

MITIGATION OF Cu(II) PHYTOTOXICITY TO RICE (*ORYZA SATIVA*) IN THE PRESENCE OF TiO₂ AND CeO₂ NANOPARTICLES COMBINED WITH HUMIC ACID

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Abstract: Engineered nanoparticles (NPs) and natural organic matter (NOM) in the environment may interact with background contaminants such as heavy metals and modify their bioavailability and toxicity. In the present study, the combined influences of 2 common NPs (TiO₂ and CeO₂) and humic acid (HA; as a model NOM) on Cu(II) phytotoxicity to rice were investigated by a 3-d root elongation assay performed on filter paper media. The results showed that the adsorption coefficients of bare TiO₂ and CeO₂ NPs (100 mg/L) toward Cu²⁺ are 2.65 and 4.37, respectively, at an initial concentration of 10 mg/L, suggesting that Cu(II) could be strongly adsorbed by NPs, whereas HA-coated TiO₂ and CeO₂ NPs further enhanced the adsorption coefficients to 4.37 and 6.85, respectively. In addition, compared with Cu-alone treatment, the addition of bare TiO₂ and CeO₂ NPs (1000 mg/L) increased the length of rice root by 32.5% and 39.0%, respectively; however, the presence of HA-coated TiO₂ and CeO₂ NPs increased the root length by 90.2% and 100.1%, respectively, which indicated that the mitigation effect of HA-coated NPs on Cu(II) phytotoxicity was more visible than that of bare NPs. The results demonstrated that coexistence of NPs and HA significantly alleviated Cu(II) phytotoxicity as a result of a decrease in bioavailable soluble Cu(II) concentration, which contributes to an understanding of the potential behavior of NPs in the environment. *Environ Toxicol Chem* 2015;34:1588–1596. © 2015 SETAC

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INTRODUCTION

Engineered nanoparticles (NPs) have attracted considerable attention because of their unique properties and widespread uses in a variety of areas, such as the construction industry, plant protection and fertilization, waste treatment, and photocatalytic applications [1–3]. Inevitably, however, these NPs may enter environmental systems [4]. In recent years, potentially adverse effects of NPs on ecosystems have been investigated intensively, resulting in a greater understanding of their environmental and health risks, but only when they act alone. In fact, because of their large surface areas and strong adsorption capacity, NPs released into natural systems may react with multiple contaminants, such as various natural components and pre-existing pollutants in the environment, and subsequently may modify the behavior and bioavailability of the associated pollutants [4,5]. Knowledge of the interaction between NPs and other coexisting contaminants in the environment is limited.

Heavy metals are common pollutants in waters and soils, and their bioavailability and toxicity may be altered in the presence of NPs. Some studies have reported the enhancement of bioaccumulation or toxicity to some aquatic organisms of heavy metals when they coexist with NPs [6–9]. A prevailing explanation for NP-enhanced bioaccumulation and/or toxicity is that NPs can serve as a carrier, facilitating the uptake of metals by organisms. Nonetheless, it was also reported that the bioavailability and toxicity of Cd(II) to the green alga *Chlamydomonas reinhardtii* was reduced in the presence of

TiO₂ NPs, which decreased its ambient soluble Cd(II) concentration [10]. This result, however, was contrary to the result observed by Zhang et al. in *Cyprinus carpio* [11]. These contradictory results may indeed be attributed to differences in test organisms. However, the effects of NPs on the behavior and toxicity of metals need further investigating for a better comprehension of the behaviors and risks of NPs in the environment.

Natural organic matter (NOM) is ubiquitous in the environment, and humic acid (HA) is a fraction of NOM that can serve as a complexing agent, affecting the mobility and bioavailability of contaminants such as metals [12]. The surfaces of bare NPs can be readily coated by HA [13], which may alter the surface properties and adsorption capacity of NPs for toxicants [14]. It has been reported that the removal efficiencies of toxic Hg(II), Pb(II), and Cd(II) by Fe₃O₄ and TiO₂ NPs in water can be enhanced with the addition of HA [15,16]. The coexistence of NOM and NPs may thus change the behavior and toxicity of metals. Kim et al. [17] showed that the bioavailability and toxicity of Cu to *Daphnia magna* were enhanced by the presence of multiwalled carbon nanotubes interacting with NOM. However, Worms et al. [18] found that HA combined with carboxyl-CdSe/ZnS quantum dots decreased Pb and Cu intracellular contents in *C. reinhardtii*. Hence, the combined effects of NPs and NOM on the bioaccumulation and toxicity of metals are complicated and need further study.

Plants represent a potential pathway for NP transport and bioaccumulation into food chains [19]. Previous studies have focused on the intrinsic toxicity of NPs to plants, and some published studies have considered the effects of interactions between NPs and other pollutants on overall phytotoxicity. Although Wang et al. [20] found that 6 types of NPs (kaolin, montmorillonite, hydroxyapatite, Fe₃O₄, α -Fe₂O₃, and γ -Fe₂O₃)

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alleviated Cd-induced root growth inhibition in 4 plant species, the potential influence of NPs on the bioavailability and phytotoxicity of other pollutants is largely unknown and needs special investigation. Moreover, to the best of our knowledge, there has been no research to date on the combined effects of NPs and NOM on the bioavailability and phytotoxicity of metals. Therefore, to explore the influence of NPs and NOM on the toxicity of metals to plants, the adsorption of Cu(II) on bare and HA-coated TiO₂ and CeO₂ NPs was investigated in the present study, and the combined effects of NPs and HA on the phytotoxicity of Cu(II) to the rice (*Oryza sativa*) were analyzed. The results of the present study shed light on the potential effects of NPs on the bioavailability and phytotoxicity of metals, especially in the presence of NOM.

MATERIALS AND METHODS

Chemicals

Engineered TiO₂ NPs (anatase, <20 nm, purity > 99.9%) and CeO₂ NPs (<50 nm, purity > 99.9%) were purchased from Nachen Science & Technology. To prepare Cu(II) solutions, CuSO₄·5H₂O (AR, purity > 99.0%), supplied by Sinopharm Chemical Reagent, was used. Aldrich HA sodium salt (H16752; Sigma-Aldrich) was selected to be the model dissolved NOM. Specifically, 1000-mg/L HA stock solutions were prepared by dissolving HA sodium salt powder in Milli-Q water, stirring the solutions overnight, and filtering the solutions through 0.45-μm cellulose membranes (Xingya Purification Materials). A total organic carbon (TOC) tester (Multi N/C 2100; Analytikjena) was used to determine the TOC content of the prepared HA solutions. The Cu(II) standard solution was purchased from o2si smart solutions. All solutions were prepared using Milli-Q ultrapure water (18.2 MΩ; Millipore).

Preparation of HA-coated TiO₂ and CeO₂ complexes

Five grams each of TiO₂ NPs and CeO₂ NPs were mixed into 1 L of the HA solutions (100 mg/L and 1000 mg/L; pH 7.0) and shaken (150 rpm, 25 °C) for 3 d, after which the mixtures were centrifuged at 3500 g for 30 min. The resultant precipitates were washed thoroughly with Milli-Q water and lyophilized in a freeze-dryer (Alpha1-4LSC; Marin Christ) at -56 °C and 0.280 mbar pressure for 24 h. Then, the dried precipitates were gently ground in the agate mortar, and the powder was stored in a vacuum desiccator at room temperature. The residual HA concentrations in the supernatants were determined by TOC measurement. The amounts of adsorbed HA were calculated by mass balance because of the negligible solute loss. The initial concentrations of the HA solutions were 100 mg/L and 1000 mg/L, and the HA-coated NPs obtained were termed TiO₂(or CeO₂)-2%HA and TiO₂(or CeO₂)-20%HA. The percentage numbers stand for the mass ratios of HA to NPs used in the loading process.

