and around 3400 cm^{-1} for the nanoparticles were assigned to Ti–O–Ti and OH stretching, respectively. Compared with that of nano-TiO_2, the new peak at around 1390 cm^{-1} for TiO_2–HA complexes indicated the titanium–acetate complexation.

The ultraviolet absorption spectrum (Fig. S2, Supporting Information) shows that no nanoparticle absorbance was obtained. The absorbance intensity of HA increased with decreasing wavelength. Cu addition significantly increased absorption intensity at the wavelength less than 240 nm. No apparent peaks for Cu and nano-TiO_2 were observed in the 3D fluorescence spectra (Fig. 2). The HA fingerprint peak (EX/EM = 265–270 nm/430–440 nm) was called the humic-like fluorescence peak, attributed to the $\pi \rightarrow \pi^*$ transfer of aromatic C=C groups in HA caused by substitution of benzene or polyphenols, and intensity of this peak was decreased by Cu. UV absorbance intensity and fluorescent characteristic of HA were altered by nano-TiO_2.

3.2. Adsorption of Cu^{2+} onto nanoparticles in the presence of HA

Fig. 3 shows that both nano-TiO_2 and TiO_2–HA complexes rapidly adsorbed Cu. The adsorption equilibrium was reached within the first 60 min. The nano-TiO_2 and TiO_2–HA complexes in the Cu only solution decreased the dissolved Cu concentration by 64% and 59% of the initial Cu concentration (30 μg/L), respectively. The decline proportions were 66% and 63% in the mixture of Cu and HA, respectively. The Cu adsorption amount of nano-TiO_2 was decreased by HA coating but increased by dissolved HA.

3.3. Ti and Cu bioaccumulation in D. magna

The accumulated Ti and Cu in *D. magna* after 48 h exposure are shown in Fig. 4(a) and (b), respectively. The accumulated Ti of *D. magna* was significantly decreased by Cu, and further reduced in the presence of HA. The high accumulated Cu in *D. magna* in the Cu solution demonstrated that Cu was taken into *D. magna*. Moreover, the nano-TiO_2 and TiO_2-HA complexes reduced the Cu accumulation compared to the Cu alone solution. The HA coating weakened the reducing effect of nano-TiO_2 on Cu accumulation in *D. magna*. However, this abated effect was not obvious. The accumulated Cu in *D. magna* exposed in the Cu alone solution significantly decreased from 273.25 ± 45.02 μg/g dry weight to 175.53 ± 31.57 μg/g dry weight because of dissolved HA. The dissolved HA eliminated the reducing effect of nano-TiO_2 on Cu accumulation.
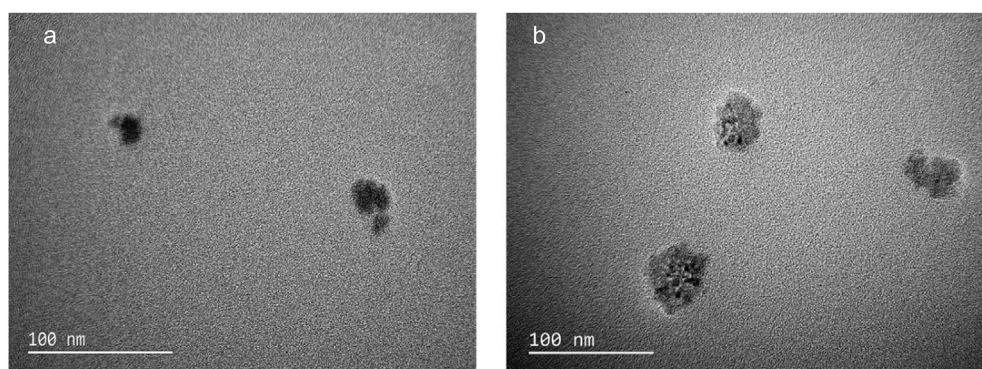

**Fig. 1.** TEM image of a single nanoparticle in SM7. (a) nano-TiO_2 and (b) TiO_2–HA complexes.

Table 1
Physicochemical properties of nano-TiO$_2$ and TiO$_2$–HA complexes.

