Environmental Science & Technology

Article

Cite This: Environ. Sci. Technol. 2018, 52, 2558−2565

pubs.acs.org/est

Stress Responses of Aquatic Plants to Silver Nanoparticles

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Supporting Information

ABSTRACT: Silver nanoparticles (AgNPs) are increasingly used in consumer products, biotechnology, and medicine, and are released into aquatic ecosystems through wastewater discharge. This study investigated the phytotoxicity of AgNPs to aquatic plants, *Egeria densa* and *Juncus effusus* by measuring physiologic and enzymatic responses to AgNP exposure under three release scenarios: two chronic (8.7 mg, weekly) exposures to either zerovalent AgNPs or sulfidized silver nanoparticles; and a pulsed (450 mg, one-time) exposure to zerovalent AgNPs. Plant enzymatic and biochemical stress responses were assessed using superoxide dismutase (SOD) and peroxidase (POD) activity, malondialdehyde (MDA) concentrations and chlorophyll content as markers of defense and phytotoxicity, respectively. The high initial pulse treatment resulted in rapid changes in physiological characteristics and silver concentration in plant tissue at the beginning of each AgNPs exposure (6 h, 36 h, and 9 days), while continuous AgNP and sulfidized AgNP chronic treatments gave delayed responses. Both *E. densa* and *J. effusus* enhanced their tolerance to AgNPs toxicity by increasing POD and SOD activities to scavenge free radicals but at different growth phases. Chlorophyll did not change. After AgNPs exposure, MDA, an index of membrane damage, was higher in submerged *E. densa* than emergent *J. effusus*, which suggested that engineered nanoparticles exerted more stress to submerged macrophytes.

INTRODUCTION

The extensive application of commercially manufactured silver nanoparticles (AgNPs) as antifungal, antibacterial, antiviral, and antimicrobial agents inevitably leads to AgNP release into the environment and may pose a risk to both the natural environment and organisms.[1,2] The toxicological literature on nanomaterial impacts suggests that AgNP exposure and loading could lead to significant changes in important ecosystem processes, such as primary productivity,[3,4] decomposition,[5] and nitrogen cycling.[6] Through simulation modeling efforts, it is recognized that AgNPs enter natural environments by means of wastewater discharge as both biosolids and effluent.[7−9] Therefore, the effects of nanoparticles on aquatic plants are of great concern because of their direct interface with both nanoparticles and their dissolved byproducts.[10] Other studies suggest that aquatic plants, as an important component of the aquatic ecosystem, should be included when evaluating the overall toxicological impact of engineered nanoparticles (ENPs) in the environment.[11]

While mechanisms of toxicity for nanoparticles have not yet been completely deciphered for all ENPs, in most cases toxicity appears to act primarily through a process of particle attachment to biological entities followed by reactions.[12] Possible reactions include disruption of membranes or potential membrane damage,[13] formation of reactive oxygen species,[14,15] cellular uptake and subsequent genotoxicity,[16] oxidation of proteins,[17] interruption of energy transduction,[11] and release of toxic constituents.[14]

A wide range of organisms—including bacteria,[18] algae,[19] fish,[19,20] and animal and human cells[20,21]—have been examined for their responses to AgNP exposure, but few toxicological studies focused on aquatic plants.[11] To date, some studies have shown that AgNPs may be detrimental to the germination and growth of aquatic plants.[11,22−24] The majority of toxicological experiments on macrophytes have been laboratory based, where high-dose treatments were applied to monoculture. Although investigations of this sort are essential in understanding the mechanisms and processes of toxicity, their applicability to natural may be limited.[24,25]

Received: November 14, 2017
Revised: January 5, 2018
Accepted: January 30, 2018
Published: January 30, 2018

DOI: 10.1021/acs.est.7b05837
Environ. Sci. Technol. 2018, 52, 2558−2565

A number of challenges lead to the difficulties in extrapolating from laboratory to ecosystem scales. The first comes from the disconnect between high-concentration short-term exposures in the lab vs low-concentration chronic exposures that aquatic plants are more likely to experience in ecosystems. Another challenge comes from the heteroaggregation and chemical transformation of AgNP with natural substances in natural systems, which can form aggregates larger than channel protein openings or plasmodesmata, which may exclude them from entering the cytoplasm.[26] For example, AgNPs were found to transport apoplastically and clump near plasmodesmata in *Arabidopsis thaliana*.[27] For those smaller particles that entered the symplast (inside of the plasma membrane), subsequent chemical transformations could make it difficult to confirm toxicity. Moreover, it can be challenging to detect when toxic effects begin at concentrations below those that have obvious inhibitory effects on plant growth, cause die-back, or senescence, end points which indicate that plants have already been seriously and irreversibly damaged. Conversely, while the growth and appearance of aquatic plants often do not show obvious signs of inhibition, this does not necessarily mean that there is no phytotoxicity or physiological stress on the plant. Therefore, the evaluation of phytotoxicity resulting from AgNPs exposure to aquatic plants in natural aquatic environments is more nuanced than in controlled laboratory conditions, and can benefit from assays that can detect biochemical changes, such as enzymatic responses to stress.