NP characterization

Hydrodynamic diameter and zeta potential of bare and HA-coated NPs (100 mg/L) were analyzed by a Zetasizer-Nano ZS instrument (Malvern Instruments) at 25 °C after sonication (100 W, 40 kHz, 30 min) into Milli-Q water (pH 7.0). Specific surface area was determined using the Brunauer-Emmett-Teller method (Tristar II 3020; Micromeritics). Surface functional groups were analyzed by Fourier transform infrared spectroscopy (FTIR; IRPrestige-21, Shimadzu; for additional details about FTIR analysis, see the Supplemental Data).

Adsorption of Cu(II) onto bare and HA-coated NPs

Adsorption isotherms were performed in 50-mL polypropylene tubes at pH 7.0 by varying initial Cu(II) concentration (0.05 mg/L, 0.1 mg/L, 0.2 mg/L, 0.5 mg/L, 1 mg/L, 2 mg/L, 5 mg/L, and 10 mg/L) in the presence of 100 mg/L and 1000 mg/L NPs, respectively. Each mixture of Cu(II) and NPs was produced in triplicate. The tubes containing 30 mL reaction solutions were shaken at 150 rpm in a reciprocating shaker and kept in the dark at 25 °C. According to the results of our preliminary study, Cu(II) adsorption equilibrated within 30 min (Supplemental Data, Figure S1). The duration of the equilibrium isotherm experiment was thus shortened to 2 h. After 2 h of equilibrium, tubes were centrifuged (24 610 g, 10 min) and filtered (0.22-μm glass filter). The residual aqueous Cu(II) concentrations in the supernatants were determined by flame atomic adsorption spectrophotometry (MKII M6; Thermo Electron). The amount of adsorbed Cu(II) was calculated by mass balance between the initial (nominal) and final solution concentrations. Control experiments were carried out without the adsorbent and there was negligible adsorption by the tube walls (<3%).

Phytotoxicity test

Phytotoxicity was investigated by a root elongation assay, as described in the US Environmental Protection Agency guideline [21] and Ma et al. [22], with some modifications. Rice seeds (Wuwangnong Group) were sterilized in 10% (v/v) sodium hypochlorite solution for 10 min, thoroughly rinsed 3 times with sterilized Milli-Q water, and then soaked in Milli-Q water for 2 d at 25 °C in the dark. The uniform germinated seeds were then sown on Whatman filter paper moistened with 5 mL test solutions (pH 7.0) in 9-cm glass Petri dishes (15 seeds/dish). The tests were carried out in the following order. The chemicals (Cu, HA, and bare and HA-coated NPs) were first tested individually, and then in combinations. Detailed test steps were as follows. First, to evaluate the potential toxic effects of Cu(II), NPs and HA alone, seeds were exposed to a series of concentrations of Cu(II) (0 mg/L, 0.2 mg/L, 0.5 mg/L, 1 mg/L, 2 mg/L, 5 mg/L, and 10 mg/L) and then to bare and HA-coated NPs (0 mg/L, 100 mg/L, 200 mg/L, 500 mg/L, and 1000 mg/L), as well as to HA (0 mg/L, 10 mg/L, 20 mg/L, 50 mg/L, and 100 mg/L). Second, to investigate the effects of NPs and HA on the phytotoxicity of Cu(II), seeds were treated with 2 mg/L Cu(II) plus bare NPs at 0 mg/L, 100 mg/L, 200 mg/L, 500 mg/L, and 1000 mg/L, and also plus HA at 0 mg/L, 10 mg/L, 20 mg/L, 50 mg/L, and 100 mg/L. Third, to assess the combined influences of NPs and HA, seeds were exposed to 2 mg/L Cu(II) in the presence of bare NPs (100 mg/L and 1000 mg/L) with HA (100 mg/L); seeds were also treated with 2 mg/L Cu(II) with 100 mg/L and 1000 mg/L bare and HA-coated NPs, respectively. The control seeds were treated with pure Milli-Q water for comparison. The pH of the test solutions was 7.0. The mixed test solutions were allowed to equilibrate for 2 h before they were added to the Petri dishes. Each treatment was performed in triplicate. The Petri dishes were covered, sealed with tape to minimize evaporation and placed in a growth chamber at 25 °C in the dark. After 3 d, root length was measured with a millimeter ruler.

Statistical analysis

Statistical analysis was performed using the statistical package SPSS (Ver 20.0) for Windows. The data are mean values ± standard deviation (SD) of 3 replicates in hydrodynamic

size, zeta potential, and root elongation tests. The statistical significance of the differences among root length values from the different treatments was evaluated by one-way analysis of variance with Fisher's least significant difference) post hoc test, and $p < 0.05$ was considered to be a significant difference.

RESULTS AND DISCUSSION

Characterization of bare and HA-coated NPs

Selected physicochemical properties of bare and HA-coated NPs are given in Table 1. Both the specific surface area and the pore volume of TiO₂ NPs and CeO₂ NPs decreased after HA coating, probably because of the surface adsorption and pore blocking of HA; but the effect of HA on the aggregation of TiO₂ and CeO₂ NPs was not significant considering the hydrodynamic diameter.

According to the results of zeta potential measurements (Table 1), bare TiO₂ NPs were negatively charged, but CeO₂ NPs were positively charged in Milli-Q water at neutral pH. The zeta potentials of both TiO₂ NPs and CeO₂ NPs were reduced by surface-bound HA, and adsorbed HA reversed the zeta potential of CeO₂ NPs from positive to negative at a high mass ratio (CeO₂-20%HA). One explanation for this is that HA contains both strong and weak acidic groups such as carboxyl and phenolic hydroxyl groups, and they continuously deprotonate according to their acid dissociation constant (pK_a) distribution. Carboxyl groups and phenolic groups have pK_a values of 3.62 and 9, respectively, which means that the carboxyl groups will be unprotonated but the phenolic groups will be protonated at neutral pH [23,24]. Because of their total amount ratio [25], HA molecules are partially deprotonated at neutral pH, making HA negatively charged in total. Hence, as the CeO₂ NP surface is positively charged and HA molecules are partially deprotonated at neutral pH, this charge neutralization can be expected. Surface-bound HA enhanced the stability of TiO₂ NP suspension because of the increased negative charge but reduced the stability of CeO₂ NPs suspension at low mass ratio as a result of charge neutralization. Charge neutralization also was reported to accelerate the sedimentation rate of ZnO NPs in the presence of HA at low concentrations [26]. The effect of HA coating can be attributed to the negatively charged adsorbed HA molecules on the surface of NPs at neutral pH.

