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Biotic and Abiotic Interactions in Aquatic Microcosms Determine Fate and Toxicity of Ag Nanoparticles: Part 2—Toxicity and Ag Speciation

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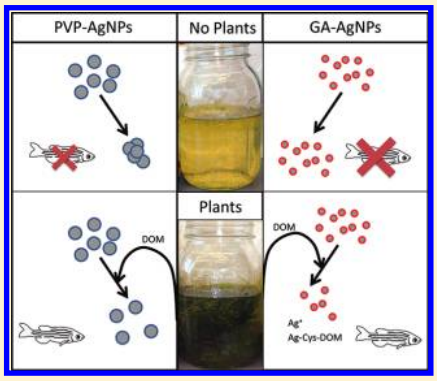
Supporting Information

ABSTRACT: To study the effects of complex environmental media on silver nanoparticle (AgNP) toxicity, AgNPs were added to microcosms with freshwater sediments and two species of aquatic plants (*Potamogeton diversifolius* and *Egeria densa*), followed by toxicity testing with microcosm surface water. Microcosms were designed with four environmental matrices in order to determine the contribution of each environmental compartment to changes in toxicity: water only (W), water + sediment (WS), water + plants (WP), and water + plants + sediment (WPS). Silver treatments included AgNPs with two different coatings, gum arabic (GA-AgNPs) or polyvinylpyrollidone (PVP-AgNPs), as well as $AgNO_3$. Water samples taken from the microcosms at 24 h postdosing were used in acute toxicity tests with two standard model organisms, early life stage zebrafish (*Danio rerio*) and *Daphnia magna*. Speciation of Ag in these samples was analyzed using Ag L3-edge X-ray absorption near edge spectroscopy (XANES). Silver speciation patterns for the nanoparticle treatments varied significantly by coating type. While PVP-AgNPs were quite stable and resisted transformation across all matrices (>92.4% Ag(0)), GA-AgNP speciation patterns suggest significantly higher transformation rates, especially in treatments with plants (<69.2% and <58.8% Ag(0) in WP and WPS, respectively) and moderately increased transformation with sediments (<85.6% Ag(0)). Additionally, the presence of plants in the microcosms (with and without sediments) reduced both the concentration of Ag in the water column and toxicity for all Ag treatments. Reductions in toxicity may have been related to decreased water column concentrations as well as changes in the surface chemistry of the particles induced by organic substances released from the plants.



INTRODUCTION

Ag nanoparticles (AgNPs) have been increasingly used for their antimicrobial properties, with production estimates in the United States between 2.8 and 20 tons per year.[1] While environmental concentrations are currently unavailable for AgNPs, and potential environmental exposures are poorly constrained, the increasing use of AgNPs has raised concern

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over their likely release into aquatic ecosystems.[2,3] Whether directly released as AgNPs, or via formation of secondary incidental nanoparticles, environmental exposures are projected to increase and could affect aquatic ecosystems.

The mechanism of ionic Ag toxicity to aquatic organisms is well characterized. Exposure to Ag disrupts ionoregulation via reduction of Na^+/K^+ ATPase activity and can cause significant mortality to fish and aquatic invertebrates at $\mu g\ L^{-1}$and even ng L^{-1} levels.[4,5] In recent years, several studies have investigated AgNP toxicity in vivo in aquatic organisms. Lethal and sublethal toxic end points for fish are reached in the high $\mu g\ L^{-1}$ or low mg L^{-1} range[6,7] and the mid to low $\mu g\ L^{-1}$ range for daphnids,[8,9] and in general, AgNPs are found to be significantly less toxic on a Ag mass basis than $AgNO_3$.[10] While the mechanisms of AgNP toxicity to aquatic organisms are less clear, Ag ion release is a significant factor.[10] Some studies suggest that AgNP toxicity is mediated through a "Trojan-horse" mechanism in which the toxicity is mechanistically identical to ionic Ag, however, the interaction of AgNPs with the media and organism results in differential uptake and biodistribution.[11] Alternatively, other studies suggest that AgNP toxicity is a combination of ionic Ag toxicity and a NP-specific toxic mechanism such as oxidative stress.[6,8,12]

Factors that mediate the interactions of AgNPs with the media and organism in this case include surface charge, size, aggregation state, and dissolution rate.[10] These determinants are in turn highly dependent on factors such as ionic strength, pH, and organic matter quantity and composition.[13−16] Altering any of these environmental factors could alter the uptake and toxicity of AgNPs. Thus, exposure scenarios performed in simple media or clean water conditions are not likely to be reflective of NP toxicity in the environment.