Here we describe the long-term effect of pulsed and chronic additions of engineered silver nanoparticles into freshwater emergent wetland mesocosms over a 12-month period to determine their bioavailability to wetland plant species as well as determine plant enzymatic and biochemical stress responses. Specifically, this study focuses on responses in superoxide dismutase (SOD) and peroxidase (POD) enzyme activity, malondialdehyde (MDA) concentration and chlorophyll content as markers of defense and phytotoxicity.

The stress from toxic materials has been shown to reduce plant metabolic activity, e.g., photosynthesis,[28] and also induce the production of reactive oxygen species (ROS) in plant cells.[29,30] These reactive oxygen free radicals may oxidize double bonds on fatty acid tails of membrane phospholipids in a process known as lipid peroxidation[15,30] and damage membranes resulting in a reduction of plant growth and potentially death.[31,32] To avoid the harmful effects of ROS, a set of antioxidant defense mechanisms in plant cells have evolved by increasing antioxidant enzyme activities, altering lipid peroxidation, and increasing antioxidant defense capacity, such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and others.[33−35] Malondialdehyde (MDA) is an end product of lipid peroxidation and is commonly used as a biomarker to index oxidative injury.[36,37] Under environmental stress, chlorophyll content may change, influence the functioning of the photosynthetic apparatus, and thus affect whole plant metabolism.[28] Therefore, these enzymatic responses,[33,38−41] chlorophyll content,[42] and MDA concentration[43] have been suggested to be a reliable marker of metal toxicity in macrophytes and to determine long-term plant biochemical responses to different exposure regimes.

In this study, we used 12 wetland mesocosms built on a slanted elevation design to simulate natural wetland ecosystems interfacing an aquatic and terrestrial environment and examined two aquatic plant species (submerged *Egeria densa*, Brazilian waterweed and emergent *Juncus effusus*, soft rush) in control mesocosms or exposed to one of three treatments: one-time pulse addition of zerovalent AgNPs, P−Ag(0)−NPs; chronic low-concentration weekly additions of either zerovalent AgNPs, C−Ag(0)−NPs, or an equivalent mass of silver as sulfidized silver nanoparticles, C−Ag_2S−NPs. Physiological responses and silver accumulation over time were measured to investigate the phytotoxicity of AgNPs to both the submerged and emergent plant species. The goals of the study were to determine: (1) what are the phytotoxicities of AgNPs to different growth forms of aquatic plants, submerged and emergent; and (2) how physiological and enzymatic responses of plants differ in their response to chronic, pulsed and sulfidized AgNPs exposure scenarios.

MATERIALS AND METHODS

Mesocosms. Twelve mesocosm containers previously designed and constructed[44] were refurbished for the experiment. New EPDM rubber (ethylene propylene diene terpolymer) liner was installed tightly fit to the bottom of each mesocosm structure. This liner was then overlain by a second liner made of polyethylene (Permalon PlyX-150; Reef Industries, Texas, U.S.A.). The rubber liner ensures containment, while the polyethylene liner was a less expensive and readily replaceable barrier that isolated the water, soil, and treatment substance from the EPDM liner. The soil mixture was from a local gardening supplier and was formulated based on previously constructed mesocosms.[25] The soil was filled in each of the 1.2 m wide mesocosms to a depth of ∼20 cm along a 2.8 m long slope descending to a 0.8 m long level floor to simulate lake shore or riparian river bank geomorphology. The mesocosm facility was housed in a framed greenhouse with a canopy installed in the winter to protect the system from expansion and damage due to freezing. In the summer, the canopy was removed and mesocosms received incoming rain.

