*International Journal of Phytoremediation*, 14:429–442, 2012
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ISSN: 1522-6514 print / 1549-7879 online
DOI: 10.1080/15226514.2011.620903

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# ACCUMULATION AND PHYTOTOXICITY OF ENGINEERED NANOPARTICLES TO *CUCURBITA PEPO*

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***The effect of bulk and engineered nanoparticle (NP) Ag, Au, Cu, Si, and C at 250 and 750 mg/L on zucchini biomass, transpiration, and element content was determined. The pH of bulk and NP solutions prior to plant growth frequently differed. Nanoparticle Cu solution pH was significantly higher than bulk Cu, whereas for Ag and C, the NPs had significantly lower pH. Plants were unaffected by Au, regardless of particle size or concentration. NP Ag reduced plant biomass and transpiration by 49–91% compared to equivalent bulk Ag. NP Si at 750 mg/L reduced plant growth and transpiration by 30–51% relative to bulk Si. Bulk and NP Cu were phytotoxic but much of the effect was alleviated by humic acid. The shoot Ag and Cu content did not differ based on particle size or concentration. The accumulation of bulk Au was greater than the NP, but humic acid increased the accumulation of NP and bulk Au by 5.6-fold and 80%, respectively. The uptake of NP Si was 5.6–6.5-fold greater than observed with the bulk element. These findings show that the NPs may have unique phytotoxicity or accumulation patterns and that solution properties can significantly impact particle fate and effects.***

**KEY WORDS:** engineered nanoparticles, nanotoxicology, phytotoxicity, nanotechnology

## INTRODUCTION

The development and implementation of engineered nanoparticles (NPs), substances with at least two dimensions less than 100 nm, has increased dramatically in recent years. Nanotechnology was a $4 billion industry in 2005 and is projected to reach $1 trillion by 2015 (Roco 2005). Because of their small size, NPs possess a range of unique chemical and physical properties that differentiate them from the corresponding bulk materials (Handy et al. 2008). Some of these unique properties result from the drastically increased surface area to volume ratio, which often leads directly to greater reactivity, but also from our increasing ability to engineer specific characteristics onto the particles (Klaine et al. 2008). The resulting NPs can possess enhanced solubility, catalytic or conductive potential, and unique magnetic or optical properties (Auffan et al. 2009). Consequently, there are now

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more than 1300 nanotechnology-containing products that are commercially available, with common examples found in food packaging, cosmetics, medical devices, fuel cells, agents of groundwater/soil remediation, and as components of pesticide/fertilizer formulations (Zhang and Elliot 2006; Klaine et al. 2008; Ma et al. 2010).

The use of nanotechnology has and will continue to increase at a rapid rate, but a comprehensive understanding of the impact of these materials on biological and ecological systems has lagged behind (Weisner et al. 2009). Although there have been numerous controlled laboratory studies measuring the toxicological effects of various NPs on receptors ranging from bacteria to mammals (Elgrabi et al. 2008; Griffit et al. 2008; Jiang et al. 2009; Wang et al. 2009), this literature is incomplete. In addition, there is a significant shortage of environmentally based fate and transport data on engineered nanomaterials, and this lack of understanding confounds accurate assessment of NP exposure, hazard, and risk (Weisner et al. 2009). One area of research with a notable lack of data is the impact of engineered nanomaterials on terrestrial plants and specifically agricultural crops. This knowledge gap is of particular concern given the current use of NPs in pesticide and fertilizer formulations, and the fact that nanoparticle contamination of agricultural crops (i.e., the food chain) may represent a significant and uncharacterized pathway of human exposure to these materials.

Recently, a number of controlled laboratory studies have sought to address the effects of NPs on plants. Several investigations have focused on the impact of engineered nanomaterials on seed germination or seedling root elongation, but with highly variable exposure conditions, toxicological endpoints and disparate results, drawing conclusions is difficult. For example, Lin and Xing (2007) studied the impact of five NPs at 2000 mg/L on the germination of six plant species and noted only two instances (out of 30) of toxicity. Zheng et al. (2005) observed that NP $TiO_2$ at concentrations up to 4000 mg/L enhanced spinach germination, whereas López-Moreno et al. (2010) reported that exposure to nanoparticle $CeO_2$ had no impact on germination but did result in genotoxicity. Similar variability in the literature exists for exposure assays measuring root elongation. Information on NP uptake by plants or on the effects of those particles on physiological parameters, such as growth or transpiration, is rather limited. Stampoulis et al. (2009) noted that exposure of zucchini (*Cucurbita pepo* subspecies *pepo*) to NP Ag, Cu, or multi-walled carbon nanotubes at 1000 mg/L for 15–17 days resulted in 25–40% reductions in biomass and transpiration as compared to unexposed or corresponding bulk material controls. Musante and White (2010) reported similar trends in NP Ag and Cu phytotoxicity for squash (*C. pepo* subspecies *ovifera*), although species specific differences existed in the magnitude of biomass and transpiration reductions, as well as in the extent of element accumulation. With regard to carbon nanomaterials, Cañas et al. (2008) reported that plants exposed to carbon nanotubes accumulated the material on outer root surfaces but that in spite of species specific toxicity, uptake was not observed. Conversely, Lin et al. (2009) reported uptake and translocation of $C_{70}$ by rice, although no toxicity was evident. More recently, Chen et al. (2010) showed that $C_{60}(OH)_{20}$ (fullerols) readily permeated cell walls, and although the particles were then excluded by the plasma membrane, measurable cell damage resulted. This sampling of the literature summarizes the conflicting data regarding NP toxicity and accumulation by terrestrial plants and highlights the need for further investigation.

