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RESEARCH ARTICLE

Phytotoxicity and accumulation of zinc oxide nanoparticles on the aquatic plants *Hydrilla verticillata* and *Phragmites Australis*: leaf-type-dependent responses

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Abstract The phytotoxicity and accumulation of zinc oxide nanoparticles (ZnO NPs) on aquatic plant *Hydrilla verticillata* and *Phragmites australis* were investigated using mesocosms. The percentage of dissolved Zn in the ZnO NP treatment solutions was measured along with plant shoot growth, antioxidant enzyme activity, chlorophyll content, and Zn content. The dissolution rate of ZnO NPs in Hoagland solution was inversely related to the concentration. The submerged aquatic plant *H. verticillata*, growth was reduced during the early stages of the experiment when exposed to the highest ZnO NP concentration (1000 mg/L), whereas the emerged aquatic plant *P. australis* began to show significantly reduced growth after a few weeks. The measurements of chlorophyll content, antioxidant enzyme activity, and Zn accumulation showed that *P. australis* was adversely affected by NPs and absorbed more Zn than *H. verticillata*. The results indicated that physiological differences among aquatic plants, such as whether they use leaves or roots for nutrient and water uptake, led to differences in nanoparticle toxicity. Overall, High ZnO NP concentrations caused significant phytotoxicity on aquatic plants, and low concentrations caused unpredictable phytotoxicity. Therefore, the use and disposal of zinc oxide nanoparticles should be carefully monitored.

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Introduction

Nanotechnology-based engineered nanoparticles (NPs) are now an important part of the industry and are used in numerous applications. There is a growing scientific concern that NPs may confer environmental risks that are not observed with macroscopic particles (Srivastava 2014), but the risks posed by manufactured NPs are still debated (Ruffini Castiglione et al. 2011). It is important to ascertain the safety of engineered NPs for current and future applications. Investigations into the toxicity of NPs have been conducted using microbial, whole animal, and cellular models (Song et al. 2013a). However, the toxicity of NPs for higher plants remains largely unexplored (Ruffini Castiglione et al. 2011), and few studies have investigated the effects of NPs on aquatic plants (Srivastava 2014). Zinc oxide (ZnO) NPs have been used in a variety of products such as semiconductors, catalysts, paints (Reed et al. 2012), sunscreen, and antibacterials (Moorer and Genet 1982). Only a few studies have been conducted to assess the effects of ZnO NPs on higher plants (Shaymurat et al. 2012), but no studies have utilized aquatic plants.

This research uses mesocosms to investigate the phytotoxicity of ZnO NPs on two types of aquatic plants: the submerged aquatic plant *Hydrilla verticillata* (L.f.) Royle, and the emerged aquatic plant *Phragmites australis* Trin. Different mesocosms were developed for each plant species to test the phytotoxic effects of ZnO NPs. The uptake of ZnO NPs and its effects on shoot growth and physiological

responses such as antioxidant enzyme activities and chlorophyll content were measured.

Materials and methods

Nanoparticles

Zinc oxide nanoparticles were obtained from Sigma-Aldrich (product CAS Number 1314-13-2; Dorset, UK). The properties of the supplied ZnO NPs were as follows: nanopowder, purity ≥97 %, particle size <50 nm, surface area >10.8 m^2/g, and contains 6 % Al as dopant.

Plant species

The aquatic plants *H. verticillata* (L.f.) Royle and *P. australis* Trin. were chosen for research. *H. verticillata* (L.f.) Royle is a common submerged aquatic plant, which exhibits strong accumulation of heavy metals from contaminated environments (Srivastava et al. 2006). *P. australis* Trin. is a common emerged aquatic plant. We chose these two representative species to compare results between submerged and emerged aquatic plants.

Experimental design

H. verticillata seedlings were grown for over a month in water tanks at room temperature filled with 5 % Hoagland solution made with distilled water (Keeley and Bowes 1982). Roots were secured with gravel (0.3–0.6-cm diameter). Subsequently, individual plants (average 5 cm height), including roots and attached gravel, were transplanted into cylinder-shaped clear glass mesocosms (5-cm inner diameter and 13-cm height) filled with 5 % Hoagland solution (10-cm depth) made with distilled water but excluding zinc sulfate ($ZnSO_4$).