Each mesocosm was partitioned into three zones along an inundation gradient: a submerged aquatic zone, a transition zone which was periodically flooded, and a rarely flooded upland zone. In the aquatic zone, the submerged species *Egeria densa* was planted in a grid pattern on April 1, 2013, 134 days before dosing. Native to South America, the female plants of this dioecious species occur sparsely in its natural habitat. As an introduced species to North America, *E. densa* reproduces vegetatively, and essentially all plants are male throughout the continent. *E. densa* leaves are produced in whorls of four to eight, 1−4 cm long and 2−5 mm broad, with an acute apex. In the transition zone, two rows of emergent plants of *Juncus effuses*, eight plants each, were planted on the upper edge of the aquatic zone, and *Carex lurida* and *Lobelia cardinalis* were planted landward on February 7, 2013, 187 days before dosing. At the same time, the upland zones were filled with *Panicum virgatum*, *Lolium perenne*, and *Chasmanthium latifolium*. (Mellow Marsh Farm, Siler City, NC). These choices represent typical wetland combinations found in wetlands in the southeastern U.S. To ensure homogeneous plant growth and density before dosing treatment, *E. densa* was replanted where missing from the mesocosms between June 25 and July 30, 2013.

Experimental Treatments. In addition to controls, three treatments were designed to reflect a difference of AgNP dosing intensity and the effects of freshly synthesized vs relevantly aged particles with all treatments receiving a total of 450 mg Ag over the course of the year. The three treatments were (1) AgNP Pulse Treatment, P−Ag(0)−NPs, AgNP added as a one-time

DOI: 10.1021/acs.est.7b05837
Environ. Sci. Technol. 2018, 52, 2558−2565

pulse of 450 mg Ag in 609 L; (2) AgNP Chronic Treatment, C−Ag(0)−NPs, AgNP added as 8.7 mg Ag weekly for a year, which totaled 450 mg Ag yr^{-1}; and (3) Weathered AgNP Chronic Treatment, C−Ag_2S−NPs, sulfidized AgNPs added at 8.7 mg Ag weekly for a year, which totaled 450 mg Ag yr^{-1}. The characterizations of the pristine AgNP stocks used in this experiment have been previously described.[25,44] Additional physical properties including TEM (transmission electron microscope) diameter, pH, zeta potential, and polydispersity of the nanoparticles are 3.9 ± 1.7 nm, 8.3, − 46.1 ± 2.0 mV, 0.344 ± 0.040 for AgNPs; 24.2 ± 0.6 nm, 6.9, − 51.8 ± 1.9 mV, 0.159 ± 0.004 for sulfidized AgNPs. Treatments commenced at 0800 h on August 13, 2013, (day 0). Treatment stock was mixed with 1 L of mesocosm water from the mesocosm to be treated, mixed, and then added using a modified Mariotte bottle. The bottle tip was kept 1 cm below water surface and moved about in a grid pattern to add treatment material as uniformly as possible to the water column.

Monitoring and Sampling. Before treatment began, a range of physical and chemical properties of treatments and control were analyzed, and there were no significant differences in any treatments in any of the measured parameters prior to starting treatments. During sampling, water samples were collected from a 10 cm depth for silver concentration at 6 h (August 13, 2013), 36 h (August 14, 2013), 9 days (August 22, 2013), 54 days (October 6, 2013), and 324 days (July 3, 2014) after dosing. Plant samples were taken from distal 20 cm branches of *E. densa* and from 10 cm proximal culms of *J. effusus* at the same schedule as water sampling. All plant samples were immediately placed in an ice box and stored in 4 °C for the measurements of POD and SOD activities, MDA and chlorophyll content, and silver concentration in tissues.

Silver in Water Column and Plant Tissue. Water column silver was determined by inductively coupled plasma mass spectrometry using an ICP-MS (Agilent 7500cx, Santa Clara, CA, U.S.A.). For aquatic plant tissue, the branches of each plant were cleansed with deionized water to remove adhered soil particles and algal debris, and then dried in a forced air oven at 70 °C for 48 h. The silver concentrations of plant tissues were determined in a nitric acid digest. Each sample was analyzed by atomic absorption spectroscopy (Perkin−Elmer 5100, Norwalk, CT, U.S.A.) equipped with graphite furnace (HGA-600, Perkin−Elmer 5100, Norwalk, CT, U.S.A.).

Assays for SOD and POD. A 0.3 g sample of plant tissue for both *E. densa* and *J. effusus* was cleaned with distilled water and homogenized using a 5-ml extraction solution (0.05 M phosphate buffer, pH 7.8), then centrifuged at 4 °C and 10 000*g* for 15 min. The supernatants were isolated and stored at 4 °C for further assays for SOD and POD activities. SOD activities were determined spectrophotometrically at 560 nm by measuring the inhibition in the photochemical reduction of nitroblue tetrazolium.[45] POD activities were determined spectrophotometrically at 470 nm by measuring the optical density of guaiacol oxidation products during particular intervals of time.[46]