Of particular interest in this study was the role of aquatic plants and sediments along with their associated organic matter in mediating AgNP toxicity. While the amelioration of ionic Ag toxicity by organic matter has been well documented, the effect of organic matter on AgNP toxicity has gone largely unstudied. Organic matter has been shown to result in increased NP toxicity due to stabilization of the NP in aqueous solution.[17,18] In addition, the type and source of organic matter can control the extent and nature of its influence on AgNP toxicity.[14]

We used a microcosm approach to test whether the presence of sediments, plants, or the combination of both and their associated dissolved organic matter (DOM) elevates or reduces the acute toxicity of AgNPs to two model aquatic organisms: zebrafish (*Danio rerio*) and *Daphnia magna*. We hypothesize that the presence of DOM will alter the speciation, complexation and aggregation state of Ag, and that these changes will influence the toxicity of Ag to zebrafish and *Daphnia magna*. To understand how speciation affects toxicity, we used X-ray absorption near edge spectroscopy (XANES) to determine Ag speciation, which is likely to influence bioavailability and toxicity of Ag to the organisms. We also studied the aggregation state of AgNPs and complexation of dissolved Ag using asymmetrical flow field flow fractionation coupled with inductively coupled plasma mass spectrometry (AF4-ICP-MS). The results of the AF4-ICP-MS analysis of samples are reported in the companion manuscript to this paper and will be referenced in this study.[19] Finally, to examine the potential importance of organic matter quality on AgNP toxicity, we measured fluorescence excitation−emission matrix (EEMs) spectra to identify changes in dissolved organic matter pools before and after additions of AgNPs.

MATERIALS AND METHODS

Silver Nanomaterial Synthesis and Characterization. Gum arabic (GA) AgNPs were prepared and characterized as described in Cheng et al.[20] Polyvinylpyrrolidone (PVP) AgNPs were prepared and characterized as described in Yang et al.[21] $AgNO_3$ and KNO_3 were obtained from Sigma-Aldrich (Sigma Aldrich Co., St. Louis, MO).

Microcosm Design. Microcosms were constructed using 1-quart mason jars (Ball Corporation, Broomfield, CO). Initially, 48 replicate microcosms were prepared by adding 600 mL deionized water to 200 g field moist soil (187 g dry). A blended soil was used for these experiments (Sands and Soils, Durham, NC) having 64% sand, 23% silt, and 13% clay, and loss on ignition of 5.1%. Over 3 months, microcosms were incubated at 22 °C in the dark to allow sediment and water quality to equilibrate. Four different environmental matrices were then established with 12 replicates each: water only (W), water + sediment (WS), water + plants (WP), and water + plants + sediments (WPS). To establish sediment free matrices, water was decanted from 24 of the microcosms into separate quart ball jars without disturbing the sediment. For matrices with plants, 3 g of freshly harvested *Potamogeton diversifolius* and 6 g of *Egeria densa* from untreated wetland mesocosms maintained in the Duke Forest (Durham, NC) were added to each microcosm.

Microcosm Dosing. Microcosms were dosed on the day environmental matrices were established. Four treatments were prepared in triplicate for each matrix: control, $AgNO_3$, GA-AgNP, and PVP-AgNP. Treatments were applied directly to the water column of each microcosm at 2 mg L^{-1} Ag for the Ag treatments, followed by gentle stirring. A 2 mg L^{-1} dose concentration was chosen based on preliminary laboratory studies suggesting it as a dose high enough to ensure some mortality for all three types of Ag and also high enough to give us samples we could analyze via XANES. Control, GA-AgNP, and PVP-AgNP also received 0.32 mg L^{-1} KNO_3 to control for the NO_3^- added with the $AgNO_3$. After dosing, microcosms were maintained in a growth chamber for 24 h at 25 °C on a light cycle with 18 h light: 6 h dark with cool fluorescent lamps with a photosynthetic photon flux of 100 $\mu mol\ m^{-2}\ s^{-1}$. At 24 h, water samples were taken from the microcosms for acute toxicity testing, water quality and Ag characterization, and quantification by decanting the water.