P. australis seedlings were grown in pots filled with thoroughly washed and autoclaved coarse sand with smooth surface gravel, containing Osmocote Plus (13 N +13P+ 13 K+2MgO; Scotts International B.V.). The Osmocote concentration was calculated to contain 0.5 % of the total nitrogen content of soil by weight (Cheng et al. 2007). The initial water depth was 1 cm, which was increased to 5 cm as the plants grew. After one and half months, individual *P. australis* plants (average 12-cm height) were transplanted into cylinder-shaped clear glass mesocosms (5-cm inner diameter and 13-cm height) filled with new sand and gravel (3 cm of sand at bottom and 2 cm of gravel on top) containing Osmocote, and covered with a 7-cm layer of 5 % Hoagland solution without zinc sulfate. The tops of the both species mesocosms were not sealed to allow sufficient air flow, and distilled water was added every time evaporation reduced the water level by 0.5 cm.

ZnO NPs were added to the mesocosms to reach specified concentrations, from environmentally realistic nanoparticle concentrations (0.01, 0.1, and 1 mg/L) to high concentrations (10, 100, and 1000 mg/L) (Buffet et al. 2012; Das et al. 2012). The plants were randomly placed in a growth chamber according to Organization for Economic Cooperation and Development (OECD) guidelines (OECD 2003). Plants were grown at the following conditions: temperature, 24 °C; humidity, 70 %±5 %; photoperiod, 18-h light; and light intensity, 350 $\mu E/m^2/s$.

Measurement methods

Ecophysiological measurements

Plant growth in the form of shoot elongation was measured every 7 days for 5 weeks. For *H. verticillata*, the main stem was stretched carefully using rubber-coated tweezers, and the length was measured with a ruler. The shoot length of *P. australis* was measured directly. To measure chlorophyll contents in both species, plant leaves were carefully wiped with laboratory-grade tissue paper and chlorophyll was measured using a SPAD 502 (Minolta Co., Japan). Plant extracts were prepared from 0.1 g of leaf tissue that was ground to powder in liquid nitrogen using a mortar and pestle. The powder was resuspended in 50 mM phosphate buffer (Song and Lee 2010). Then, superoxide dismutase (SOD) activity was measured using the previously published method of Peskin and Winterbourn (Peskin and Winterbourn 2000) and the manufacturer's recommended protocol for preparing the water-soluble tetrazolium salt used in the SOD assay (Dojindo, Japan). Plant leaf cell diameters (long axis) were measured on ten upper cells from thin sections of two different young leaves (Dalla Vecchia et al. 2005) in each mesocosms.

Zinc content measurements

Zinc contents in plants were measured by digesting 0.1 g of dried plant tissue with HNO_3 and 70 % perchloric acid at 200 °C. Then, the amples were filtered through Whatman 44 filter paper. The Zn contents in the samples were analyzed using inductively coupled plasma mass spectrometry (ICP-MS; Agilent Technologies 7500 series, USA). Dissolved Zn contents in treatment solutions were analyzed by preparing 50-mL tubes containing 5 % Hoagland solution without $ZnSO_4$. The tubes were covered, well shaken, and placed in the growth chamber for 5 weeks under the identical environmental conditions as the experimental plants. After 5 weeks, the samples were centrifuged at 4000 rpm for 10 min (Lee et al. 2010). The supernatants were filtered through Whatman GF/A glass microfiber filters and acidified by the addition of 0.5 % HNO_3. Then, the solution was diluted with

ten times amount of DW, and Zn contents were analyzed using ICP-MS.

Statistical analysis

A one-way ANOVA was conducted to identify significant differences between treatments. When a significant difference was detected, a post hoc Tukey's studentized range (HSD) test was performed and assessed using SAS 9.1 (SAS Institute Inc., USA). Differences were considered significant when $p < 0.05$.