The goal of the current study was to evaluate the impact of particle size, concentration, and solution chemistry on NP toxicity to and accumulation by *Cucurbita pepo* subspecies *pepo* (zucchini). The impact of particle size on solution pH was also determined. Previous work has shown that labor intensive 15–17 day hydroponic assays with daily monitoring of biomass and transpiration were effective in demonstrating NP-specific toxicity. As such, efforts were made in this study to shorten and simplify the hydroponic assay so as to

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facilitate more rapid yet accurate toxicological assessment. Plants were exposed to bulk and NP Ag, Au, Cu, Si, and C (fullerenes) for 7 days at 0, 250, and 750 mg/L in Hoagland's solution with or without 100 mg/L humic acid. Solution pH, plant biomass, transpiration, and element content were determined.

## MATERIALS AND METHODS

### Seeds and Particles

*Cucurbita pepo* subspecies *pepo* (zucchini; cultivar variety Costata Romanesco) seeds were acquired from Johnny's Selected Seeds (Albion, ME) and were surface sterilized by rinsing in 5% sodium hypochlorite solution. The seeds were added to moist germination paper and after one week, germinated seedlings were added to 35-mL amber vials filled with 25% Hoagland's solution (MP Biomedicals, Solon, Ohio) (Hoagland and Arnon 1950; Stampoulis et al. 2009). After 14 d of incubation, similarly-sized plants were selected for the hydroponic exposure assay.

The source and properties of the bulk and nanoparticles used are described in Table 1. The individual bulk and nanoparticle treatments were prepared in one-liter amber glass jars at 0, 250, and 750 mg/L of 25% Hoagland's solution or 25% Hoagland's solution amended with 100 mg/L humic acid (Alfa Aesar, Ward Hill, MA). The solutions were shaken vigorously and allowed to sit under laboratory conditions for 5–6 days. The pH of each treatment was determined. These experiments were designed to directly compare the toxicity and accumulation of specific engineered nanoparticles to the corresponding bulk material under defined initial conditions; as such, information on the degree of particle aggregation and/or dispersion during the exposure period was not obtained.

### Exposure Assay

A batch hydroponic phytotoxicity assay was initiated to measure the impact of engineered nanoparticles and the corresponding bulk materials in the biomass, transpiration, and elemental content of zucchini. Triplicate zucchini plants were individually weighed,

**Table 1** Source and properties of bulk and nanoparticles (NP) used in the hydroponic exposure assay

| Particle | Source | Particle size and Purity |
|---|---|---|
| Ag | | |
| Bulk | Sigma-Aldrich, St. Louis, MO, USA | <250 $\mu$m; 99.99% purity |
| NP | Sigma-Aldrich, St. Louis, MO, USA | <100 nm; 99.5% purity |
| Au | | |
| Bulk | Alfa-Aesar, Ward Hill, MA | 0.8–1.5 $\mu$m; 99.96% purity |
| NP | Sigma-Aldrich, St. Louis, MO, USA | <100 nm; 99.9% purity |
| C | | |
| Bulk | Calgon Carbon Corp., Pittsburgh, PA, USA | F200 12 × 40 mesh granular activated carbon |
| NP | Strem Chemicals Inc, Newburyport, MA | 99.0% pure $C_{60}$ Fullerene |
| Cu | | |
| Bulk | Sigma-Aldrich, St. Louis, MO, USA | 99.999% pure powder |
| NP | Melorium Technologies, Rochester, NY, USA | <50 nm; 99.985% purity |
| Si | | |
| Bulk | Strem Chemicals Inc, Newburyport, MA | 99% pure powder |
| NP | Sigma-Aldrich, St. Louis, MO, USA | <100 nm; ≥98% purity |

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marked, and added to single one-liter treatment jars. The plants were incubated in a growth room at 25°C with a 12 h photoperiod for 9 days. Unexposed control plants were also prepared. Due to resource limitations, the incubation period for plants exposed to nanoparticle or bulk Si was 10 days. Because of the large volume, individual treatment solutions did not need to be replenished during the exposure period. Abiotic evaporative losses from treatments were negligible compared to transpiration. At harvest, the mass of each marked zucchini plant was determined. The stem was then severed with a razor blade and a portion of the shoot system that was never in contact with the treatment solutions was stored in a Nasco Whirl Pak® bag at -18°C. To determine the impact of particle exposure on overall plant transpiration, the mass of each of the solution-containing jars was also recorded. The mass of the empty jar was subtracted from this value, and the total volume of solution transpired was converted to a daily mass adjusted transpiration value (DMATV). Specifically, the DMATV is the total transpired volume of solution for a treatment divided by the relative mass of each of the replicate plants in the treatment, all divided by the number of exposure days (9 or 10). Thus, the units for the DMATV are mL transpired/plant/day. The pH of all solutions was also determined at harvest.