The FTIR spectra of bare and HA-coated NPs are shown in Figure 1. The peaks of approximately 3423 cm⁻¹ and 1629 cm⁻¹ for bare and HA-coated TiO₂ NPs were assigned to a hydroxyl group stretching on the surface of NPs and water [13], the peak intensity of which increased after HA coating. A new peak of approximately 1390 cm⁻¹ appeared and increased with the HA coating, suggesting the formation of a Ti-acetate complex between the -COOH of HA and the

Ti of TiO₂ [13]. As for CeO₂ NPs, there were no obvious striking changes in the peak of approximately 3423 cm⁻¹ as a result of the HA coating, but the peaks of approximately 2926 cm⁻¹ and 2856 cm⁻¹ (asymmetric and symmetric C-H stretching of aliphatic -CH₂) increased after HA coating. The bands in the frequency region from 1200 cm⁻¹ to 1700 cm⁻¹ were assigned to carbonate species [27]. The band of approximately 1629 cm⁻¹ corresponded to the bending of H-O-H, which partly overlapped the O-C-O stretching band [27]. A significant peak shift from 1335 cm⁻¹ of bare CeO₂ to 1375 cm⁻¹ of the CeO₂-HA complex could be the result of strong interactions of -COOH with CeO₂ NPs. The coating of these anionic functional groups could be the reason for the increased negative charges on NPs in the presence of surface-bound HA.

Adsorption of Cu(II) onto bare and HA-coated NPs

The adsorption isotherm was determined using 2 h of equilibrium time, and the results are presented in Figure 2. The Freundlich adsorption model is widely used for adsorption isotherm fitting, and previous studies have shown that Cd(II) adsorption onto TiO₂ NPs [16] and Cr(VI) adsorption onto CeO₂ NPs [28] were best explained by a Freundlich model. Therefore, the Freundlich model, as follows, was used for data fitting

$$q_e = KC_e^{1/n}$$

where q_e and C_e are equilibrium adsorbed (mg/g) and nonadsorbed (mg/L) Cu(II) concentrations, respectively; K is the adsorption coefficient ([L/gⁿ]); and $1/n$ is used as an indicator of isotherm nonlinearity. A higher K implies a higher selectivity or adsorption. The Freundlich parameters were obtained by curve fitting using SigmaPlot (Ver 12.5) and are given in Table 2. The K values of CeO₂-2%HA and TiO₂-2%HA for Cu(II) were higher than those of bare TiO₂ and CeO₂ NPs, respectively, indicating that the HA coating enhanced the adsorption capacity of NPs. Note that the K values of Cu(II) adsorbed onto TiO₂-20%HA and CeO₂-20%HA were respectively near to or smaller than that adsorbed onto TiO₂-2%HA and CeO₂-2%HA, suggesting that the adsorption capacity is not proportional to the amount of surface-bound HA. The interaction of NPs with HA might lead to 2 opposite effects on the adsorption of toxic substances. On the one hand, the interaction may increase the available adsorption probability attributable to the HA-introduced anionic functional groups on the NP surfaces. The HA-introduced anionic functional groups not only could directly bind around the aqueous metal ions through chemical complexation, but also could increase the charge negativities of the NPs, which could enhance metal adsorption by electrostatic attraction. In addition, surface-bound

Table 1. Selected characteristics of bare and humic acid (HA)-coated nanoparticles

Sample ^a	HA content (%)	SSA _{BET} (cm ² /g)	Pore volume (cm ³ /g)	Hydrodynamic diameter ^b (nm)	Zeta potential (mV)
TiO ₂	N/A	57.66	0.14	252.80 ± 4.23 B	-14.17 ± 4.38 A
TiO ₂ -2%HA	1.14	53.09	0.13	241.83 ± 7.89 C	-31.20 ± 1.47 B
TiO ₂ -20%HA	4.83	53.49	0.14	255.63 ± 1.10 A	-35.23 ± 2.07 B
CeO ₂	N/A	98.61	0.31	284.93 ± 13.76 B	24.23 ± 0.68 A
CeO ₂ -2%HA	1.59	84.45	0.29	351.20 ± 18.16 A	9.64 ± 1.01 B
CeO ₂ -20%HA	5.71	86.92	0.29	282.63 ± 3.90 B	-27.13 ± 2.83 B

^aThe percentages stand for the mass ratios of HA to nanoparticles used in the loading process.

^bThe data were measured at pH 7.0 in Milli Q water with 100 mg/L of the test substances ($n = 3$). Capitalized letters indicate the significance in each group of TiO₂ and CeO₂ treatments individually ($p < 0.05$).

SSA_{BET} = specific surface area measured using the Brunauer-Emmett-Teller method.

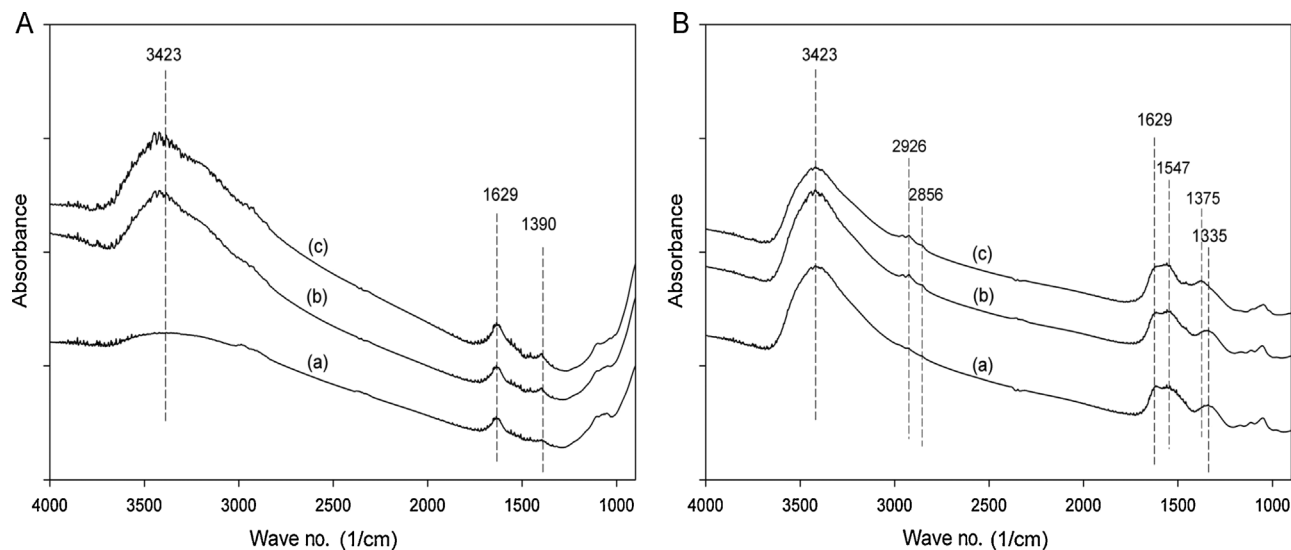


Figure 1. Fourier transform infrared spectroscopy (FTIR) spectra of bare and humic acid (HA)-coated TiO₂ nanoparticles (NPs; **A**) and CeO₂ NPs (**B**). Line a represents bare NPs, line b represents NP-2%HA, and line c represents NP-20%HA.

HA could improve dispersion of the NPs through steric and electrosteric repulsion, which would then expose more accessible adsorption sites and contribute to adsorption enhancement. On the other hand, increasing surface-bound

HA may reduce the available adsorption sites for toxicants because of competition and/or blockage by adsorbed HA fractions [29], as well as a decrease in NP dispersion and specific surface area.