Results and discussion

ZnO NPs on Hoagland solution without zinc sulfate showed rapid agglomeration and immediately precipitated. Hoagland solution contains many ions, which cause more NP agglomeration than that caused by distilled water (Song et al. 2013b). Table 1 presents data on the dissolved zinc contents of each treatment solution. For the treatment with 0.01 mg/L ZnO NPs, zinc was not detected. The ICP-MS limit of resolution for Zn was approximately 2 parts per trillion; therefore, detection rate would be much higher than the concentration even if the 0.01 mg/L treatment showed less than 1 % of dissolved Zn. One hypothesis is that during ICP analysis, each treatment solution is acidified by the addition of 0.5 % HNO_3. And then, it was diluted with DW to prevent corrosion of pipes in the ICP-MS. This might cause the concentration of Zn below the detection level. However, still the concentration of the diluted solution should be higher than the detection limit but was not detected. For other treatments, the treatment solution containing 10 mg/L ZnO NPs had the highest rate of Zn dissolution, whereas treatment solutions containing high concentrations of ZnO NPs (100 and 1000 mg/L) had lower rates of dissolution. Previous studies report that solutions containing higher NP concentrations have greater agglomeration (Song et al. 2013b; Yoon et al. 2011). This suggests that the higher concentration treatments in our study precipitated more and dissolved less than lower concentration treatments. Other studies report approximately 4 % of dissolved Zn for 400 mg/L ZnO NPs (Lee et al. 2010), and 10–25 % of dissolved Zn for 100 mg/L ZnO NPs at 20 °C (Reed et al. 2012). Our study showed the highest percent of dissolved Zn (23 %) in the 10 mg/L ZnO NP treatment, and the lowest percent of dissolved Zn (0.8 %) in the 1000 mg/L ZnO NP treatment.

Table 1 Dissolved zinc contents of treatment solutions

Treatment (mg/L)	Dissolved zinc (mg/L)
Control	ND
0.01	ND
0.1	0.01 ± 0.00
1	0.11 ± 0.01
10	2.29 ± 0.11
100	4.45 ± 0.13
1,000	8.48 ± 0.67

Values represent mean ± SE of three replicates

As we used Hoagland solution which is believed to cause more agglomeration than distilled water and can observe rapid precipitation, most toxicity of ZnO NPs would be caused by dissolved Zn^{2+} ion, which is also reported as the primary source of toxicity of ZnO NPs (Aruoja et al. 2009; Franklin et al. 2007; Kasemets et al. 2009; Wu et al. 2010). Also, in growing media, ZnO NPs tend to dissolve more compared to distilled water and Zn^{2+} ions becomes major source of toxicity (Reed et al. 2012). Therefore, dissolved Zn is believed to be the source of phytotoxicity of our research. However, still there are chances that ZnO NPs directly affect plants without ionization, for instance, aggregation of NPs on the root surface of plants causing physical damage to roots (Wang et al. 2015), physically blocking cell wall pore and water transport capacity (Asli and Neumann 2009) and alteration in mineral uptake (Hong et al. 2015), etc. However, such direct effects of ZnO NPs without dissolution are limited and need further research studies to define undissolved effects of ZnO NPs.

H. verticillata displayed significantly reduced growth during the early experimental stages when exposed to the highest ZnO NP concentration (1000 mg/L; Fig. 1a). Treatment with lower ZnO NP concentrations (including 100 mg/L) did not significantly affect *H. verticillata* growth during the early experimental stages. Growth was slightly affected during the third week for treatments containing 0.1 and 1 mg/L ZnO NPs. By the fourth week, *H. verticillata* exposed to 1000 mg/L ZnO NPs showed better growth, and growth was similar to that of other treatments by the fifth week. These results indicate that *H. verticillata* plants exposed to high ZnO NP concentrations can recover after a period of adaptation.

It is difficult to explain the growth patterns of *H. verticillata* during the third week of the experiment with 0.1 and 1 mg/L ZnO NPs. If the negative growth effects were due to contamination, they should continue to be observed during the subsequent weeks. All mesocosms were randomly placed in the growth chamber and relocated often, so environmental factors could not be the cause. One hypothesis is that ZnO NP concentrations greater than 10 mg/L agglomerate more and becomes less available than 1 mg/L concentrations, whereas ZnO NP concentrations below 0.1 mg/L do not significantly affect plants. Higher-concentration NP solutions agglomerate more easily than lower-concentration solutions (Song et al. 2013b; Yoon et al. 2011). A previous study of ZnO NPs also detected a larger hydrodynamic diameter of NPs in higher-concentration solutions (Reed et al. 2012), which indicates