The shoot tissue of all plants except the carbon treatments were oven-dried at 100°C for 72 hours and digested on a hot block with concentrated $HNO_3$ for 1 h at 115°C. The digested shoot tissues were analyzed by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) for Au, Ag, Cu, and Si.

### Statistical Analysis

The relative change in biomass (final mass/initial mass) for each replicate plant was determined for all treatments. The statistical significance of differences in relative biomass change among the concentrations (0, 250, 750 mg/L) of a specific element and particle size was determined by a One Way Analysis of Variance (ANOVA) followed by a Student Newman Keuls Multiple Comparison Test (SNK MCT). The statistical significance of differences as a function of particle size (bulk against nanoparticle) for a given element were determined by a Student t-test ($p < 0.05$, unless otherwise noted). The statistical significance of differences in elemental content and transpiration volume (DMATV) among the concentrations (0, 250, 750 mg/L) of a specific element were determined by a One Way ANOVA with a SNK MCT.

## RESULTS AND DISCUSSION

### Solution pH Measurements

The pH of all solutions was measured 5–6 days after preparation but before plant addition, as well as after plant harvest. The initial solution pH measurements are shown in Table 2. The addition of 100 mg/L humic acid significantly raised the pH of all treatments except the granular activated carbon. Across all treatments, the pH of the Hoagland's solutions ranged from 4.47–6.24 (mean of 5.34), whereas the humic acid amended solutions ranged from 6.20–6.63 (mean of 6.44). This finding is not particularly surprising given the known buffering capacity of humic acid. Pertusatti and Prado (2007) reported that the maximum buffering capacity of a commercially available humic acid was at 6.0. More meaningful pH solution information can be found by comparing the bulk and nanoparticle treatments for a specific element at a specific concentration. For example, in the Ag

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**Table 2** Initial solution pH measured 5–6 days after preparation but prior to plant addition

| Treatment | Concentration (mg/L) | Hoagland's | Hoagland's + 100 mg/L humic acid |
|---|---|---|---|
| Control | NA | 5.74 x | 6.06 y |
| Ag | | | |
| NP | 250 | 5.62 Ax | 6.28 Ay |
| Bulk | 250 | 5.10Bx | 6.51 By |
| NP | 750 | 5.00 Ax | 6.79 Ay |
| Bulk | 750 | 5.17 Ax | 6.41 By |
| Cu | | | |
| NP | 250 | 5.73 Ax | 6.63 Ay |
| Bulk | 250 | 5.04 Bx | 6.45 By |
| NP | 750 | 6.19 Ax | 6.77 Ay |
| Bulk | 750 | 5.22 Bx | 6.41 By |
| Au | | | |
| NP | 250 | 5.20 Ax | 6.32 Ay |
| Bulk | 250 | 5.12 Ax | 6.52 By |
| NP | 750 | 5.20 Ax | 6.38 Ay |
| Bulk | 750 | 5.12 Bx | 6.43 Ay |
| Si | | | |
| NP | 250 | 5.21 Ax | 6.36 Ay |
| Bulk | 250 | 5.09 Ax | 6.33 Ay |
| NP | 750 | 4.47 Ax | 6.20 Ay |
| Bulk | 750 | 5.56 Bx | 6.39 By |
| C | | | |
| NP | 250 | 5.28 Ax | 6.43 Ay |
| Bulk | 250 | 6.24 Bx | 6.43 Ax |
| NP | 750 | 5.22 Ax | 6.45 Ay |
| Bulk | 750 | 6.12 Bx | 6.28 Ax |

1-Within columns by element and concentration, values followed by different capital letters (A,B) are significantly different (Student t-test, $p < 0.05$).

2-Within rows (across solution type), values followed by different lowercase letters (x,y) are significantly different (Student t-test, $p < 0.05$).

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Hoagland's treatment, the pH of NP and bulk solutions were statistically equivalent at 750 mg/L but at 250 mg/L, the Ag NP solution pH was significantly greater than that of the corresponding bulk Ag. Conversely, in the Si Hoagland's treatment, the pH of NP and bulk solutions were statistically equivalent at 250 mg/L but at 750 mg/L, the Si bulk solution pH was significantly greater than that of the corresponding NP Si. For the Cu and C treatments, the pH was differentially affected by the bulk and nanoparticles at both concentrations; for Cu, the NP solution pH measurements were significantly higher than with the bulk Cu, whereas for the C treatments, the fullerene solutions had significantly lower pH than observed with the bulk activated carbon. The presence of humic acid at 100 mg/L had varying effects. For the Cu and Si treatments, humic acid raised all solution pH values but the relative differences based on particle size were largely unaffected. For the Ag and Au treatments, the effect of humic acid was less clear. However, the effect of humic acid on the pH of fullerene solutions was significant and notable; the pH was raised by 1.1–1.2 units and became statistically indistinguishable from the bulk C treatment.