Determination of MDA. Lipid peroxidation in leaf tissues was measured in terms of MDA concentrations in the leaf tissues of *E. densa* and *J. effusus*. Extraction was performed by homogenizing 0.3 g leaf tissue with 5 mL 10% (w/v) trichloroacetic acid (TCA), after which the homogenate was centrifuged at 10 000*g* for 10 min. The supernatant contains the MDA extract, which was stored at 4 °C before analysis. MDA levels were estimated according to the corrected TBA (thiobarbituric acid) method.[47]

Chlorophyll Content. A 0.2 g of plant tissue from each of *E. densa* and *J. effusus* was extracted with 10 mL anhydrous ethanol−acetone (1:2) mixture for 48 h in the dark.[48] The absorbance of the supernatant was measured spectrophotometrically at 663 and 645 nm. The total chlorophyll contents were calculated from the absorbance readings as described by Porra.[49]

Statistical Analyses. Comparisons among levels of each of the three main effects, species, treatment, and time are achieved through PROC MIXED procedure using SAS 9.4 package. Main effects as well as interactions were tested in the model statement, where denominator degrees of freedom were calculated using the between-within method (DDFM = BW). Time was designated as a repeated measure with an unstructured covariance matrix (TYPE = UN) that resulted in preferred goodness of fit, small Akaike information criterion (AIC). Least square means, accounting for other main effects, are compared to reflect the three-factor experiment. We used the more conservative studentized maximum modulus method (ADJUST = SMM) as adjustment for multiple comparisons among the least-squares means. Differences were considered significant when *t*-test *p* value was below type I error at α = 0.05. For all figures, data were presented as arithmetic mean ± standard error of the mean (SEM) of the three replicates for each treatment at specific time.

RESULTS AND DISCUSSION

Silver in the Water Column and in Plant Tissue. After 6 h of dosing, silver concentrations in the water column in the mesocosms dropped from our initial target dosing concentration of 750 $\mu g\ L^{-1}$ down to 467 ± 195 $\mu g\ L^{-1}$ for the P−Ag(0)−NPs treatment, which was significantly higher in the pulsed treatment than concentrations found in the chronic C−Ag(0)−NPs treatment (13.14 ± 0.84 $\mu g\ L^{-1}$) and C−Ag_2S−NPs treatment (16.19 ± 3.04 $\mu g\ L^{-1}$) (Figure 1A). We utilized closest measured Ag concentrations (1 week or less) rather than time-weighted concentrations when making our comparisons to the responses of plant enzymes. The concentrations of Ag peaks in the water column for the P−Ag(0)−NPs treatment and the chronic release treatments (C−Ag(0)−NPs treatment and C−Ag_2S−NPs treatment) all occurred near the first plant sample collection, 6 h past initial dosing commenced. Subsequently, silver concentrations kept decreasing and declined most rapidly in the P−Ag(0)−NPs treatment. By day 54, silver concentrations decreased to below 10 $\mu g\ L^{-1}$ in all Ag treatments, but silver concentration for the C−Ag(0)−NPs treatment (4.64 ± 3.03 $\mu g\ L^{-1}$) and C−Ag_2S−NPs treatment (6.42 ± 2.97 $\mu g\ L^{-1}$) rose to levels slightly higher than that in the P−Ag(0)−NPs treatment (1.94 ± 1.06 $\mu g\ L^{-1}$). By day 324, Ag concentrations in all treatments remained quite low, Ag concentration for the C−Ag(0)−NPs treatment (2.33 ± 1.03 $\mu g\ L^{-1}$) and C−Ag_2S−NPs treatment (2.99 ± 1.57 $\mu g\ L^{-1}$) were still higher than that in the single P−Ag(0)−NPs treatment (0.23 ± 0.14 $\mu g\ L^{-1}$) (Figure 1A).

For *E. densa* tissue, all AgNP treatments resulted in increases of silver in *E. densa* after AgNP exposure. Ag concentrations in tissue ranged from 2.38 ± 0.15 $\mu g\ g^{-1}$ to 19.50 ± 4.72 $\mu g\ g^{-1}$ in the C−Ag(0)−NPs treatment, 2.01 ± 0.87 $\mu g\ g^{-1}$ to 6.27 ± 2.85 $\mu g\ g^{-1}$ in the C−Ag_2S−NPs treatment, 1.20 ± 0.13 $\mu g\ g^{-1}$ to 17.75 ± 3.58 $\mu g\ g^{-1}$ in the P−Ag(0)−NPs treatment, and was undetectable in the control. Despite great dissimilarities in