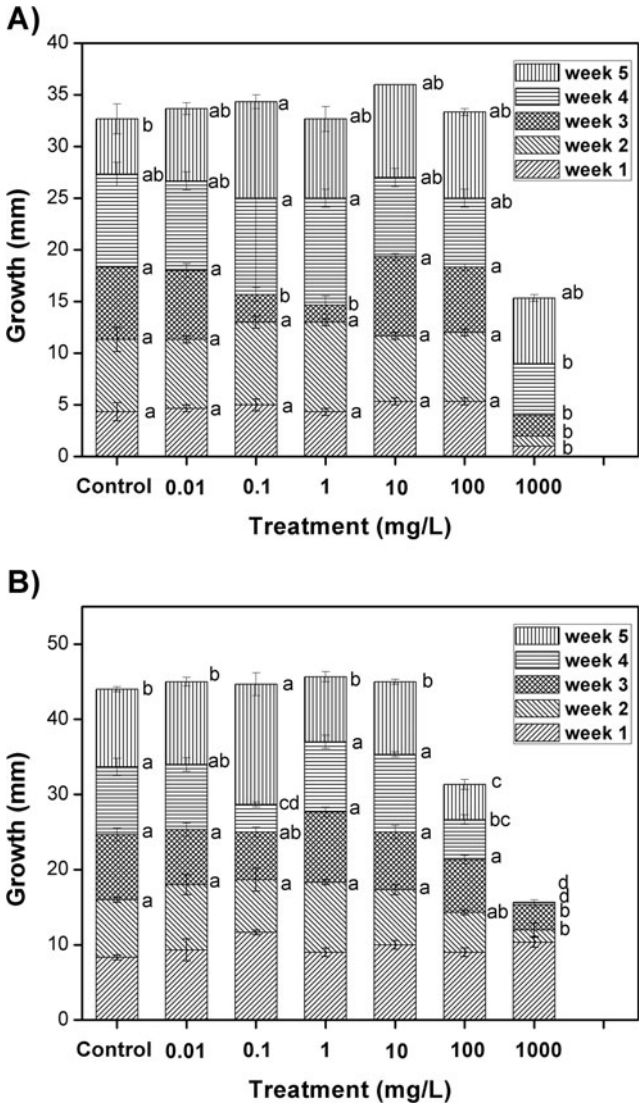

Fig. 1 Weekly growth of **a** *Hydrilla verticillata* and **b** *Phragmites australis*. Values represent mean ± SE of three replicates. *Bars* with *different letters* are significantly different at the $p < 0.05$ level, whereas those with the *same letters* are not

higher agglomeration. *H. verticillata* is a submerged plant with minor roots, which would be more sensitive to suspended particles and ions than to precipitated particles. These considerations might explain the observed growth responses. Overall, the results and analyses indicate that the phytotoxicity of NPs in an aquatic environment is difficult to predict and involves many factors.

The results of the experiments with *P. australis* differed from those with *H. verticillata. P. australis* growth was not significantly affected during the early stages of the experiment, but growth began to decline during later stages (Fig. 1b). *P. australis* exposed to 1000 mg/L ZnO NPs exhibited significantly reduced growth during the second to fifth weeks. Plants exposed to 100 mg/L ZnO NPs also exhibited reduced growth after 4 weeks. *H. verticillata* has only minor roots, and most nutrients and water are taken up by leaves and stem (Chen and Barko 1988); therefore, the response to dissolved ZnO NPs is immediate, but precipitated NPs deposited at the bottom of the mesocosms would not affect the plants. By contrast, *P. australis* has a more extensive root system, and plants would be more sensitive to precipitated NPs deposited in the gravel and sand. Since sand was not enough to support aboveground biomass of *P. australis*, gravel was used to fix the plant. As result, NPs precipitated at pore of gravel and even pore of sands. As mentioned before, Hoagland solution contains many ions, which cause NP agglomeration (Song et al. 2013b). Therefore, roots of *P. australis* would have direct contact with precipitated ZnO NPs, which would be much a higher concentration than original treatment concentration. The results showed that *P. australis* exposed to 1000 mg/L ZnO NPs exhibited complete growth inhibition after 5 weeks. The results indicate that plants showed different toxicity reactions by leaf types, whether they are submerged or emerged. The submerged plant with minor roots is only affected by NPs with direct contact with leaves. On the other hand, emerged plants with developed root systems will be affected by precipitated and agglomerated NPs. There are possibilities that ZnO NPs would physically block pore of plant leaves and roots and cause toxicity of plants (Asli and Neumann 2009). This might also be the reason that *H. verticillata*, which every leaves has direct contact with NPs, showed more immediate responses. However, in our microscopic pictures for cell size analysis, we were not able to find any attached NPs on plant leaves. Still, as the pictures were taken 5 weeks after NP treatment, early status of plant leaves is not known. There might be some possibilities of such physical interference. Nonetheless, dissolved Zn values and precipitation of ZnO NPs indicate different toxicity reactions by leaf types, whether they are submerged or emerged.