The reasons for and significance of many of the particle size-dependent pH differences noted above are unclear. The findings on Cu are of interest because Musante and White

(2010) observed that the greater initial amount of Cu nanoparticles added to Hoagland's solution, the less total Cu found in solution 10 days later. This counterintuitive finding is indicative of the complex redox chemistry of Cu in solution and was hypothesized to be the result of elemental Cu oxidation in solution, which consumes protons, increases pH, and subsequently drives the precipitation of ionic Cu to salts. The higher the initial element concentration added, the greater the subsequent pH change and more aggressive the ionic precipitation. The observation in the current study that the pH of Cu NP solutions (5–6 days after preparation) is significantly greater than the corresponding bulk element solutions and that the pH of the NP solutions is directly related to initial concentration supports the hypothesized chemical reaction proposed by Musante and White (2010). The finding that fullerenes result in significantly lower solution pH than activated carbon and the Hoagland's control is also noteworthy. Andrievsky et al. (2002) suggested that fullerenes possess negative surface charges from protolytic processes and can subsequently polarize water molecules on a local scale to the extent of charge transfer, resulting in released protons and decreased pH. Interestingly, the addition of humic acid completely alleviates pH decrease caused by the fullerenes.

Interpretation and discussion of the post-plant growth pH results (SI Table 1) is confounded by a number of factors, including individual treatment effects on biomass, transpiration and other undetermined physiological parameters that may have unknown consequences on root exudation and indirectly on solution pH. The pH of control solutions postplant growth were 4.19 and 4.40 in the Hoagland's and Hoagland's with humic acid treatments (significantly different; $p < 0.05$), respectively. This observation is not surprising given that plant roots are known to exude a number of organic acids, metabolites, and even H+, all of which will lower pH in solution (Marschener 1998; Dakora and Phillips 2002). This evolved mechanism of rhizosphere acidification will promote nutrient availability and acquisition (Marschener 1998). In comparing bulk against nanoparticle solutions, several differences are noteworthy. In the Ag treatments, the pH of the bulk solutions are 4.15–4.17, significantly lower than the NP Ag solutions at 6.84–6.98. The addition of humic acid decreased this particle-size difference somewhat but the NP solutions were still significantly lower than with bulk Ag. Similar results were observed with the C treatments; the fullerene solution pHs ranged from 3.98–4.09, significantly lower than the bulk activated carbon at 4.98–7.18. Again, the number of variables potentially impacting pH during and after plant growth make a thorough discussion of these results far too speculative. However, it should be noted that none of the particle-size specific pH effects mentioned above result in conditions that would were either dramatically different from the control plants or were of a magnitude expected to directly impact plant growth.

## Plant Biomass and Transpiration

The effects of nanoparticle and bulk elements on zucchini biomass after 7-d of exposure varied with particle type, size, and solution properties (i.e., humic acid) (Table 3). The biomass is expressed as relative change in mass during the exposure period and is calculated by dividing the final mass by the initial mass. Exposure to bulk and nanoparticle Au at 250 and 750 mg/L had no impact on zucchini biomass, regardless of the presence of 100 mg/L humic acid. Conversely, exposure to Cu at 250 and 750 mg/L in Hoagland's solution reduced plant biomass by more than 50%, regardless of particle size. Interestingly, the phytotoxicity of bulk and nanoparticle Cu was minimized by the presence of 100 mg/L humic acid. Similar to Au, exposure to bulk and nanoparticle Si at 250 mg/L had no effect

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**Table 3** Relative change in zucchini biomass (final mass/initial mass) after 7-d exposure to 0–750 mg/L nano- or bulk particles

| | 0 mg/L | 250 mg/L | 750 mg/L |
|---|---|---|---|
| Cu | | | |
| Hoagland's Cu Bulk | 2.01 A[1] s | 1.05 B x[2] s[3] | 0.93 B x s |
| Hoagland's Cu NP | 2.01 A | 0.89 B x s | 0.90 B x s |
| Humic + Hoagland's Cu Bulk | 2.08 A s | 1.35 A x s | 1.18 A x s |
| Humic + Hoagland's Cu NP | 2.08 A | 1.17 A x s | 1.08 A x s |
| Au | | | |
| Hoagland's Au Bulk | 2.01 A | 1.60 A x s | 1.56 A x s |
| Hoagland's Au NP | 2.01 A | 1.53 A x s | 1.63 A x s |
| Humic + Hoagland's Au Bulk | 2.08 A s | 1.52 A x s | 1.43 A x s |
| Humic + Hoagland's Au NP | 2.08 A | 1.56 A x s | 1.26 A x s |
| Si | | | |
| Hoagland's Si Bulk | 2.01 A s | 1.87 A x s | 2.45 A x s |
| Hoagland's Si NP | 2.01 A | 1.94 A x s | 1.72 A y s |
| Humic + Hoagland's Si Bulk | 2.08 A s | 2.48 A x s | 2.11 A x s |
| Humic + Hoagland's Si NP | 2.08 A | 1.88 A x s | 1.42 A x s |
| C | | | |
| Hoagland's GAC | 2.01 A s | 1.49 B x s | 1.12 B x s |
| Hoagland's Fullerenes | 2.01 A | 1.48 A x s | 1.76 A x s |
| Humic + Hoagland's GAC | 2.08 A s | 2.05 A x t | 2.60 A x s |
| Humic + Hoagland's Fullerenes | 2.08 A | 1.37 A y s | 2.13 A y s |

3-Within rows, values followed by different capital letters (A,B) are significantly different (One way ANOVA followed by a Multiple Comparison Test).