Animals: Care and breeding. *Danio rerio.* Adult zebrafish were obtained from Ekkwill Waterlife Resources (Ruskin, FL). Cultures were maintained at Duke University. In the laboratory, zebrafish were maintained in 60 mg L^{-1} Instant Ocean, at 28 °C and pH 6.5−7.5 in an AHAB flow-through system (Aquatic Habitats, Apopka, FL) on a 14:10 light:dark cycle. Fish were fed *Artemia franciscana* nauplii and a mix of Cyclop-eeze (Argent Chemical Laboratories, WA) and Ziegler's Adult Zebrafish Complete Diet (Aquatic Habitats). Embryos were collected by natural spawning and rinsed with 30% Danieau water before dosing.[22]

Daphnia magna. Cultures were maintained at the Clemson University Institute of Environmental Toxicology. *Daphnia* were cultured in reconstituted moderately hard water (MHW) using the U.S. Environmental Protection Agency recipe: 190 L 18 MΩ deionized water, 11.4 g $CaSO_4$, 11.4 g $MgSO_4$, 0.76 g KCl, 18.24 g $NaHCO_3$.[23]

Exposure. For the toxicity experiments, dilutions were necessary for zebrafish (GA-AgNPs and $AgNO_3$ treatments)

and *Daphnia* (all treatments) as the treatments resulted in mortality rates too high to evaluate the potential of plants and/or sediments to alter toxicity. For zebrafish acute toxicity testing, both undiluted samples and samples diluted 50% with 2X MHW were used. Zebrafish embryos were screened for normal development at the 4−8 cell stage and immediately dosed individually with 0.2 mL in 96-well plates, with $n = 24$ embryos for each microcosm sample. For *Daphnia*, 48 h static nonrenewal bioassays were conducted using 1 in 10 dilutions of microcosm water in MHW. For each microcosm sample, five *D. magna* neonates (less than 24 h old) were dosed in 30 mL of diluted sample in glass beakers. Exposures were run in triplicate for a total $n = 15$ neonates for each microcosm sample.

Dissolved Organic Carbon (DOC) Quantification. To measure dissolved organic carbon—operationally defined as that fraction of C in natural organic matter that passes through an ashed 0.7 μm glass fiber filter—filtered samples were analyzed for nonpurgeable organic carbon on a Shimadzu TOC-V CPH (Shimadzu, Columbia, MD).

Fluorescence Excitation−Emission Matrices (EEMs) Analysis. Samples for analysis by ultraviolet−visible (UV−vis) absorbance and fluorescence were filtered through ashed 0.7 μm glass fiber filters immediately following incubation termination and stored at 4 °C until analysis within seven days of collection. Analysis of UV−vis absorbance and fluorescence was conducted in 1-cm path length quartz cuvettes on samples diluted 2-fold with lab-grade deionized (DI) water (Dracor, NC) to minimize interference from the inner-filter effect. Absorbance spectra were measured using a USB4000-USB-ISS-UV/vis spectrophotometer (OceanOptics, FL). Fluorescence excitation−emission matrices (EEMs) were measured using a Fluorolog-321 spectrofluorometer with a synapse charge-coupled device (CCD) detector in ratio mode (Horiba Jobin Yvon, NJ). Intensities in EEMs are reported in Raman Units (RU) following normalization to the area under the water Raman peak at excitation of 350 nm from the blank EEM.