The measured chlorophyll contents in plants support these hypotheses (Fig. 2). *H. verticillata* exposed to 1000 mg/L ZnO NPs showed significantly different chlorophyll contents after 3 weeks, but similar chlorophyll contents as other treatments on week 5 (Fig. 2a). *H. verticillata* exposed to 100 mg/L ZnO NPs showed significantly higher chlorophyll contents on week 5, which could explain the reduced growth observed for this treatment at week 5 (Fig. 1a). *H. verticillata* plants with higher chlorophyll contents had darker leaves that appeared unhealthy. Additional experiments were performed to check chlorophyll contents in detached leaves of *H. verticillata* immersed in 0 °C water. The chlorophyll contents of leaves changed from 7.1 to 7.9 in detached leaves (values represent mean of ten replicates). A previous study reports increased chlorophyll contents in *H. verticillata* after heavy metal treatment (Gupta et al. 1996). *P. australis* showed significantly reduced chlorophyll contents after 5 weeks (Fig. 2b). During the first 3 weeks of the experiment, *P. australis* chlorophyll contents did not show any significant

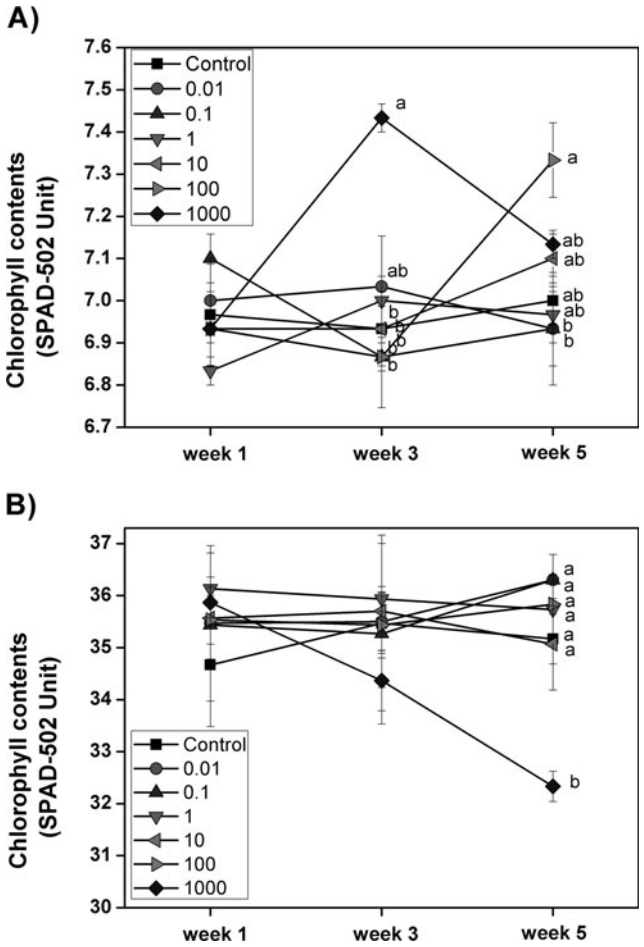

Fig. 2 Weekly chlorophyll contents (relative units) of **a** *Hydrilla verticillata* and **b** *Phragmites australis*. Values represent mean ± SE of three replicates. *Symbols* with *different letters* are significantly different at the $p < 0.05$ level, whereas those with the same letters are not

differences except for plants treated with 1000 mg/L ZnO NPs, which became faded and yellowish. This result indicates that *P. australis* plants exposed to 1000 mg/L ZnO NPs are under stress, consistent with the growth results (Fig. 1b).