Samples	Elemental content		S$_{BET}$ (m^2/g)	Average particle size in water (nm)	Zeta potential[a] (mV)
	C (%)	H (%)			
HA	38.6	3.53	–	308.0 ± 26.87	−8.17 ± 0.97
Nano-TiO$_2$	0.34	0.47	55.291	872.1 ± 8.63	−7.20 ± 0.17
TiO$_2$–HA complexes	0.99	0.30	51.355	314.6 ± 0.28	−13.07 ± 0.21

[a] pH levels of HA solution, nano-TiO$_2$ suspension, and TiO$_2$–HA complex suspension were 7.87, 7.84, and 7.72, respectively.
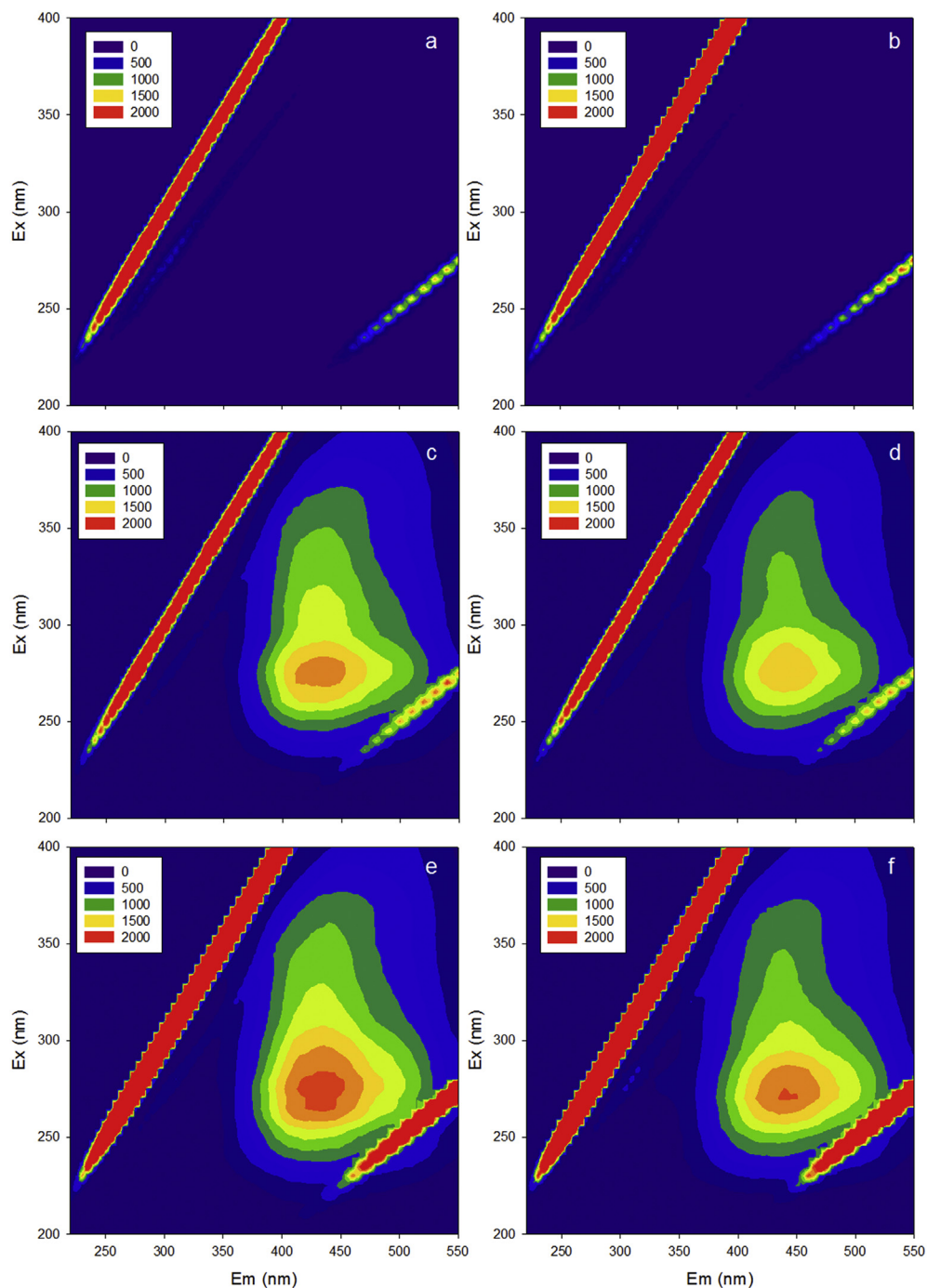
Fig. 2. 3D fluorescence spectra of Cu (a), nano-TiO$_2$ (b), HA (c), HA + Cu (d), HA + TiO$_2$ (e) and HA + Cu + nano-TiO$_2$ (f).
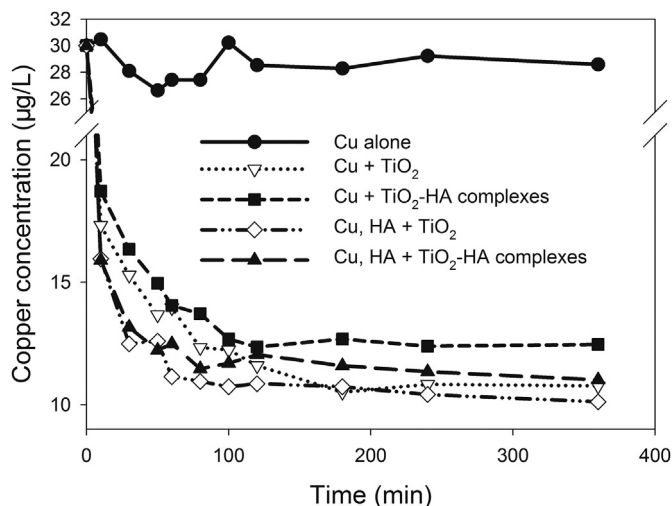
Fig. 3. Cu concentration in the supernatant after Cu^{2+} absorption onto nanoparticles in the solution.