4-Within columns and solution type, values followed by different lowercase letters (x,y) are significantly different (Student t-test; $p < 0.05$).

5-Within columns and across solution type, values followed by different lowercase letters (s,t) are significantly different (Student t-test; $p < 0.05$).

on plant growth in the presence or absence of 100 mg/L humic acid. However, at 750 mg/L, nanoparticle Si reduced zucchini biomass by 30% relative to bulk Si powder (significantly different; $p < 0.05$). This trend in NP Si toxicity was also evident in the presence of humic acid, although large replicate variability in this treatment negated differences of statistical significance. The effect of exposure to bulk and nanoparticle Ag on relative zucchini biomass is shown in Figure 1. Nanoparticle Ag at 250 mg/L decreased plant growth by 49 and 59% relative to bulk Ag and control treatments, respectively; at 750 mg/L, the reductions in plant mass were 55 and 64%, respectively. The presence of 100 mg/L humic acid minimized NP Ag phytotoxicity at 250 mg/L but at 750 mg/L, nanoparticle Ag reduced plant biomass by 70%. Exposure to fullerenes had no impact on zucchini biomass, regardless of solution type (Table 2). However, granular activated carbon at 250 and 750 mg/L reduced plant biomass by 26 and 44%, respectively. The presence of humic acid at 100 mg/L completely alleviated the phytoxicity of granular activated carbon.

Similar to the biomass data, the effect of bulk and nanoparticle element exposure on zucchini transpiration varied with particle type and size, as well as solution properties (Table 4). Exposure to bulk and nanoparticle Au at 250 and 750 mg/L had no impact on zucchini transpiration volume, although the presence of 100 mg/L humic acid resulted in transpiration volume decreases at 750 mg/L regardless of particle size. Exposure to bulk and nanoparticle Si had non-significant impacts on transpiration volume relative to unexposed zucchini. However, at 750 mg/L, NP Si exposure reduced zucchini transpiration

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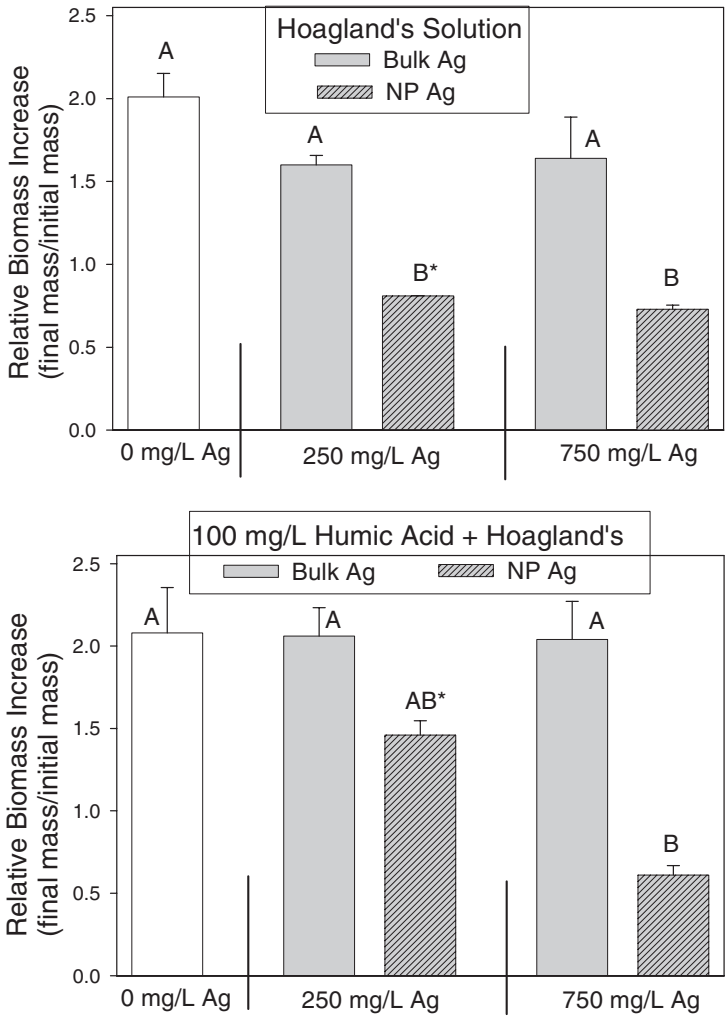


**Figure 1** Relative biomass increase (final mass/initial mass) of zucchini exposed to 0–750 mg/L nano- or bulk particles in Hoagland's solution with (bottom) or without (top) 100 mg/L humic acid. Within a graph, bars with different letters are significantly different (One way ANOVA with Student Newman Keuls MCT). Across graphs, bars at the same particle size and concentration that have an asterisk (*) are significantly different (Student t-test; $p < 0.05$). Error bars are standard errors of the mean.