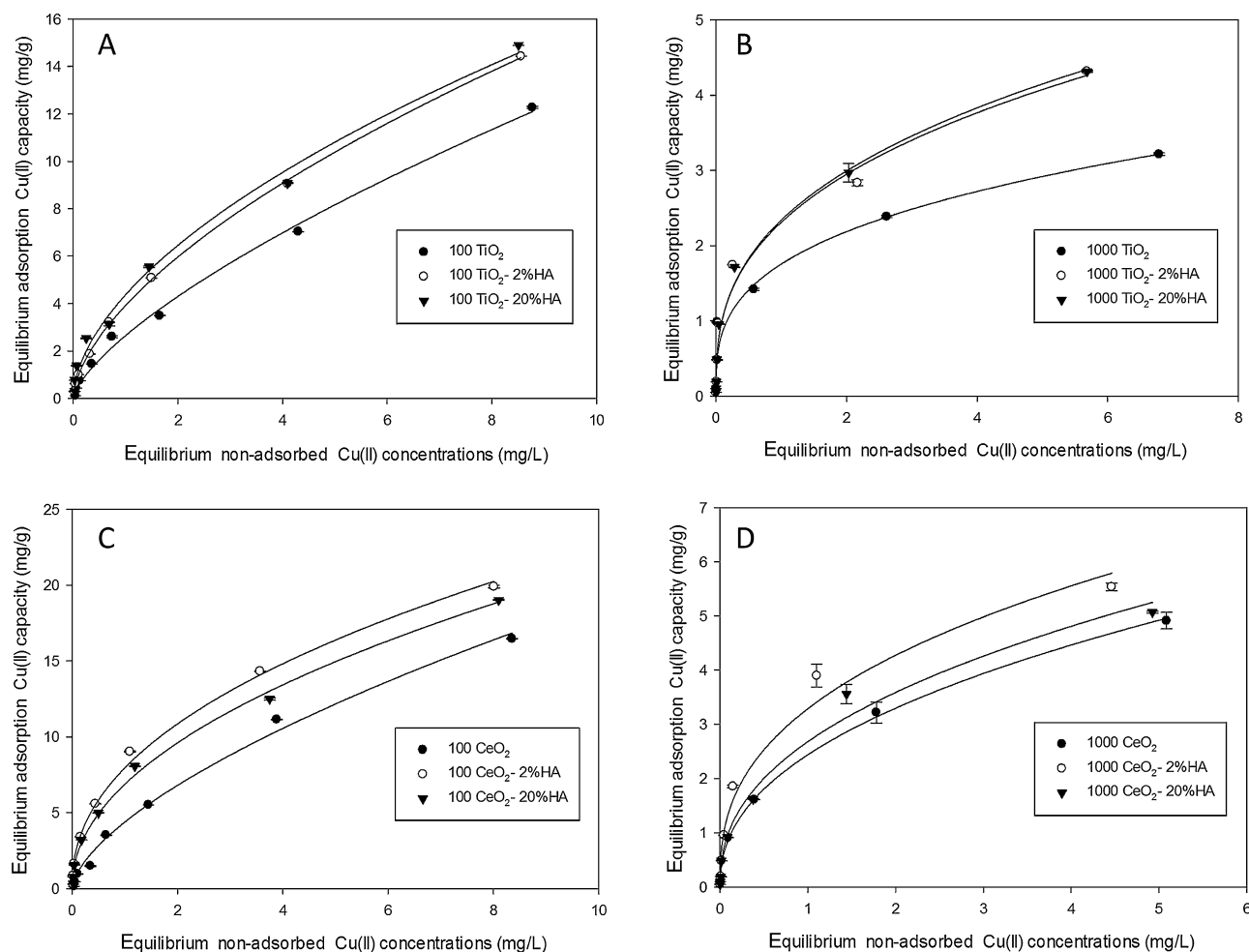


Figure 2. Freundlich adsorption curves of Cu(II) onto bare and humic acid (HA)-coated TiO₂ nanoparticles (NPs; **A**, **B**) and CeO₂ NPs (**C**, **D**) at pH 7.0. Initial concentrations of NPs were 100 mg/L (**A**, **C**) and 1000 mg/L (**B**, **D**). Values are mean $\pm$ standard deviation of triplicate samples.

Table 2. Freundlich adsorption model parameters for Cu(II) onto bare and humic acid (HA)-coated nanoparticles

Sample ^a	100 mg/L				1000 mg/L			
	<i>K</i>	1/ <i>n</i>	<i>r</i> ²	<i>p</i>	<i>K</i>	1/ <i>n</i>	<i>r</i> ²	<i>p</i>
TiO ₂	2.65 ± 0.15	0.70 ± 0.03	0.9960	<0.0001	1.75 ± 0.12	0.32 ± 0.04	0.9644	<0.0001
TiO ₂ -2%HA	3.93 ± 0.06	0.60 ± 0.01	0.9996	<0.0001	2.31 ± 0.13	0.35 ± 0.03	0.9790	<0.0001
TiO ₂ -20%HA	4.37 ± 0.25	0.56 ± 0.03	0.9929	<0.0001	2.34 ± 0.10	0.36 ± 0.03	0.9881	<0.0001
CeO ₂	4.37 ± 0.26	0.64 ± 0.03	0.9937	<0.0001	2.44 ± 0.05	0.44 ± 0.02	0.9974	<0.0001
CeO ₂ -2%HA	7.95 ± 0.31	0.45 ± 0.02	0.9937	<0.0001	3.29 ± 0.18	0.38 ± 0.04	0.9769	<0.0001
CeO ₂ -20%HA	6.85 ± 0.24	0.48 ± 0.02	0.9958	<0.0001	2.68 ± 0.13	0.42 ± 0.03	0.9867	<0.0001

^aThe percentage numbers stand for the mass ratios of HA to nanoparticles used in the loading process.

K = adsorption coefficient ([L/gⁿ]); 1/*n* = an indicator of isotherm nonlinearity.

Phytotoxicity of Cu(II) or bare and HA-coated NPs

The effects of Cu(II), dissolved HA, and bare and HA-coated NPs on rice root growth are shown in Figure 3. Root length was negatively related to the exposure concentration of Cu(II) in solutions (Figure 3A). The median effective concentration (EC50; i.e., the concentration that reduced root growth of rice seedlings by 50% based on Cu(II) concentration in solution) was 1.6 mg/L. We found that dissolved HA slightly promoted rice root growth even at high concentrations (50 mg/L and 100 mg/L; Figure 3B), which was likely because of the role of HA as a

nutrition source [30]. In terms of NPs, both bare TiO₂ and CeO₂ NPs suspensions (up to 1000 mg/L) had no obvious phytotoxicity on rice seedling growth (Figure 3C and D). However, the HA-coated NPs slightly promoted root growth at high concentrations (e.g., TiO₂-20%HA at 200–1000 mg/L, CeO₂-2%HA at 200 mg/L and 500 mg/L, and CeO₂-20%HA at 200–1000 mg/L; Figure 3C and D), which may be attributed to the dissolved HA released from HA-coated NPs. Many studies have reported the effects of TiO₂ and CeO₂ NPs on organisms. In terms of plants, TiO₂ and CeO₂ NPs usually show low toxicity to or positive effects on many plant species. For example, no toxic