DOI: 10.1021/acs.est.7b05837
Environ. Sci. Technol. 2018, 52, 2558−2565

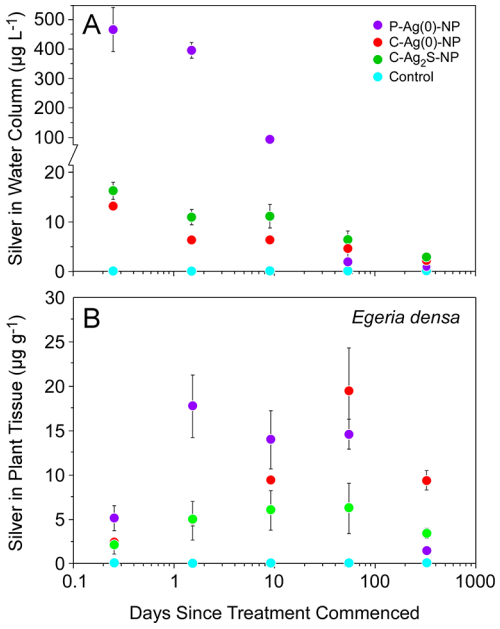

Figure 1. Silver (Ag) concentrations in (A) the water column by pulsed (P) and chronic (C) treatment and days from treatment and (B) *Egeria densa* silver tissue concentrations by treatment and days from treatment. Values are mean ± SEM for $n = 3$.

the form of silver and dosing procedures, tissue Ag concentrations in all treatments were all higher ($p \leq 0.0037$) than those in the control (Figure 1B). At the beginning of AgNPs exposure (6 h, 36 h, and 9 days), silver concentrations in *E. densa* tissue in the P−Ag(0)−NPs treatment were obviously higher than those in the chronic dosing treatments. With continuous exposure and the accumulation of AgNPs, silver concentrations in *E. densa* tissue in C−Ag(0)−NPs treatment gradually increased and reached higher concentrations than those in other treatments after dosing 54 days. Metabolism of *E. densa* growing in a temperate climate can be curtailed in both summer and winter.[50] However, at 36 h in the height of hot summer, the pulse treatment P−Ag(0)−NPs provided a concentrated medium that allowed for ample uptake by the plants. The exceptionally high concentration probably exceeded the level for normal plant active uptake, which is related to its growth vitality. However, the chronic treatments did not result in extra uptake through passive channels like in the pulse treatment. The C−Ag(0)−NP and C−Ag_2S−NP treatments in *E. densa* did not reach their peaks until day 54 in October, when weather conditions turned favorable for the accelerated metabolism. During the experiment, the Ag concentration in *E. densa* tissue were lowest after dosing for 6 h (August) for all three treatments in the first year. In the second year (after dosing 324 days, July), Ag concentration in *E. densa* tissue were significantly decreased compared with those by day 54 ($p < 0.001$). It was probably because Ag transferred to the sediment through decay of plant tissue in the winter. A significant interaction ($p = 0.0064$) between treatment and time existed in our study. The highest Ag concentrations in *E. densa* tissue over time was 17.75 ± 3.58 μg g^{-1} in the P−Ag(0)−NPs treatment after dosing for 36 h, 19.50 ± 4.72 μg g^{-1} in the C−Ag(0)−NPs treatment by day 54 and 6.27 ± 2.85 μg g^{-1} in the C−Ag_2S−NPs treatment by day 54 (Figure 1B), which lagged behind Ag concentration peaks in the water column at 6 h (Figure 1A). Delayed uptake trends reflect the bioavailability of the nanoparticles.

During the whole experiment, Ag concentrations in *J. effusus* aboveground tissue in all treatments were at or below the method detection limit (MDL equivalent to 2 μg g^{-1}) of graphite furnace Atomic Absorption Spectroscopy. Roots retained higher concentrations of Ag but only limited sampling was done to reduce plant growth damage. This result suggested that AgNPs in aquatic environments will exert less stress to emergent than submerged macrophytes in this kind of lentic systems. The soil-rooted emergent plants, flooded intermittently, uptake nutrients, and metals mostly from sediment via roots, but nanoparticle mobility in soil was found to be very low,[44] which restricted the absorption and transportation of silver in our emergent plants. Bao et al.[51] research found that the minuscule AgNP in emergent *J. effusus* culm tissue may be attributable to a lack of acropetal transport of AgNP beyond the root cell wall. In addition, our early mesocosm tests revealed predominant AgNP accumulation in root tissue (4.49−8.87 μg g^{-1}) versus that of culms (0.42−0.72 μg g^{-1}) in *J. effusus*, which helps explain our low aboveground AgNP concentrations.