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by 51% (significant at $p < 0.05$) relative to the bulk Si-treated plants. The presence of humic acid instigated bulk Si phytotoxicity at 750 mg/L, eliminating particle size differences in transpiration volume. Exposure to Cu reduced plant transpiration by 66–76%, regardless of particle size or concentration. Humic acid minimized the phytotoxicity of bulk Cu but the transpiration volume at 750 mg/L NP Cu was significantly reduced relative to unexposed controls. The phytotoxicity of NP Ag was clearly evident as measured by transpiration volume. In Hoagland's solution, zucchini transpiration was reduced by 85–91% relative to bulk Ag or un-exposed plants. In agreement with the biomass data, NP Ag phytotoxicity at 250 mg/L was minimized by 100 mg/L humic acid but reductions in transpiration at

**Table 4** Daily mass adjusted transpiration volume (mL/plant/day) during 7-d exposure to 0–750 mg/L nano- or bulk particles

| | 0 mg/L | 250 mg/L | 750 mg/L |
|---|---|---|---|
| Ag | | | |
| Hoagland's Ag Bulk | 14.5 A[1] s | 15.8 A x[2] s[3] | 20.8 A x s |
| Hoagland's Ag NP | 13.0 A s | 1.90 B y s | 1.90 B y s |
| Humic + Hoagland's Ag Bulk | 14.5 A | 7.76 A x s | 14.6 A x s |
| Humic + Hoagland's Ag NP | 13.0 A | 7.42 AB x | 2.94 B y t |
| Cu | | | |
| Hoagland's Cu Bulk | 14.5 A s | 4.04 B x s | 3.47 B x s |
| Hoagland's Cu NP | 13.0 A s | 4.72 B x s | 3.17 B x s |
| Humic + Hoagland's Cu Bulk | 14.5 A | 4.97 A x s | 6.30 A x s |
| Humic + Hoagland's Cu NP | 13.0 A | 3.60 A x s | 2.95 B x s |
| Au | | | |
| Hoagland's Au Bulk | 14.5 A s | 15.2 A x s | 15.6 A x s |
| Hoagland's Au NP | 13.0 A s | 17.0 A x s | 16.1 A x s |
| Humic + Hoagland's Au Bulk | 14.5 A | 13.0 A x s | 4.82 A x t |
| Humic + Hoagland's Au NP | 13.0 A | 11.3 A x s | 4.42 A x s |
| Si | | | |
| Hoagland's Si Bulk | 14.5 A s | 17.1 A x s | 18.1 A x s |
| Hoagland's Si NP | 13.0 A s | 17.4 A x s | 8.81 A y s |
| Humic + Hoagland's Si Bulk | 14.5 A | 6.60 A x s | 6.66 A x t |
| Humic + Hoagland's Si NP | 13.0 A | 10.1 A x s | 9.66 A x s |

1-Within rows, capital values followed by different capital letters (A,B) are significantly different (One way ANOVA followed by a Multiple Comparison Test).

2-Within columns and solution type, values followed by different lowercase letters (x,y) are significantly different (Student t-test; $p < 0.05$).

3-Within columns and across solution type, values followed by different lowercase letters (s,t) are significantly different (Student t-test; $p < 0.05$).

750 mg/L NP Ag were 77–80%. Zucchini exposed to 250 mg/L fullerenes transpired 44% less than the bulk activated carbon treated plants but this effect was not evident at 750 mg/L (SI Figure 1). In the presence of humic acid, exposure to bulk activated carbon resulted in dramatic increases in transpiration volume; the levels were 3.3–6.5 times greater than fullerene or unexposed plants.

The element- and particle-size specific toxicity of nanoparticles to plants has recently been reviewed by Ma et al. (2010) and some of the results from the current work are in line with findings discussed in that review article. The non-toxic nature of Au, regardless of particle size, is supported by the literature where no reports of phytotoxicity could be located. Similarly, no reports of Si nanoparticle phytotoxicity could be found in the literature. In line with our current findings, Stampoulis et al. (2009) found that zucchini exposed to 1000 mg/L bulk and NP Si experienced no negative effects on biomass. Thus, the finding of no biomass effects at the lower concentrations used in the current study is not surprising. The observation of significantly reduced transpiration volume upon exposure to 750 mg/L NP Si relative to the corresponding bulk material is interesting and also agrees with Stampoulis et al. (2009), which showed that for Ag nanoparticles, transpiration volume was a more sensitive toxicological parameter than overall plant mass. Our results also show that exposure to fullerenes had no impact on plant biomass but caused a slight yet statistically significant reduction in transpiration. The literature on the phytotoxicity of fullerenes is not extensive; Ma and Wang (2010) noted that exposure to fullerenes at 2 and