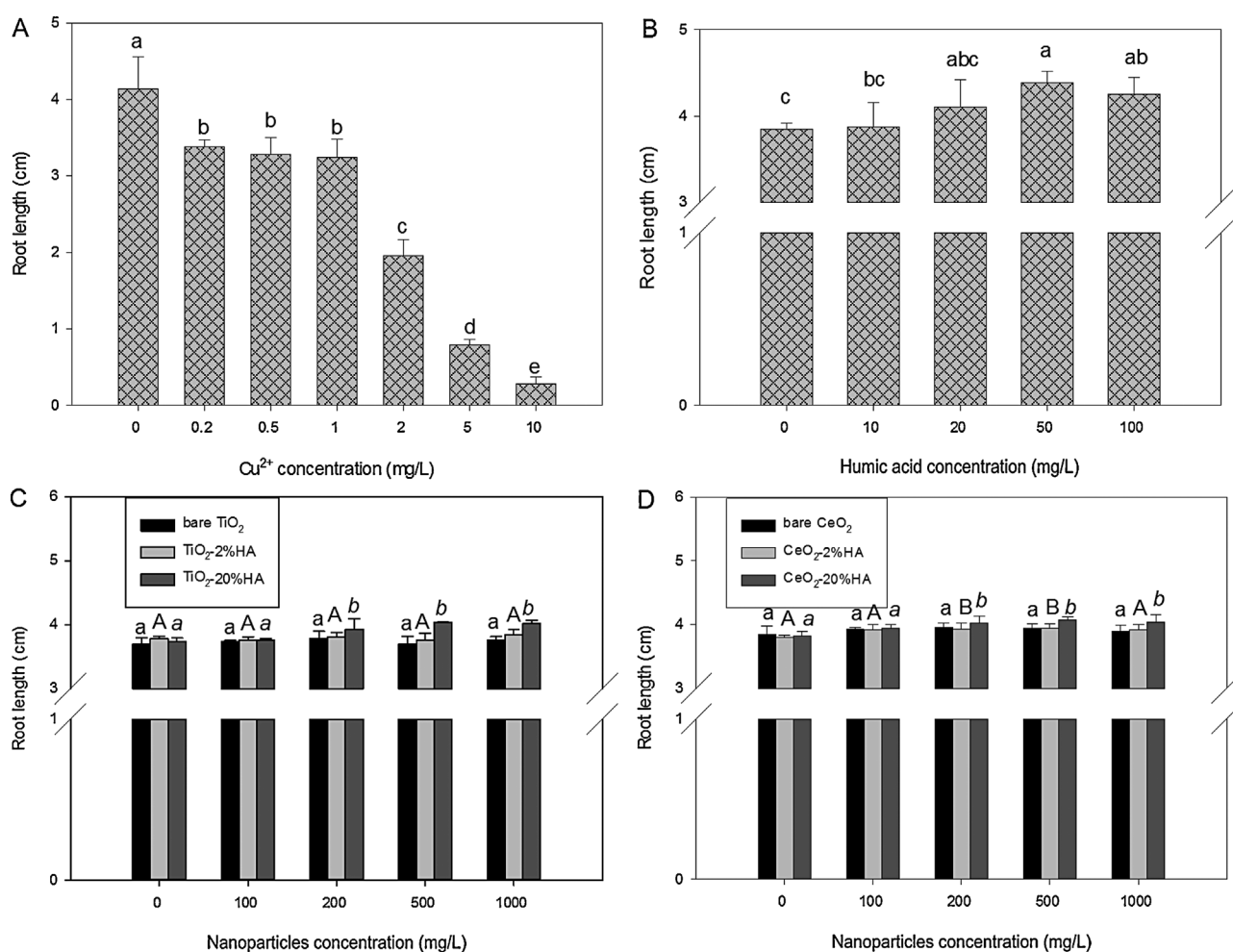


Figure 3. Root elongation of rice seedlings treated with Cu(II) (A), dissolved humic acid (HA; B), bare and HA-coated TiO₂ nanoparticles (NPs; C), and bare and HA-coated CeO₂ NPs (D). The control groups were treated with pure Milli-Q water. The values are mean ± standard deviation of triplicate samples. Different letters represent significant differences between the treatment means (*p* < 0.05, Fisher's least significant difference).

effect on cucumber (*Cucumis sativus*) root growth was observed with TiO₂ NP concentrations up to 4000 mg/L, whereas shoot growth was promoted by TiO₂ NPs [31]. In addition, CeO₂ NPs (at 2000 mg/L) were reported to have no obvious effect on root growth of several plant species, including rape (*Brassica napus*), radish (*Raphanus sativus*), wheat (*Triticum aestivum*), cabbage (*Brassica oleracea*), tomato (*Lycopersicon esculentum*), and cucumber; lettuce (*Lactuca sativa*) was an exception [22]. However, negative effects of TiO₂ and CeO₂ NPs on plants were also reported in some studies. For example, TiO₂ NPs were reported to inhibit maize (*Zea mays*) growth as a result of inhibition of root hydraulic conductivity from physical blockage [32]. Genotoxicity of TiO₂ NPs in onion (*Allium cepa*) and tobacco (*Nicotiana tabacum*) has been observed [33], and potential genotoxicity of CeO₂ NPs in soybean (*Glycine max*) also has been reported [34]. The effects of TiO₂ and CeO₂ NPs may depend on NP characteristics, plant species and growth stages, environmental conditions, and exposure methods.

Phytotoxicity of Cu(II) in the presence of bare NPs and dissolved HA

As can be seen in Figure 4A, rice root growth was promoted in the presence of bare TiO₂ and CeO₂ NPs, compared with Cu treatment alone, with significant differences emerging at high NP concentrations. Compared with Cu-alone treatment, TiO₂ NPs at 500 mg/L and 1000 mg/L increased rice root length by 40.0% and 32.5%, respectively; CeO₂ NPs at 200 mg/L, 500 mg/L, and 1000 mg/L increased root length by 51.1%, 33.3%, and 39.0%, respectively, whereas both TiO₂ and CeO₂ NPs at 100 mg/L did not significantly promote root length. The results indicated that both TiO₂ and CeO₂ NPs could alleviate the toxicity of Cu(II) to the root growth of rice seedlings, which was consistent with a previous study showing that Cd-induced root growth inhibition in 4 plants was mitigated by the presence of NPs [20]. Compared with the Cu-alone treatment, the presence of HA (100 mg/L) significantly increased the root length (Figure 4B), apparently because of the decrease in bioavailable Cu(II) concentration after complexing with HA.