3.4. Inhibition ratio of D. magna

The inhibition ratio was calculated on the basis of the number of *D. magna* that remained immobile within 15 s after 48 h exposure. The inhibition ratio of *D. magna* in all of the treatments was below 10% because of the low Cu concentration [Fig. 4(d)]. The Cu exposure resulted in a notable rise in the inhibition ratio of *D. magna*, and this finding indicated that Cu was the main toxicant to *D. magna* in this study. The correlation between the inhibition ratio and other *D. magna* biomarkers was analyzed using IBM® SPSS® Statistics 20.0. The results showed that the inhibition ratio was positively correlated with Na^+/K^+-ATPase activity ($p = 0.03$).

Therefore, the Cu toxicity to *D. magna* in this study was mainly manifested as membrane damage. Nano-TiO_2 and TiO_2–HA complexes had no obvious effect on the inhibition ratio caused by Cu, whereas dissolved HA significantly reduced the Cu toxicity.

3.5. Biomarkers in D. magna

Nano-TiO_2 and TiO_2–HA complexes did not significantly affect the MT content in SM7 [Fig. 4(c)]. MT content in the Cu solutions was not significantly higher than that in the control solution. However, nano-TiO_2 significantly increased the MT content of *D. magna* in the Cu solution, and thus indicated that the Cu and nano-TiO_2 interaction enhanced the MT induction in *D. magna*. This enhancement was weakened by surface-bound HA and dissolved HA.

The toxicity exposure caused a similar variation on the SOD and Na^+/K^+-ATPase activities in *D. magna* [Fig. 5(a) and (b)]. The SOD and Na^+/K^+-ATPase activities had no obvious change in *D. magna* exposed to nanoparticles, but were significantly increased by Cu. Accordingly, Cu induced oxidative stress and membrane damage in *D. magna*. SOD and Na^+/K^+-ATPase activity inductions by Cu were weakened by nanoparticles. Nano-TiO_2 decreased SOD and Na^+/K^+-ATPase activities in the Cu only solution by approximately 39% and 35%, respectively, and 30% and 12% in the mixture of Cu and HA, respectively. SOD and Na^+/K^+-ATPase activities caused by TiO_2–HA complexes were decreased by approximately 13% and 4% in the Cu alone solution and by 15% and 9% in the mixture of Cu and HA, respectively. In conclusion, surface-bound HA and dissolved HA weakened the effect of nano-TiO_2 on Cu toxicity.