The superoxide dismutase activity results (Table 2) confirm that *P. australis* plants exposed to 1000 mg/L ZnO NPs are under stress. SOD values are markers that reflect heavy metal stress (Song and Lee 2010). The results in Table 2 show that low ZnO NP concentrations do not induce plant stress, whereas high ZnO NP concentrations induce high levels of plant stress. These results are consistent with those for chlorophyll contents and growth.

H. verticillata extracts turned out to be not suitable for our SOD analysis due to the tissue water content. This was unexpected because *H. verticillata* was used for a previous study of antioxidant enzyme activity (Srivastava et al. 2006). This difference might be related to differences of extraction buffer used, that while we used phosphate buffer, the reference used potassium phosphate buffer with other components (Srivastava et al. 2006). Therefore, we introduced leaf cell

Table 2 Superoxide dismutase (SOD) activity in *Phragmites australis* extracts

SOD (U/mg protein)	Week 5
Control	65.8 ± 0.6^{c}
0.01	66.1 ± 1.8^{bc}
0.1	65.7 ± 0.5^{c}
1	64.8 ± 1.1^{c}
10	71.8 ± 0.5^{b}
100	69.2 ± 1.9^{bc}
1,000	80.4 ± 1.1^{a}

Values represent mean ± SE of three replicates. Values with different letters are significantly different at the $p < 0.05$ level, whereas those with the same letters are not

diameter measurements to evaluate effects of dissolved heavy metal (Zn) on submerged plant (Dalla Vecchia et al. 2005). However, there were no significant differences among treatments (Table 3) indicating ZnO NPs or dissolved Zn does not have any effect on cell enlargement or cell division. These results are consistent with those for chlorophyll contents and growth that *H. verticillata* showed less affected growth and chlorophyll contents by NPs compared to *P. australis*.

Zinc contents after 5 weeks of ZnO NP treatment were higher in *P. australis* than in *H. verticillata* (Table 4). *H. verticillata* has minor roots and uptakes primarily suspended NPs rather than precipitated NPs. By contrast, *P. australis* has more extensive roots and uptakes primarily precipitated NPs. It is still uncertain how precipitated NPs can be absorbed by plants (by direct uptake of ZnO NPs or by dissolving of precipitated ZnO NPs or by decomposition of bonding between ions in Hoagland solution/Osmocote and zinc), but the results such as growth, physiological responses, and zinc contents indicate that precipitated NPs is affecting *P. australis*. The percentage of Zn accumulation was lower for treatments with higher ZnO NP concentrations. This result might be related to the rate of Zn dissolution, which was lower for higher ZnO NP concentrations. Also, plants under heavy metal stress have lower metabolic rates and absorb less

Table 3 Long axis cell diameter of *Hydrilla verticillata* 5 weeks after ZnO nanoparticle treatment

Treatment	Cell diameter (μm)
Control	28.2 ± 0.9
0.01	27.7 ± 1.7
0.1	26.7 ± 1.5
1	28.1 ± 2.1
10	27.8 ± 1.6
100	27.2 ± 2.2
1,000	27.4 ± 1.8

Values represent mean ± SE of 60 replicates. No significant differences were observed

Table 4 Zinc accumulation in plants after exposure to zinc oxide nanoparticles for 5 weeks

Zinc content (mg/kg)	*Hydrilla verticillata*	*Phragmites australis*
Control	ND	0.02 ± 0.01^{c}
0.01	0.08 ± 0.01^{c}	0.22 ± 0.01^{c}
0.1	0.10 ± 0.01^{c}	0.57 ± 0.31^{bc}
1	0.11 ± 0.01^{c}	1.17 ± 0.03^{ab}
10	0.20 ± 0.03^{b}	1.43 ± 0.03^{a}
100	0.35 ± 0.01^{a}	1.47 ± 0.03^{a}
1,000	0.34 ± 0.01^{a}	1.67 ± 0.22^{a}

Values represent mean ± SE of three replicates. Values with different letters are significantly different at the $p < 0.05$ level, whereas those with the same letters are not