In contrast, submerged aquatic macrophytes, such as *E. densa*, totally submerged and drifting in the water column are able to take up nutrients and metals from both sediment by roots and from water column by stems, leaves, and adventitious roots.[52] Furthermore, agglomerated ENPs can be resuspended in water column due to many natural processes, e.g., turbulence in rivers,[53] or suspension by natural organic matter.[15,31,54−56] Compared with emergent plants, submerged macrophytes must also exhibit much faster mass transfer rates to acquire enough CO_2 for photosynthesis and other dissolved gases from the water.[32] These mass transfer rates are enabled by an underdeveloped or absent epidermis and/or protective lipophilic cuticle.[32] Consequently, well-dispersed ENPs can interact directly with the cellulose cell wall. This can facilitate AgNPs absorption by submerged plants thus exerting more ENPs stress to submerged macrophytes.

Enzymatic Activity and Physiological Responses. Submerged *E. densa* and emergent *J. effusus*, both had pronounced SOD activities 6 h after dosing with AgNPs and decreased quickly at day 9 (Figure 2). However, the SOD activities significantly differed between species ($p < 0.0001$), among treatments ($p < 0.0001$) and among times ($p < 0.0001$). *J. effusus*, being an aquatic wetland emergent plant, had significantly less SOD activity than those of the submerged species *E. densa*. The SOD activities of all treatments were significantly different from controls ($p \leq 0.0088$). Analyses of SOD activity also showed considerable differences between species in their activities at various times. Significant interactions for SOD was found between treatment and time ($p \leq 0.0001$). SOD activities were highest in the sulfidized AgNPs treatment for *E. densa* at 6 h (35.34 ± 1.95 unit g^{-1}) and in the chronic AgNPs treatment for *J. effusus* at 6 h (27.78 ± 3.90 unit g^{-1}) (Figure 2). After dosing for 9 days, the SOD activities of the two aquatic plants in different treatments matched control values and gradually reached a lower uniform activity level. By day 324, SOD activities were not discernible between treatments and controls for both species (Figure 2).

The POD activities of *E. densa* and *J. effusus* in all treatments mesocosms increased steadily and reached their highest values by day 9 or day 54 for the different treatments in the first year and fell to the level of the controls by day 324 (July in the second year) (Figure 3). Throughout the 324 days of the

DOI: 10.1021/acs.est.7b05837
Environ. Sci. Technol. 2018, 52, 2558−2565

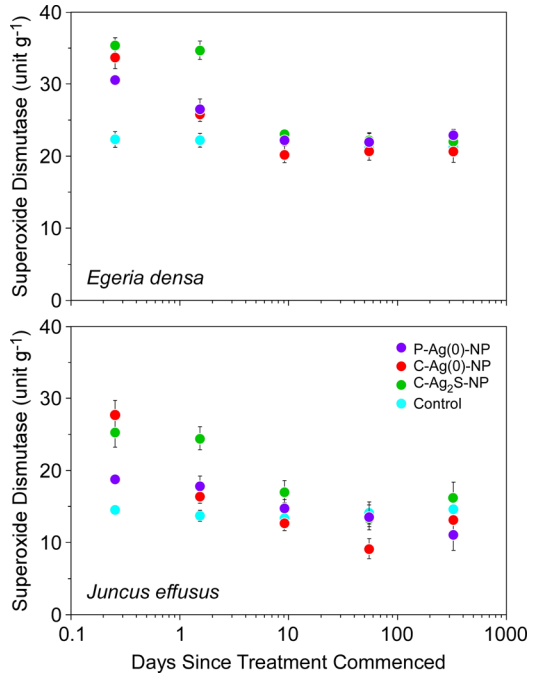

Figure 2. SOD activities in *Egeria densa* and *Juncus effusus* varied by pulsed (P) and chronic (C) treatment and by time. Values are mean ± SEM for $n = 3$.

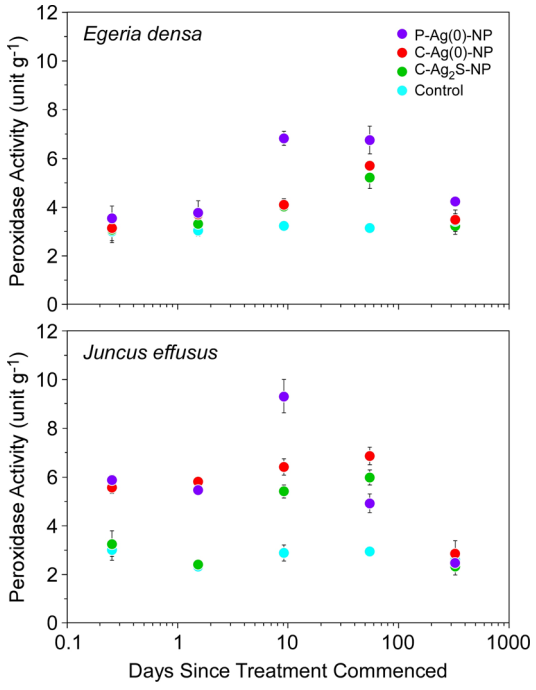

Figure 3. Peroxidase (POD) activities in *Egeria densa* and *Juncus effusus* varied by pulsed (P) and chronic (C) treatment and by time. Values are mean ± SEM for $n = 3$.