4. Discussion

4.1. Effect of nano-TiO_2 on Cu toxicity

In Fig. 4(a), the accumulated Ti in *D. magna* was significantly
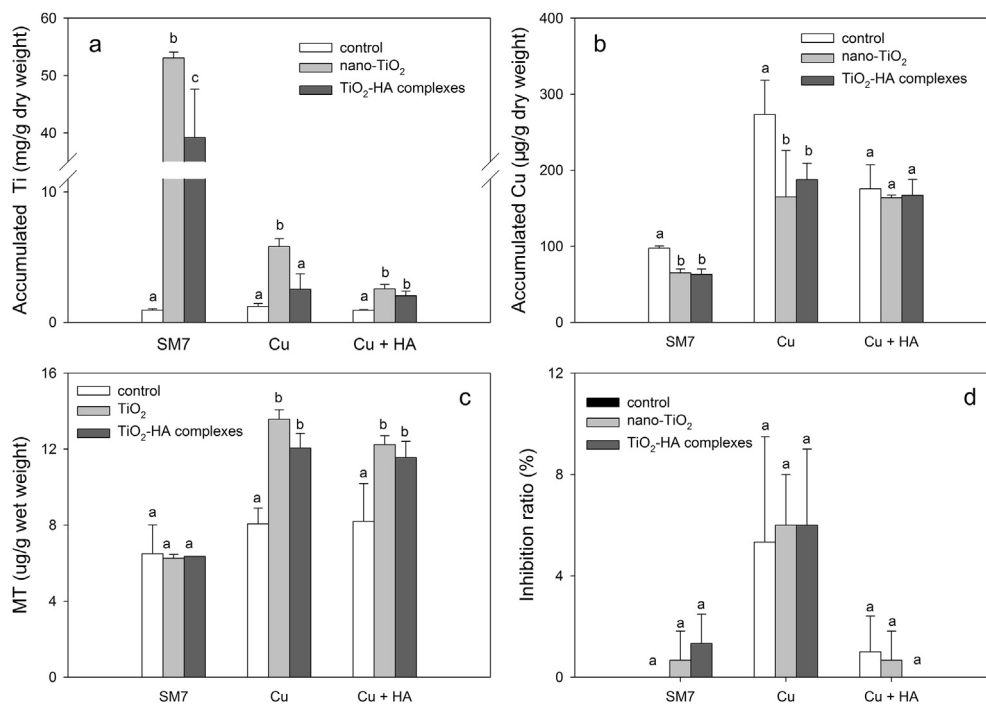
Fig. 4. Ti bioaccumulation (a), Cu bioaccumulation (b), MT content (c), and inhibition ratio rate (d) of *D. magna* after 48 h of being exposed to the different Cu solution in the absence and presence of nanoparticles. Mean ± standard deviation (n = 3). The mean values sharing the different letter are significantly different ($p < 0.05$, one-way ANOVA) among the nanoparticle treatments within the same Cu solution.
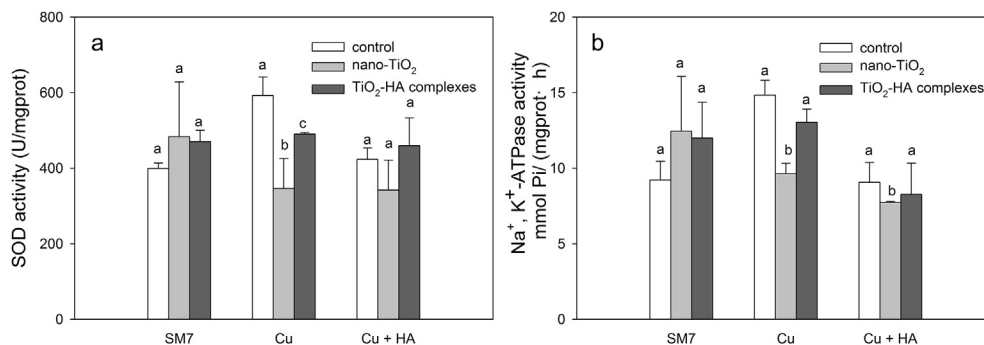
Fig. 5. SOD (a) and Na$^+$/K$^+$-ATPase (b) activities of *D. magna* after 48 h of being exposed to the Cu alone solution or the mixed solutions of Cu and HA in the absence and presence of nanoparticles. Mean ± standard deviation (n = 3). The mean values sharing the different letter are significantly different (*p* < 0.05, one-way ANOVA) among the nanoparticle treatments within the same Cu solution.