Zn. Especially for *P. australis* exposed to 1000 mg/L ZnO NPs, the leaves became dehydrated and dry, indicating a severe disruption of water transport from roots. Therefore, if Zn contents had been analyzed during week 3, other treatments might have shown much lower levels than those with 1000 mg/L ZnO NPs. However, our replicate numbers were limited due to space constraints in our growth chamber. Future research can expand these first experiments and perform time series analysis of Zn contents in plants exposed to ZnO NPs. Also, such series analysis can confirm whether plants are absorbing NPs after precipitation. *P. australis* control plants also showed a low level of Zn accumulation. This could result from Zn in the sand and gravel matrix used to anchor the roots. However, this cannot be verified because we could not measure Zn content of the sand because it already includes precipitates of ZnO NPs. The observation that Zn contents in *P. australis* were higher than those in *H. verticillata* explains why ZnO NPs had more adverse effects on *P. australis* than on *H. verticillata*. Treatments with low ZnO NP concentrations showed higher Zn accumulation rates, and plants accumulated more Zn than measured in the treatment solutions (Table 1 and 3). These results suggest that even low NP concentrations would accumulate in plants and cause phytotoxicity.

There was no indication of aluminum (Al) contamination in the bottle of commercially obtained ZnO NPs. Therefore, Al toxicity was not considered during our study. However, we subsequently learned that the commercially obtained ZnO NPs contain 6 % of Al as dopant. Al has phytotoxicity (USEPA 2003) and could cause certain effects on plants. The typical range of Al in soil is from 1 to 30 % (USEPA 2003). The Al concentration in our studies (maximum 60 mg/L) would not be anticipated to cause significant phytotoxicity. Previous research indicated that plants are affected by Al in soil solutions with pH >9 (Ma et al. 2003). Hoagland solution has pH 6. Therefore, we conclude that the Al levels in our treatments would not significantly affect the observed results.

ZnO NPs in Hoagland solution dissolved better at lower concentrations than at higher concentrations. Previous studies indicate that NP dissolution depends on the solution (Lee et al. 2010) and temperature (Reed et al. 2012). Our results indicate that further research should be conducted to test environmentally relevant solutions. Mesocosm research cannot reflect natural aquatic environments, which include physical effects of wind and flow. Because these physical forces would affect the behavior of NPs (Song et al. 2013b), more realistic mesocosm research should be designed for future studies.

H. verticillata growth was significantly reduced by treatment with the highest ZnO NP concentration (1000 mg/L) during the early experimental stages, whereas *P. australis* growth began to significantly decline after a few weeks. These results reflect physiological differences in the aquatic plants regarding whether they use leaves or roots for nutrient and water uptake. The plant with minor roots that takes up nutrients and water into leaves (*H. verticillata*) was not affected by precipitated ZnO NPs and showed reduced phytotoxicity with increasing time. By contrast, the plant that takes up nutrients and water into roots (*P. australis*) was affected by precipitated NPs and showed greater phytotoxicity with increasing time. Chlorophyll contents and the SOD values (*P. australis*) also show similar patterns. Plant Zn contents also support these results and show that *P. australis* absorbs more Zn than *H. verticillata*. Overall, ZnO NPs had significant phytotoxicity at high concentrations. Although environmentally realistic ZnO NP concentrations did not show significant phytotoxicity, exposure of *H. verticillata* to low ZnO NP concentrations caused significant growth reductions during the third week. The observed high accumulation rates in plants exposed to low ZnO NP concentrations indicate that NP phytotoxicity is unpredictable. Therefore, ZnO NPs must be used carefully, and disposal must be strictly limited to landfills that prevent outflow to the surrounding environment.

Conclusions

In Hoagland solution, the dissolution rate of ZnO NPs was inversely related to the concentration. However, since low concentration treatment has more dissolution rate, even low concentrations would cause toxic effects on plants. The plants showed different toxicity reactions by leaf types that the submerged aquatic plant *H. verticillata* growth was reduced during the early stages of the experiment when exposed to the highest ZnO NP concentration (1000 mg/L), whereas the emerged aquatic plant *P. australis* began to show significantly reduced growth after a few weeks. Also, chlorophyll content, antioxidant enzyme activity, and Zn accumulation showed that *P. australis* was adversely affected. Between aquatic plants, whether they use leaves or roots for nutrient and water uptake led to differences in nanoparticle toxicity. High ZnO

NP concentrations caused significant phytotoxicity on aquatic plants, and low concentrations caused unpredictable phytotoxicity. Therefore, the use and disposal of zinc oxide nanoparticles should be carefully monitored.

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