experiment, POD in the control remained low and invariant. Similar to SOD, significant POD differences were detected between species, among treatments and over time ($p \leq 0.0001$, Figure 3). *J. effusus* showed relative higher POD activities than *E. densa* ($p = 0.0001$). Significant interactions existed between treatment and time ($p \leq 0.0001$), and between treatment and species ($p \leq 0.0001$) for POD. POD activities were the highest under the P−Ag(0)−NPs treatment by day 9 (Figure 3). By day 54, POD activities under the C−Ag(0)−NPs treatment and C−Ag_2S−NPs treatment were highest for both aquatic plants. However, POD activities of both *E. densa* and *J. effusus* were maximum in the P−Ag(0)−NPs treatment. Following P−Ag(0)−NPs treatment, the two chronic treatments, C−Ag(0)−NPs and C−Ag_2S−NPs, had similar ($p = 1.0000$) POD activities, but higher ($p \leq 0.0330$) than the controls (Figure 3).

The enzymatic activity results indicated that increasing reactive oxygen species (ROS) produced by AgNPs led to oxidative stress.[29,30] This in turn induced synthesis of antioxidant enzymes to scavenge free radicals and enhance the peroxides defense system in both aquatic plant species to reduce the organism's environmental stress induced by AgNPs. In the second year (after dosing 324 days), environmental stress reduced with the reduction of Ag concentration both in water and tissue so that antioxidant enzyme activity decreased and values were similar to controls. However, it should be noted that antioxidant enzymes played different roles for different species at different time after dosing. For example, SOD responded in a matter of hours after dosing in both aquatic species and then declined, while POD responded almost immediately in *J. effusus* but not until after 9 days in *E. densa*. Highest levels of SOD activities occurred at 6 h, but POD activities were highest after day 9.

The MDA contents of *E. densa* and *J. effusus* in all treated mesocosms were all increased initially, reached highest values at day 9 and then gradually decreased (Figure 4). The change in

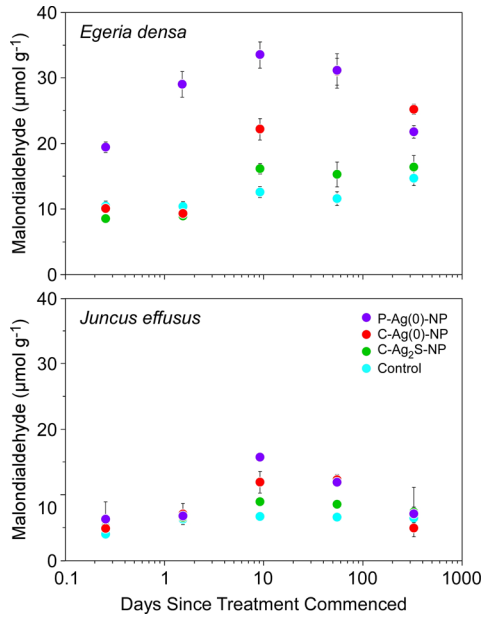

Figure 4. Malondialdehide (MDA) contents in *Egeria densa* and *Juncus effusus* varied by pulsed (P) and chronic (C) treatment and by time. Values are mean ± SEM for $n = 3$.

MDA contents of these two aquatic plants showed similar trends, but MDA contents differed among treatments ($p < 0.0001$), between species ($p < 0.0001$) and among times ($p < 0.0001$). MDA content in both *E. densa* and *J. effusus* were highest in the AgNP pulse treatment and those in the AgNP chronic treatment followed, while MDA contents in C−Ag_2S−NPs treatment were close to control values. It suggested that the addition of AgNPs had different impacts depending on which treatment P−Ag(0)−NPs > C−Ag(0)−NPs > C−