decreased by Cu. According to DLS data (Table S1, Supporting Information), average sizes of nanoparticles in the presence of Cu was larger, and thus might decrease uptake of these particles by *D. magna* (Liu et al., 2015). Fig. 4(b) shows that nano-TiO$_2$ significantly decreased Cu accumulation in *D. magna* exposed to the Cu only solution. The results were consistent with those obtained by Rosenfeldt et al., who reported the total body burden of Cu in *D. magna* (Rosenfeldt et al., 2014) and *Gammarus* (Rosenfeldt et al., 2015b) decreased in the presence of 2 mg/L nano-TiO$_2$. Liu et al. (2015) found that four TiO$_2$ nanosheets reduced Cu accumulation in *D. magna*. Various effects of nano-TiO$_2$ on heavy metal bioaccumulation have been observed in other studies. For instance, nano-TiO$_2$ greatly enhanced Cd (Zhang et al., 2007) and As (Sun et al., 2009) accumulations in carp, Cd concentration in *D. magna* (Hartmann et al., 2012) and Pb bioaccumulation in zebrafish larvae (Miao et al., 2015). Balbia et al. (2014) found that nano-TiO$_2$ did not alter Cd accumulation in marine bivalve *Mytilus galloprovincialis* after 96 h exposure.

Two sources of the accumulated heavy metals in organisms in the mixture of nano-TiO$_2$ and heavy metals are free metal ions and nano-TiO$_2$-bound metal. On the one hand, nano-TiO$_2$ decreased the concentration of dissolved metal ions in the solution by adsorption, thus decreased its accumulation in organisms. On the other hand, nano-TiO$_2$ could serve as a heavy metal carrier because of the uptake of nano-TiO$_2$–metal complexes. Therefore, the effect of nano-TiO$_2$ on heavy metal accumulation depended on the adsorption capacity for heavy metals of nano-TiO$_2$ and the uptake of nano-TiO$_2$–metal complexes by organisms. Various findings in published studies may be attributed to different exposure conditions and types of nano-TiO$_2$, heavy metals and organisms, which caused differences in the adsorption capacity of nano-TiO$_2$ and the uptake of TiO$_2$–metal complexes. The higher adsorption capacity led to the lower dissolved metal ion concentration in water. The amount of dissolved heavy metals that accumulated in organisms was low. Our previous study, which reported the effect of pure anatase nano-TiO$_2$ on Cu toxicity to *D. magna*, suggested that nano-TiO$_2$ increased Cu accumulation in *D. magna*. It showed that nano-TiO$_2$ decreased the dissolved Cu concentration in solution by approximately 20% (Fan et al., 2011). Liu et al. (2015) showed that four TiO$_2$ nanosheets decreased the dissolved Cu concentration in solution by approximately 60%–70%. The decreased extent of the dissolved Cu concentration was much higher in the latter case than in the former case. Therefore, the TiO$_2$ nanosheets decreased the Cu accumulation in *D. magna*. Moreover, the particle size of the TiO$_2$–metal complexes also affected metal accumulation. The low uptake of TiO$_2$–metal complexes likely results in a decreased metal accumulation via the complexes if TiO$_2$–metal complexes are too large