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15 mg/L had no impact on *Populusdeltoides* (cottonwood) morphology, transpiration, or biomass. Alternatively, several reports have noted phytotoxicity of water soluble fullerenes (fullerols). Chen et al. (2010) observed that $C_{60}(OH)_{20}$ effectively penetrated plant cell walls, subsequently accumulating outside the cell membrane and causing physical damage at concentrations as low as 10 mg/L. Similarly, Lui et al. (2010) noted that exposure to $C_{70}(C(COOH)_2)_{4\text{-}8}$ at 20 mg/L resulted in shortened root length, as well as a range of physiological abnormalities measured at the cellular level. Several studies have reported on the particle-size dependent phytotoxicity of Ag nanoparticles. Stampoulis et al. (2009) and Musante and White (2010) both noted reductions in plant biomass and transpiration upon exposure to Ag NP at concentrations as low as 100 mg/L. Kumari et al. (2009) described genotoxic effects, including mitotic disruption and chromosomal alterations, when *Allium cepa* was exposed to Ag NPs at levels concentrations up to 100 mg/L. Ma et al. (2010) described reduced biomass and other morphological effects when *Arabidopsis thaliana* was exposed to Ag NPs at concentrations as low as 1 mg/L.

The rationale for the humic acid treatment in the current study was two-fold. First, humic acid is a natural component of soils and soil pore water, thereby making laboratory based-bioassays more environmentally relevant. Second, reports in the literature indicate that humic acid and similar materials may impact nanoparticle dispersion and stability, which can then subsequently impact toxicity (Ghosh et al. 2008; Domingos et al. 2009). In the current study, the effect of humic acid at 100 mg/L on phytotoxicity was element- and particle-size specific; a finding supported by the literature. For example, humic acid had a negligible impact on the biomass effects noted for Au and Si nano- and bulk particles. These findings agree with that of Slaveykova and Startchev (2009), who observed that humic acid had no effect on the transformation of coated quantum dots. Alternatively, humic acid seemed to alleviate some of the phytotoxicity experienced upon exposure to bulk and NP Cu, as well as NP Ag and bulk activated carbon. These findings agree with Musante and White (2010), who observed similar reductions in bulk Cu toxicity as measured by biomass and transpiration but did not observe similar alleviation of phytotoxicity with Cu NPs at 100 and 500 mg/L. These differences may be the result of different element and humic acid concentrations, as well as differences in exposure time. The reduction in nano- and bulk particle toxicity upon humic acid amendment is likely due to element specific interactions with the large array and number of carboxylic acid and phenolic functional groups on the humic acid structure, thereby reducing direct particle exposure to plant tissues (Piccolo 2002).

## Plant Element Content

The amounts of Au, Ag, Cu, and Si in the shoot tissues of plants grown in solutions amended with these elements were determined by ICP-OES. The Ag content of zucchini shoots exposed to bulk and nanoparticle Ag was significantly greater than non-exposed plants but did not differ based on particle size, concentration or humic acid presence. High replicate variability confounded data analysis. The Ag content of nanoparticle-exposed plants ranged from 7.6–43 mg/kg, whereas bulk Ag treated plants contained 4.0–63 mg/kg. The Au content of plants exposed to bulk and nanoparticle Au was significantly greater than the un-exposed controls, which had non-detectable amounts of the element, and ranged from 0.76–5.4 mg/kg across particle size, concentration and solution type. In Hoagland's solution amended with 250 mg/kg Au, the elemental content of nanoparticle and bulk exposed plants were 0.76 and 2.4 mg/Kg, respectively (significantly different by Student

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t-test at $p = 0.059$). The Au content of plants exposed to 750 mg/L bulk and nanoparticle Au in Hoagland's solution were 1.7 and 1.3 mg/kg; these values are not significantly different from each other or from the respective 250 mg/L treatments. Humic acid amendment significantly increased the Au content of zucchini shoots exposed to 250 mg/L Au. The nanoparticle exposed shoot Au content was increased by 5.6-fold to 4.2 mg/kg (significantly different from Hoagland control at $p < 0.05$) and the bulk exposed tissue levels of Au were increased by 80% to 4.3 mg/kg (significantly different from Hoagland control at $p = 0.069$). The bulk and nanoparticle Au content at 250 mg/L in Hoagland's solution amended with humic acid did not differ significantly. However, at 750 mg/L, the shoot concentration of Au in bulk and nanoparticle exposed plants were 5.4 and 1.2 mg/kg, respectively (significantly different at $p = 0.063$). Similar to Ag, high replicate variability and non-significant results were noted with Cu-exposed plants. The Cu content of nanoparticle exposed plants ranged from 28–39 mg/kg whereas bulk Cu exposed plants contained 31–120 mg/kg; no differences of statistical significance were noted across particle size, concentration or solution type. Figure 2 shows the Si shoot content of plants exposed to bulk and nanoparticle Si in Hoagland's solution with or without humic acid. Plants exposed to 250 mg/L bulk Si contained levels of the element that were statistically equivalent to un-exposed controls. Plants exposed to NP Si contained concentrations of the element that were 5.6 and 6.5 times greater than the bulk Si exposed shoots in solutions with or without humic acid, respectively (significantly different at $p < 0.05$). Plants exposed to the bulk element at 750 mg/L in Hoagland's solution contained significantly greater Si than those from the 250 mg/L treatment but in the humic acid amended solutions, the levels at the two exposure concentrations were statistically equivalent. Similar to the lower concentration, zucchini tissues from the nanoparticle exposure at 750 mg/L were significant increased as compared to the corresponding bulk element treatment, regardless of solution type. When directly comparing elemental content across the solution type, humic acid did not significantly impact Si uptake as a function of particle size or concentration.