The results in Figure 5 show the comparison of mixed NPs and HA with single NPs or HA with respect to alleviation of Cu(II) phytotoxicity. A significant reduction in Cu(II)-induced root growth inhibition was observed with additional matrices, compared with inhibition in solutions with no matrix. Compared with Cu alone, Cu + HA, Cu + 100 mg/L TiO₂, Cu + 100 mg/L TiO₂ + HA, Cu + 1000 mg/L TiO₂, and Cu + 1000 mg/L TiO₂ + HA increased rice root length by 24.6%, 15.6%, 31.4%, 58.1%, and 90.2%, respectively; Cu + HA, Cu + 100 mg/L CeO₂, Cu + 100 mg/L CeO₂ + HA, Cu + 1000 mg/L CeO₂, and Cu + 1000 mg/L CeO₂ + HA increased root length by 68.4%, 26.7%, 84.1%, 54.1%, and 100.1%, respectively. The Cu(II)-induced root growth inhibition in the mixtures of NPs and HA solutions was significantly lower than that in the corresponding single NPs or HA solutions. These results demonstrate that coexistence of NPs and HA could effectively mitigate Cu(II) phytotoxicity. A previous study also showed that equilibrium Cd bioaccumulation in zebrafish in mixed TiO₂ NPs and HA solutions was lower than that in single TiO₂ NPs solutions [35]. Moreover, the decrease in Pb and Cu accumulation in *C. reinhardtii* induced by combined HA and carboxyl-CdSe/ZnS quantum dots was more significant than that induced by quantum dots alone [18]. The reduced toxicity or bioaccumulation of metals apparently resulted from adsorption of metals to both NPs and HA. In the mixtures of NPs and HA, however, a portion of the HA was

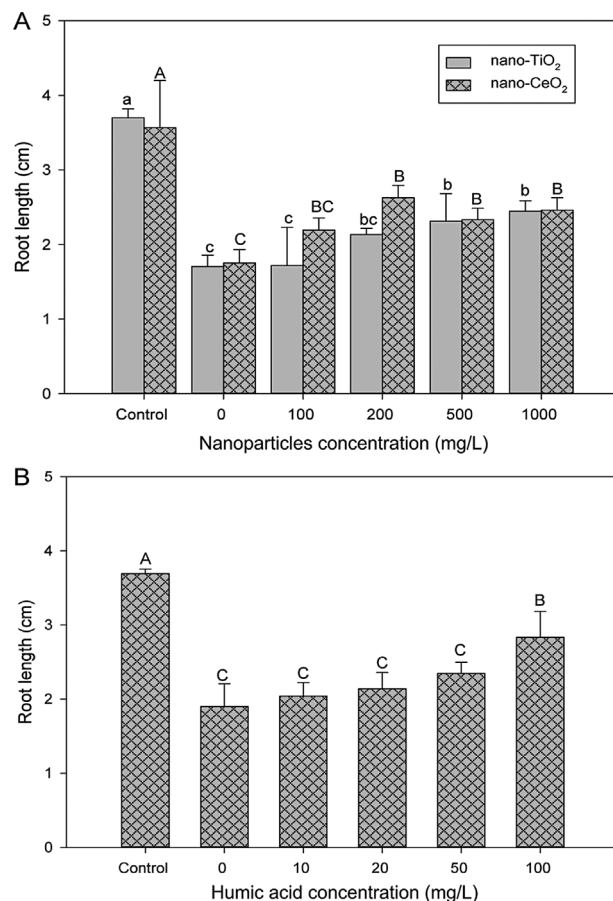


Figure 4. Root elongation of rice seedling treated with 2 mg/L Cu(II) in the presence of bare nanoparticles (A) and humic acid (B), respectively. The control groups were treated with pure Milli-Q water. The values are mean $\pm$ standard deviation of triplicate samples. Different letters represent significant differences between the treatment means ($p < 0.05$, Fisher's least significant difference).

adsorbed onto NP surfaces, leading to a decrease in dissolved HA; the surface-bound HA also could change the properties and adsorption capacity of NPs. As stated, surface-bound HA changed the dispersion, stability, and adsorption capacity of both TiO₂ and CeO₂ NPs. Therefore, the overall effects of NP and HA mixtures may be not equivalent to the sum of single NPs and HA.

Phytotoxicity of Cu(II) in the presence of bare and HA-coated NPs

To further clarify the potential effects of HA on NP-alleviated phytotoxicity of Cu(II), the influences of bare and HA-coated NPs on Cu(II) phytotoxicity were compared (Figure 6). First, 100 mg/L HA-coated TiO₂ NPs slightly enhanced the mitigation of Cu(II) phytotoxicity compared with 100 mg/L bare TiO₂ NPs (Figure 6A). Addition of 1000 mg/L TiO₂ NPs, TiO₂-2%HA, and TiO₂-20%HA increased root length by 45.2%, 64.2%, and 66.5%, respectively, compared with Cu alone (Figure 6A). As for CeO₂, 100 mg/L bare or HA-coated CeO₂ NPs had no obvious effect on Cu(II) phytotoxicity, whereas addition of 1000 mg/L CeO₂ NPs, CeO₂-2%HA, and CeO₂-20%HA increased root length by 52.6%, 85.1%, and 56.8%, respectively, compared with Cu alone (Figure 6B). The mitigation of Cu(II) phytotoxicity was proportional to the adsorption capacity of bare or HA-coated NPs for Cu(II) (Figure 2 and Table 1). Therefore, the alleviation of Cu(II)

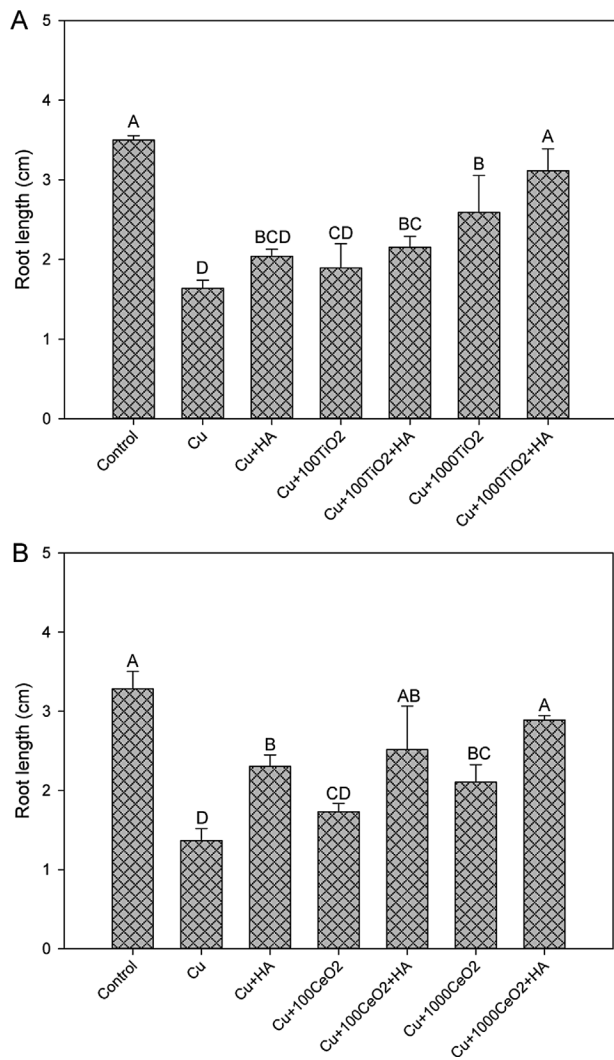


Figure 5. Root length of rice seedling exposed to 2 mg/L Cu(II) plus nanoparticles (NPs), humic acid (HA), and mixtures of NPs and HA, respectively. (A) Cu(II) plus TiO₂ NPs and/or HA. (B) Cu(II) plus CeO₂ NPs and/or HA. Initial concentration of NPs: 100 mg/L and 1000 mg/L; HA: 100 mg/L. The control groups were treated with pure Milli-Q water. The values are mean $\pm$ standard deviation of triplicate samples. Different letters represent significant differences between the treatment means ($p < 0.05$, Fisher's least significant difference).

phytotoxicity could be the result of a decrease in bioavailable soluble Cu(II).