to be taken in by organisms. Accordingly, Yang et al. (2012) indicated that the Cd^{2+} adsorption by TiO$_2$ decreased its accumulation in green alga *C. reinhardtii* because no TiO$_2$ was taken into the algal cells. *D. magna* can ingest food with particle sizes ranging from 0.4 μm to 4 μm (Gophen and Geller, 1984). However, nanoparticles may not be taken in by *D. magna* when they form large aggregates. Cu accumulation then decreased as the dissolved Cu concentration was reduced. Rosenfeldt et al. (2014) reported that nano-TiO$_2$ reduced the Cu concentration in water by 33%–82%, and the mean size of nano-TiO$_2$ reached up to 3277 nm after 24 h and 6973 nm after 72 h. As a result, nano-TiO$_2$ decreased the Cu accumulation in *D. magna* by 14 times. In this study, the dissolved Cu concentration was reduced by approximately 64% because of the nano-TiO$_2$ adsorption (Fig. 3). The average particle size of nano-TiO$_2$ was approximately 2204 nm in the Cu solution at beginning of exposure (Table S1, Supporting Information). Thus, the decrease in Cu accumulation in this study could be explained by the low dissolved Cu concentration and the large particle size of nano-TiO$_2$ in water.

MT reacts with extra Cu^{2+} in cells via oxidation and reduction; thus, Cu toxicity to cells is decreased by Cu–MT (Salgado and Stillman, 2004). SOD is an antioxidant enzyme, which catalyzes the disproportionation of O$_2^-$ to O$_2$ and H$_2$O$_2$ (Kim et al., 2011; Pinto et al., 2003). Na$^+$/K$^+$-ATPase plays an important role in membrane function (Therien and Blostein, 2000). Cu accumulation caused marked oxidative stress and cell membrane damage in *D. magna* as indicated by the higher SOD and Na$^+$/K$^+$-ATPase activities (Fig. 5). However, no significant change was found in the MT content of *D. magna* in the Cu solution compared to the control [Fig. 4(c)]. Nano-TiO$_2$ significantly enhanced the MT induction and weakened the facilitation of the SOD and Na$^+$/K$^+$-ATPase activities caused by Cu [Figs. 4(c) and 5]. These phenomena could be explained by three possibilities. First, the Cu–nanoparticle complexes were taken into *D. magna*, and Cu^{2+} adsorbed on nano-TiO$_2$ could be released within the gastrointestinal tract of *D. magna* (Rosenfeldt et al., 2014; Tan et al., 2012). Cu^{2+} was then assimilated by the intestinal cells of *D. magna*, and helped to cause damage. Second, nano-TiO$_2$ and Cu might exert synergistic effects. Nano-TiO$_2$ can affect the MT content (Bigorgne et al., 2011; Clemente et al., 2013), oxidative stress (Kim et al., 2010), and Na$^+$/K$^+$-ATPase activity (Federici et al., 2007; Valant et al., 2012). *D. magna* may be less tolerant of Cu under nano-TiO$_2$ stress (Tan and Wang, 2014). Therefore, Cu and nano-TiO$_2$ might promote MT induction, oxidative stress and membrane damage jointly. Third, different Cu species, such as free Cu^{2+} and nano-TiO$_2$-bound Cu, taken by *D. magna* might cause different Cu subcellular distributions (Yang et al., 2014) and effects on biomarkers. The results of this study were different from those obtained in our previous study (Fan et al., 2011), which demonstrated

that the alternation of biomarkers might be caused by the increased Cu accumulation. Different nano-TiO₂ used in two studies caused different amount of the accumulated TiO₂ and Cu species, such as free Cu²⁺ and nano-TiO₂ bound Cu, and thus caused different effect on biomarkers.