Few studies in the literature exist that directly compare element accumulation by plants exposed to equivalent bulk and nanoparticle solutions. The finding that exposure to Si nanoparticles resulted in significantly greater Si content than exposure to the corresponding bulk element agrees with Stampoulis et al. (2009), who observed similar patterns in particle-size dependent uptake of Ag. The mechanism driving the enhanced accumulation of Si from NP solutions are unknown but could involve greater ion dissolution due to higher surface activity or actual accumulation of the elemental nanoparticle itself. In the current study, the uptake of Ag was not particle-size dependent; these findings disagree with Stampoulis et al. (2009) but a number of differences exist between the two studies including plant age, exposure time, and exposure concentration. The accumulation of Cu was also not dependent on particle size; these findings are in line with those of Musante and White (2010). The mechanisms responsible for the decreased uptake of Au from NP solutions relative to the bulk element are unknown and are the topic of ongoing investigation. Similar to Musante and White (2010), humic acid amendment in the current study did not impact Ag, Cu, or Si accumulation by the plant. However, the uptake of Au at the low NP exposure concentration and at both concentrations of bulk Au exposure was significantly increased by humic acid amendment; the mechanisms responsible for this phenomenon are currently under investigation.

The findings of the current report add to a growing body of literature highlighting the differential toxicity, accumulation, and fate of engineered nanoparticles upon interaction with plants. The experimental design utilized in this study is significantly less labor intensive

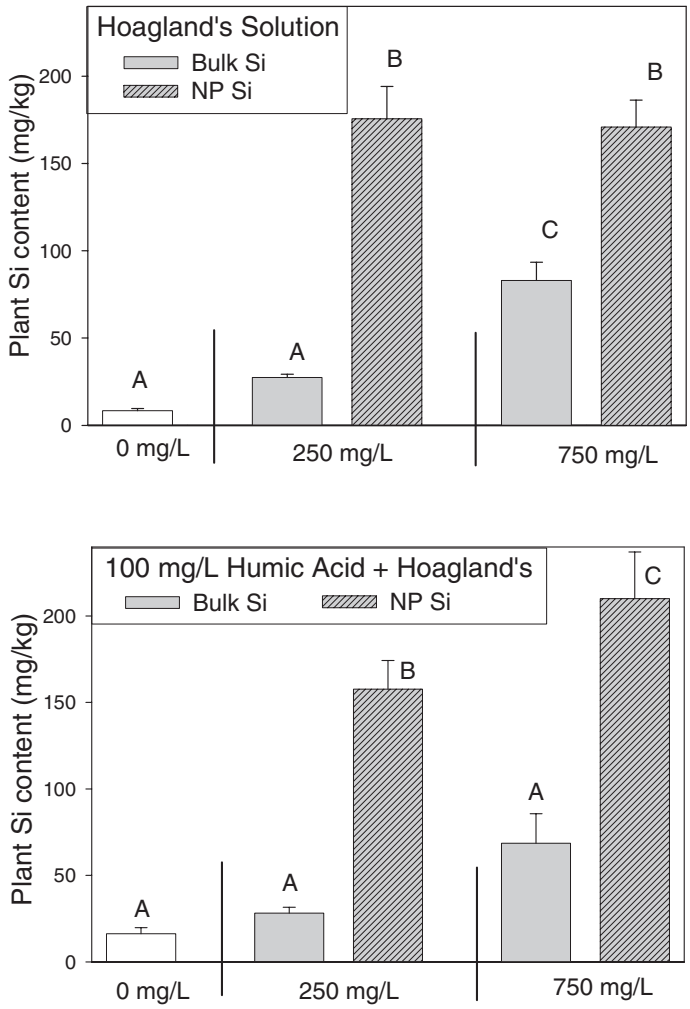


**Figure 2** Si content of zucchini exposed to 0–750 mg/L nano- or bulk particles in Hoagland's solution with (bottom) or without (top) 100 mg/L humic acid. Within a graph, bars with different letters are significantly different (One way ANOVA with Student Newman Keuls MCT). Error bars are standard errors of the mean.

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than our previous methods but has proven to be sufficiently accurate and sensitive. Given the widespread and increasing use of these materials in commercial applications such as pesticides and fertilizers, a full characterization of nanoparticle phytotoxicity, fate and transport in the planted systems is warranted.

## ACKNOWLEDGMENTS

We thank the University of New Haven Summer Undergraduate Research Fellowship (SURF) program which was awarded to J.H. in 2010 for funding this research project.

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