Engineered NPs can act as carriers to facilitate metal bioaccumulation, or they may serve as scavengers to reduce the exposure of organisms to metals. The overall effects of NPs on the bioavailability and toxicity of metals to tested organisms depend on whether the reduction in uptake of soluble metals or the additional uptake of the NP-bound metals is predominant. Most aquatic organisms such as fishes and zooplankton could actively ingest NPs through the dietary pathway (e.g., via gills or intestine). Facilitated uptake of metals may occur when metal-loaded NPs are transported via gills or intestine. As a result, the bioaccumulation and toxicity of metals may be enhanced in the presence of NPs. Several studies have provided evidence of facilitated accumulation and/or enhanced toxicity of metals in aquatic organisms by metal oxide NPs [6–9,36], carbon nanotubes [37], and quantum dots [38]. However, unicellular green alga could not internalize NP–metal complexes to a high degree, because

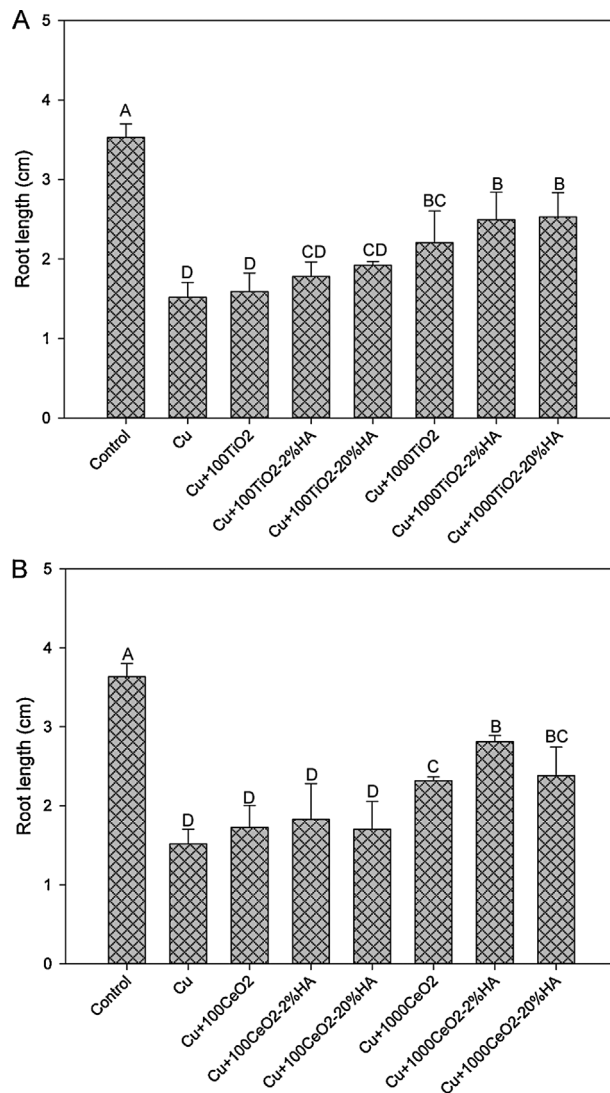


Figure 6. Root growth of rice seedling treated with 2 mg/L Cu(II) in the presence of bare and humic acid (HA)-coated TiO₂ nanoparticles (NPs; A) and CeO₂ NPs (B), respectively. Initial concentration of NPs: 100 mg/L and 1000 mg/L. The control groups were treated with pure Milli-Q water. The values are mean $\pm$ standard deviation of triplicate samples. Different letters represent significant differences between the treatment means ($p < 0.05$, Fisher's least significant difference).

of their limited size and the presence of a cell wall, which may result in a decrease in metal bioavailability to alga [39], as demonstrated in a previous study that showed that carboxyl-CdSe/ZnS quantum dots decreased Pb and Cu accumulation in walled *C. reinhardtii* but increased the intracellular content of metals in wall-less strains [18]. As for higher plants, some studies have demonstrated the uptake of many NPs containing TiO₂ and CeO₂ NPs by plants, which has been summarized in a series of review papers [19,40]. However, the root uptake of NPs in plants is limited because of the existence of a cell wall, suberin, cutin, and a casparian strip [41], and the NP-associated metals are thus less bioavailable to plants than soluble metals [20]. Therefore, in the present study, the presence of bare or HA-coated NPs reduced the phytotoxicity of Cu(II) to rice seedling as a result of adsorption, leading to a decrease in the bioavailable soluble Cu(II) concentration in the liquid medium.

CONCLUSIONS

The results of the present study showed that TiO₂ and CeO₂ NPs combined with HA can alter the sorption and toxicity of Cu(II) and potentially other heavy metals to plant species. Adsorption of Cu(II) onto TiO₂ and CeO₂ NPs resulted in a decrease in bioavailable soluble Cu(II) concentration and alleviation of Cu(II) phytotoxicity to rice seedling. The presence of surface-bound HA enhanced the adsorption capacity of TiO₂ and CeO₂ NPs for Cu(II) and thus improved the mitigation of Cu(II) phytotoxicity. The presence of mixed NPs and HA had the greatest mitigation effect because of the overall effects of NPs as well as dissolved and absorbed HA. Our results suggest that the presence of engineered NPs can change the bioavailability and toxicity of metals (and potentially other contaminants) to organisms and that NOM or other environmental components may affect the interactions of NPs and other pollutants. Hence, research should focus not only on the fate and toxicity of NPs themselves but also on the potential enhancement or reduction in the bioavailability and toxicity of other toxic pollutants when they coexist, which will help us to better understand the potential behavior of NPs in the environment.

SUPPLEMENTAL DATA

Supplemental Methods.

Supplemental Figures S1 and S2. (42 KB DOC).