4.2. Role of HA in combined effects of nano-TiO₂ and Cu

Surface-bound HA and dissolved HA decreased Ti accumulation of *D. magna* exposed to the co-existing system of nano-TiO₂ and Cu [Fig. 3 (a)], probably as a result of lower bioavailability of nano-particles with higher molecular weight after reacting with HA (Koukal et al., 2003). Dissolved HA significantly decreased Cu accumulation in *D. magna* exposed to the Cu only solution, thus decreased Cu toxicity [Fig. 4(b) and (d)]. HA significantly reduced Cu bioavailability by decreasing free Cu²⁺ available for *D. magna*, thus decreased Cu toxicity to *D. magna* (Fan et al., 2012c). Fig. 4(b) shows that nano-TiO₂ sharply decreased the Cu accumulation in *D. magna* exposed to the Cu only solution. However, these particles slightly affected Cu accumulation in the Cu and HA mixture. In other words, the reducing effect of nano-TiO₂ was eliminated in the presence of dissolved HA. Hu et al. (2011) investigated the combined effects of nano-TiO₂ and HA on the Cd accumulation in zebrafish. They found that adding a comparable concentration of nano-TiO₂ into the Cd and HA solution did not change the Cd accumulation in zebrafish, which was concordant with our results. The main existing forms of Cu in the HA solution were free Cu and HA-bound Cu. Spectral characterization demonstrated that phenolic hydroxyl of HA was related to the interaction with nano-TiO₂ through ligand exchange (Yang et al., 2009). Loosli et al. (2015) calculated the binding affinity constant (K_b) of nano-TiO₂ and HA. logK_b ranged from 5.9 to 6.6. Cu increased the UV adsorption intensity at a wavelength less than 240 nm, and indicated that Cu interacted with lone pair electrons and π electrons, such as carboxyl, hydroxyl, and aromatic structure in HA (Wu et al., 2008). Cu decreased the humic-like fluorescence peak, indicating that Cu reacted with the substituent on benzene ring in HA (Gu et al., 2014). The conditional stability constants (log K_M) of Cu and HA fractions ranged from 4.61 to 5.26 (Chen et al., 2015). The hydroxyl group of nano-TiO₂ was involved in the formation of surface complexes with Cu²⁺ (Yang and Davis, 1999):

$$Ti-(OH)(OH_2) + Cu^{2+} \rightleftharpoons Ti-(OH_2)O-Cu^+ + H^+; \quad K_{int}^s \tag{1}$$
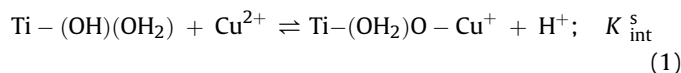
The intrinsic surface equilibrium constant (log K_{int}^s) was 0.63. The conditional stability constant of the Cu–HA complexes was comparable to that of the TiO₂–HA complexes but was several magnitudes higher than that of TiO₂–Cu. Therefore, nano-TiO₂ could not easily react with Cu in the presence of HA. However, the adsorption experiment exhibited that the dissolved HA increased the Cu adsorption on nano-TiO₂ (Fig. 3). Yang and Davis (1999) indicated that nano-TiO₂ adsorbed Cu in EDTA by the formation of ternary surface complexes between nano-TiO₂ and EDTA rather than metal-bridged ternary surface complexes. The increase of Cu adsorption might result from the adsorption of Cu–HA complexes on nano-TiO₂ rather than from the adsorption of Cu²⁺. The decrease of Ti accumulation in the presence of HA indicated that bioavailability of the TiO₂–HA–Cu complexes might be low. Based on the earlier analysis, nano-TiO₂ seldom reacted with Cu²⁺ and affected the bioavailability of Cu–HA complexes in the mixture of Cu and HA. As a result, Cu accumulation and the effect of Cu on biomarkers in *D. magna* were slightly altered by nano-TiO₂ in the HA solution.