DOI: 10.1021/acs.est.7b05837
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Ag_2S−NPs was used on an annual basis. Sulfidized nanoparticles had relatively weaker damage on aquatic plant, suggesting that the uptake and impacts of unweathered nano materials are likely to be overemphasized. The MDA contents of *J. effusus* were dramatically lower than *E. densa* ($p < 0.0001$). For *E. densa*, MDA contents ranged from 8.82 ± 0.38 μmol g^{-1} to 33.58 ± 3.50 μmol g^{-1}. For *J. effusus*, MDA contents ranged from 4.08 ± 0.21 μmol g^{-1} to 15.83 ± 2.69 μmol g^{-1}, which were almost half of those in *E. densa*. The interactions between species and time ($p = 0.0280$), between treatment and time ($p = 0.0005$), and between treatment and species ($p < 0.0001$) all showed significant for MDA contents. MDA content were the highest under the P−Ag(0)−NPs treatment and C−Ag_2S−NPs treatment by day 9, but highest under C−Ag(0)−NPs treatment by day 54 (Figure 4). It was reported that a significant increase of MDA content in green alga *Chlamydomonas reinhardtii* was measured 6 h after cultivating in the presence of TiO_2 nanomaterials.[57] However, MDA levels in *Vicia faba* were not modified following exposure to altered TiO_2 nanocomposites, because of nanoparticles' surface modification.[13] In our study, a trend of increased MDA in both of *E. densa* and *J. effuses* clearly indicated the cellular lipid peroxidation and membrane damage was induced by AgNPs. The leveling-off and decrease in lipid peroxidation after 324 days exposure may have resulted either from aggregation of AgNPs and/or the possible biomodification of AgNPs by the cells after long exposure.[58,59]

The variable chlorophyll contents of all treatment mesocosms followed the seasonal pattern for controls and showed no treatment effects. There were differences of chlorophyll content between *E. densa* and *J. effusus* over time ($p \leq 0.0149$) in each mesocosm, but treatment effects within each species were not discernible (Figure S1 of the Supporting Information). Overall, the chlorophyll contents of *E. densa* were higher than those in *J. effusus* ($p < 0.0001$, Figure S1). Despite AgNP dosing intensity and sulfidation differences, photosynthetic pigments of these two aquatic plants were insensitive to treatments when compared to controls. The highest silver concentrations in *E. densa* tissue was close to 20 μg g^{-1}, but the chlorophyll contents of *E. densa* were not lower than controls, which indicates that *E. densa* has a high tolerance to AgNPs. The significant interactions only existed between species and time ($p = 0.0001$). The highest chlorophyll contents for *E. densa* and *J. effusus* were at day 324 (in July) and lowest in day 54 (in October). Different to our results, a time-dependent decrease in total chlorophyll content was observed in an aquatic macrophyte *Spirodela polyrhiza* after respective treatment with 5 mg L^{-1} AgNPs and $AgNO_3$, which inhibited the plants ability to photosynthesize.[11] It is possible that the exposure concentrations of AgNPs in our mesocosms are much lower than in the lab monoculture experiments, and other organisms or media in the mesocosms system could directly or indirectly sorb part of AgNPs in mesocosms, which then decreased the influence of AgNPs on chlorophyll content in our aquatic plants, suggesting that chlorophyll content is not a sensitive marker for Ag stress in the natural environment.

In summary, the physiologic characteristics and silver accumulation of submerged and emergent aquatic plants showed that AgNPs caused some injury and stress to aquatic plants, but macrophytes also displayed enzymatic defenses to tolerate relatively low-concentration of AgNPs exposure. These physiologic characteristics of plants are sensitive enough to detect the toxic effects of abiotic and biotic environmental stress and can be used to directly evaluate phytotoxicity.[30,59,60] Through our use of a range of aquatic vegetation from submerged plants to emergent wetland plants, field conditions, and different AgNPs release scenarios, our mesocosms experiments were designed to follow complex natural environments influenced by the effluent often found from wastewater treatment plants. Thus, these results could easily be transferable to real world environmental conditions. Additional research is needed to determine how reproductive properties of different aquatic plants respond to different AgNPs exposure concentrations in a natural aquatic or wetland ecosystem and importantly determine the toxicity thresholds of AgNPs for different aquatic plants, especially submerged species.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b05837.

(Figure S1) The total chlorophyll content in *Egeria densa* and *Juncus effusus* (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Anna Fedders and Fabienne Schwab for setting up, maintaining the mesocosm facility, and helping in sample collections. Major funding source came from NSF (DBI-1266252), and EPA (EF-0830093). The authors are funded through the Center for the Environmental Implications of Nanotechnology (CEINT) supported by grants from the National Science Foundation (NSF) Division of Biological Infrastructure and the U.S. Environmental Protection Agency (EPA) as well as the Duke Wetland Center endowment. Any opinions, findings, conclusions, or recommendations expressed in this paper are those of the authors and do not necessarily reflect the views of the NSF or the EPA. This work has not been subjected to EPA review and no official endorsement should be inferred.

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