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REFERENCES

- Van Broekhuizen P, van Broekhuizen F, Cornelissen R. 2011. Use of nanomaterials in the European construction industry and some occupational health aspects thereof. *J Nanopart Res* 13:447–462.
- Gogos A, Knauer K, Bucheli T.D. 2012. Nanomaterials in plant protection and fertilization: Current state, foreseen applications, and research priorities. *J Agr Food Chem* 60:9781–9792.
- Kwon S, Fan M, Cooper AT. 2008. Photocatalytic applications of micro- and nano-TiO₂ in environmental engineering. *Crit Rev Environ Sci Technol* 38:197–226.
- Nowack B, Bucheli TD. 2007. Occurrence, behavior and effects of nanoparticles in the environment. *Environ Pollut* 150:5–22.
- Baun A, Hartmann NB, Grieger K, Kusk KO. 2008. Ecotoxicity of engineered nanoparticles to aquatic invertebrates: A brief review and recommendations for future toxicity testing. *Ecotoxicology* 17:387–395.
- Fan W, Cui M, Liu H, Wang C, Shi Z, Tan C, Yang X. 2011. Nano-TiO₂ enhances the toxicity of copper in natural water to *Daphnia magna*. *Environ Pollut* 159:729–734.
- Wang D, Hu J, Irons DR, Wang J. 2011. Synergistic toxic effect of nano-TiO₂ and As(V) on *Ceriodaphnia dubia*. *Sci Total Environ* 409:1351–1356.
- Hu J, Wang D, Wang J, Wang J. 2012. Toxicity of lead on *Ceriodaphnia dubia* in the presence of nano-CeO₂ and nano-TiO₂. *Chemosphere* 89:536–541.
- Wang D, Hu J, Forthaus BE, Wang J. 2011. Synergistic toxic effect of nano-Al₂O₃ and As(V) on *Ceriodaphnia dubia*. *Environ Pollut* 159:3003–3008.
- Yang W, Li Y, Miao A, Yang L. 2012. Cd²⁺ toxicity as affected by bare TiO₂ nanoparticles and their bulk counterpart. *Ecotoxicol Environ Saf* 85:44–51.
- Zhang X, Sun H, Zhang Z, Niu Q, Chen Y, Crittenden JC. 2007. Enhanced bioaccumulation of cadmium in carp in the presence of titanium dioxide nanoparticles. *Chemosphere* 67:160–166.
- Yang R, van den Berg CMG. 2009. Metal complexation by humic substances in seawater. *Environ Sci Technol* 43:7192–7197.
- Yang K, Lin D, Xing B. 2009. Interactions of humic acid with nanosized inorganic oxides. *Langmuir* 25:3571–3576.
- Yang K, Xing B. 2009. Sorption of phenanthrene by humic acid-coated nanosized TiO₂ and ZnO. *Environ Sci Technol* 43:1845–1851.
- Liu J, Zhao Z, Jiang G. 2008. Coating Fe₃O₄ magnetic nanoparticles with humic acid for high efficient removal of heavy metals in water. *Environ Sci Technol* 42:6949–6954.
- Chen Q, Yin D, Zhu S, Hu X. 2012. Adsorption of cadmium(II) on humic acid coated titanium dioxide. *J Colloid Interface Sci* 367:241–248.
- Kim KT, Edgington AJ, Klaine SJ, Cho JW, Kim SD. 2009. Influence of multiwalled carbon nanotubes dispersed in natural organic matter on speciation and bioavailability of copper. *Environ Sci Technol* 43:8979–8984.
- Worms IAM, Boltzman J, Garcia M, Slaveykova VI. 2012. Cell-wall-dependent effect of carboxyl-CdSe/ZnS quantum dots on lead and copper availability to green microalgae. *Environ Pollut* 167:27–33.
- Rico CM, Majumdar S, Duarte-Gardea M, Peralta-Videa JR, Gardea-Torresdey JL. 2011. Interaction of nanoparticles with edible plants and their possible implications in the food chain. *J Agr Food Chem* 59:3485–3498.
- Wang M, Chen L, Chen S, Ma Y. 2012. Alleviation of cadmium-induced root growth inhibition in crop seedlings by nanoparticles. *Ecotoxicol Environ Saf* 79:48–54.
- US Environment Protection Agency. 2012. Ecological effects test guidelines—OCSPP 850.4230: Early seedling growth toxicity test. EPA 712/C/010. [cited 2015 February]. Available from: <http://www.regulations.gov/#/documentDetail;D=EPA-HQ-OPPT-2009-0154-0025>
- Ma Y, Kuang L, He X, Bai W, Ding Y, Zhang Z, Zhao Y, Chai Z. 2010. Effects of rare earth oxide nanoparticles on root elongation of plants. *Chemosphere* 78:273–279.
- Fukushima M, Tanaka S, Hasebe K. 1995. Interpretation of the acid-base equilibrium of humic acid by a continuous pK distribution and electrostatic model. *Anal Chim Acta* 302:365–373.
- Choppin GR, Kullberg L. 1978. Protonation thermodynamics of humic acid. *J Inorg Nucl Chem* 40:651–654.
- Perdue E.M. 1978. Solution thermochemistry of humic substances—I. Acid-base equilibria of humic acid [J]. *Geochim Cosmochim Acta* 42:1351–1358.
- Bian S, Mundukotuwa IA, Rupasinghe T, Grassian VH. 2011. Aggregation and dissolution of 4 nm ZnO nanoparticles in aqueous environments: Influence of pH, ionic strength, size, and adsorption of humic acid. *Langmuir* 27:6059–6068.
- Mokkelbost T, Kaus I, Grande T, Einarsson MA. 2004. Combustion synthesis and characterization of nanocrystalline CeO₂-based powders. *Chem Mater* 16:5489–5494.
- Recillas S, Colon J, Casals E, Gonzalez E, Puentes V, Sanchez A, Font X. 2010. Chromium VI adsorption on cerium oxide nanoparticles and morphology changes during the process. *J Hazard Mater* 184:425–431.
- Zhang SJ, Shao T, Bekaroglu SSK, Karanfil T. 2010. Adsorption of synthetic organic chemicals by carbon nanotubes: Effects of background solution chemistry. *Water Res* 44:2067–2074.
- Khan A, Khan M.Z., Hussain F. 2013. Effect of humic acid on the growth, yield, nutrient composition, photosynthetic pigment and total sugar contents of peas (*Pisum sativum* L). *J Chem Soc Pakistan* 44:206–211.
- Servin AD, Castillo-Michel H, Hernandez-Viezas JA, Diaz BC, Peralta-Videa JR, Gardea-Torresdey JL. 2012. Synchrotron micro-XRE and micro-XANES confirmation of the uptake and translocation of TiO₂ nanoparticles in cucumber (*Cucumis sativus*) plants. *Environ Sci Technol* 46:7637–7643.
- Asli S, Neumann PM. 2009. Colloidal suspensions of clay or titanium dioxide nanoparticles can inhibit leaf growth and transpiration via physical effects on root water transport. *Plant Cell Environ* 32:577–584.
- Ghosh M, Bandyopadhyay M, Mukherjee A. 2010. Genotoxicity of titanium dioxide (TiO₂) nanoparticles at two trophic levels: Plant and human lymphocytes. *Chemosphere* 81:1253–1262.
- Lopez-Moreno ML, de la Rosa G, Hernandez-Viezas JA, Castillo-Michel H, Botez CE, Peralta-Videa JR, Gardea-Torresdey JL. 2010. Evidence of the differential biotransformation and genotoxicity of ZnO and CeO₂ nanoparticles on soybean (*Glycine max*) plants. *Environ Sci Technol* 44:7315–7320.
- Hu X, Chen Q, Jiang L, Yu Z, Jiang D, Yin D. 2011. Combined effects of titanium dioxide and humic acid on the bioaccumulation of cadmium in Zebrafish. *Environ Pollut* 159:1151–1158.

36. Tan C, Fan W, Wang W. 2012. Role of titanium dioxide nanoparticles in the elevated uptake and retention of cadmium and zinc in *Daphnia magna*. *Environ Sci Technol* 46:469–476.
37. Kim KT, Klaine SJ, Lin S, Ke PC, Kim SD. 2010. Acute toxicity of a mixture of copper and single-walled carbon nanotubes to *Daphnia magna*. *Environ Toxicol Chem* 29:122–126.
38. Zhang W, Sun X, Chen L, Lin K, Dong Q, Huang C, Fu R, Zhu J. 2012. Toxicological effect of joint cadmium selenium quantum dots and copper ion exposure on zebrafish. *Environ Toxicol Chem* 31:2117–2123.
39. Yang W, Miao A, Yang L. 2012. Cd²⁺ toxicity to a green alga *Chlamydomonas reinhardtii* as influenced by its adsorption on TiO₂ engineered nanoparticles. *Plos ONE* 7:e32300.
40. Miralles P, Church TL, Harris AT. 2012. Toxicity, uptake, and translocation of engineered nanomaterials in vascular plants. *Environ Sci Technol* 46:9224–9239.
41. Dietz KJ, Herth S. 2011. Plant nanotoxicology. *Trends Plant Sci* 16:582–589.