The nano-TiO₂ surface-bound HA was applied to further investigate the influence of HA on the combined effects of nano-TiO₂ and Cu. Figs. 4 and 5 show that the nano-TiO₂ surface-bound HA did not significantly change the effect of nano-TiO₂ on the total Cu accumulation in *D. magna*. However, HA evidently decreased the effect of nano-TiO₂ on Cu toxicity. The different species of Cu uptake by *D. magna* should be compared to further explore the mechanisms by which surface-bound HA affects Cu and nano-TiO₂. Therefore, Cu accumulation in the presence of Cu and nano-TiO₂ was divided by the Ti accumulation content. The Cu accumulation per unit Ti accumulation in the Cu only solution with nano-TiO₂ or TiO₂–HA complexes was 36.7 ± 7.3 μg Cu/mg Ti and 55.2 ± 8.4 μg Cu/mg Ti. Therefore, surface-bound HA increased the Cu accumulation per unit Ti accumulation. The hydroxyl on nano-TiO₂ surface was responsible for adsorption of HA and Cu. HA competed with Cu for hydroxyl on nano-TiO₂, reduced the nano-TiO₂ micropore surface area (Table 1), and likely caused steric hindrance to inhibit Cu adsorbed onto nano-TiO₂ (Wang et al., 2015), thereby leading to the lower Cu adsorption of the TiO₂–HA complexes in this study (Fig. 3). HA coating weakened the effect of nano-TiO₂ on free Cu concentration in the exposure medium, resulting in a more free Cu²⁺ accumulation in *D. magna*. TiO₂–HA complexes led to more free Cu²⁺ accumulation in *D. magna* than bare nano-TiO₂, and exhibited a weaker effect on the speciation of the accumulated Cu. Therefore, surface-bound HA weakened the combined effects of nano-TiO₂ and Cu on the MT content and the SOD and Na⁺/K⁺-ATPase activities.

A much greater effect of the dissolved HA was observed in Cu accumulation and biomarkers of *D. magna* exposed to Cu only solution than in the mixture of Cu and nano-TiO₂. The surface-bound HA altered the effect of nano-TiO₂ on Cu toxicity in Cu only solution, but generated slight change of nano-TiO₂ in the presence of dissolved HA. The inhibition ratio of *D. magna* was slightly affected, but was significantly reduced by dissolved HA. These results showed that the HA-altered Cu speciation was the main factor responsible for the differences in Cu toxicity to *D. magna* in the coexistence of nano-TiO₂.

The interactions among nano-TiO₂, HA and Cu were complex. Nano-TiO₂ could change Cu bioavailability as discussed in Section 4.1. HA could also alter the Cu bioavailability for biotic ligands and the surface properties of nano-TiO₂. Moreover, the dissolved HA can modify environmental conditions, such as salinity and pH, and may affect the antioxidant capacity and transport function of the membrane (Giacomin et al., 2013). In this manner, the Cu bioavailability in the mixture of HA and nano-TiO₂ might depend on organisms, exposure conditions, and nano-TiO₂ physicochemical properties. OM is ubiquitous in natural water and may induce complex influences on the combined effects of nano-TiO₂ and heavy metals. Hence, further studies should focus on the combined effects of nanomaterials and heavy metals under natural conditions.

5. Conclusion

Nano-TiO₂ inevitably interacts with other contaminants and background components in natural water. This study investigated the mechanism by which nano-TiO₂ affects Cu toxicity to *D. magna* and the role of OM in inducing combined effects. The following conclusions were obtained.

- Analyzing and comparing the studies on the combined effects of nano-TiO₂ and heavy metals, it was concluded that the effect of nano-TiO₂ on heavy metal toxicity depended on the adsorption capacity of heavy metals onto nano-TiO₂ and the uptake of nano-TiO₂–metal complexes by organisms.
- The decreasing effect of nano-TiO₂ on Cu accumulation was eliminated in the presence of the dissolved HA because

nano-TiO$_2$ slightly affected the bioavailability of free Cu and Cu–HA complexes. The surface-bound HA weakened the effect of nano-TiO$_2$ on the speciation of the accumulated Cu, such as Cu^{2+} or nanoparticle bound Cu, and thus reduced the effect of nano-TiO$_2$ on Cu toxicity.

● HA-altered Cu speciation might be the main factor responsible for the effect of HA on the co-existing system of nano-TiO$_2$ and Cu.

This study provided new insights into the combined effects of nano-TiO$_2$ and heavy metals, and revealed that HA could weaken the effect of nano-TiO$_2$ on heavy metal toxicity. However, the effects of concentration and types of OM have yet to be clarified. Further research should be performed to investigate such combined effects.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2016.08.060.

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