Changes in the quality of the fluorescent fraction of DOM were quantified as changes in the fluorescence index (FI), a proxy for DOM source,[24] and as emission intensity in the three characteristic peak regions A, C, and T related to humic and protein-like moieties.[22] The FI was calculated as the ratio of emission intensities at 470 and 520 nm at excitation 370 nm.[25] Emission intensity at peaks A, C, and T was evaluated at excitation/emission pairs 250/450, 350/450, 275/340 (nm/nm), respectively, in RU.[22]

It is not possible to quantify what fraction of DOM is fluorescent, thus analysis of the FI and emission intensity at peaks A, C, and T are qualitative measures of a shift in DOM quality. For example, high FI values are indicative of less aromatic DOM sources derived from bacterial and algal precursors, while lower FI values correlate with greater aromatic content derived from the breakdown of higher plant and soil organic matter.[24] Peaks A and C are both associated with humic soil organic matter (Supporting Information (SI) Figures S1A and S1B). Peak T is described as tryptophan-like or protein-like due to its similarity to the fluorescent signature of the indole ring of tryptophan and is often correlated with bacterial or algal processing of DOM.[26,27] However, peak T can also reflect releases of very fresh plant matter.[28,29]

Ag Speciation. The speciation of Ag containing particles in the water column was studied using Ag L3-edge X-ray absorption near edge spectroscopy (XANES), which gives insight into the oxidation state of Ag and its local atomic coordination. Approximately 100 mL of water decanted from the Ag treated microcosms were first prefiltered through a 0.7 μm glass fiber filter, and then were passed through 0.025 μm filters (VSWP Millipore) fitted on a glass vacuum filtration apparatus to separate and collect AgNPs and Ag^+ associated with particles and colloids from dissolved Ag. The 0.025 μm filter membrane was stored at −20 °C for one hour, freeze-dried, and stored in a desiccator before XANES analysis. XANES spectra of the sample filters and reference materials were collected at room temperature in fluorescence mode at the Stanford Synchrotron Radiation Lightsource (SSRL) on beamline 4−3. Background subtraction and normalization were performed on spectra using SixPack and Athena software packages following standard procedures.[30,31] Least-squares combination fitting (LCF) was performed between the samples and two reference compounds within the energy range from 20 eV before the adsorption edge (3351 eV) and 20 eV after the edge.[32,33] LCFs were ranked based on the three fitting parameters R factor, χ^2, and reduced χ^2, better fits having lower R, χ^2, and $\Delta\chi^2$ values. The most likely reference compounds (as determined from the fitting parameters) were then used in three component LCFs to test the relative contribution from each one. Errors reported for individual components are calculated by the software's least-squares fitting module.

Statistical Analysis. Matrix effects on organic matter quantity and quality and acute toxicity were determined by one-way ANOVA by treatment with posthoc analysis using Holm-Sidak comparison of means SigmaStat 4 (Systat Software Inc., San Jose, CA). Pearson's analyses were performed using JMP 9.0 (SAS Institute Inc., Cary, NC). A p-value of ≤ 0.05 was considered significant in all analyses.

RESULTS

Silver Nanomaterial Synthesis and Characterization. PVP-AgNPs had a nominal size by transmission electron microscopy (TEM) of 49.3 ± 22.5 nm while GA-AgNPs had a nominal size of 12.0 ± 9.2 nm. TEM images and analysis are available in SI Figure S1. Hydrodynamic diameter and zeta potential of the NPs were also determined and are available in Unrine et al.[19] The zeta potentials for the GA-AgNPs and PVP-AgNPs in DI water at pH 5.8 were −46.3 ± 6.8 mV and −23.0 ± 10.0 mV, respectively. In 50% synthetic moderately hard water[19] at pH 6.8, the zeta potentials were −19.4 ± 4.38 mV and 2.97 ± 10.5 mV for GA-AgNPs and PVP-AgNPs, respectively.[19]

Effect of Matrix on Water and DOM Quality. There was no pattern in pH among the different matrices, with average pH values of 6.0−6.5 (data not shown). In the control treatments, there was no detectable change in DOC concentration across all matrices. In Ag-treated microcosms, however, there was an increase in DOC in matrices containing plants (i.e., WP and WPS), with the magnitude of the increase in DOC varying with the Ag treatment. The $AgNO_3$-dosed microcosms had the largest increase in DOC, GA-AgNP-dosed microcosms had a similar increase, and PVP-AgNP-dosed microcosms had the smallest increase (Figure 1A). In WS and W matrices, there was no detectable change in DOC concentration. The increase in DOC in PVP-AgNP-treated WP microcosms was not statistically significant due to an outlier in this matrix causing an increase in variance. Due to the strong trend of an increase in DOC concentration in WP and WPS matrices, discussion of the

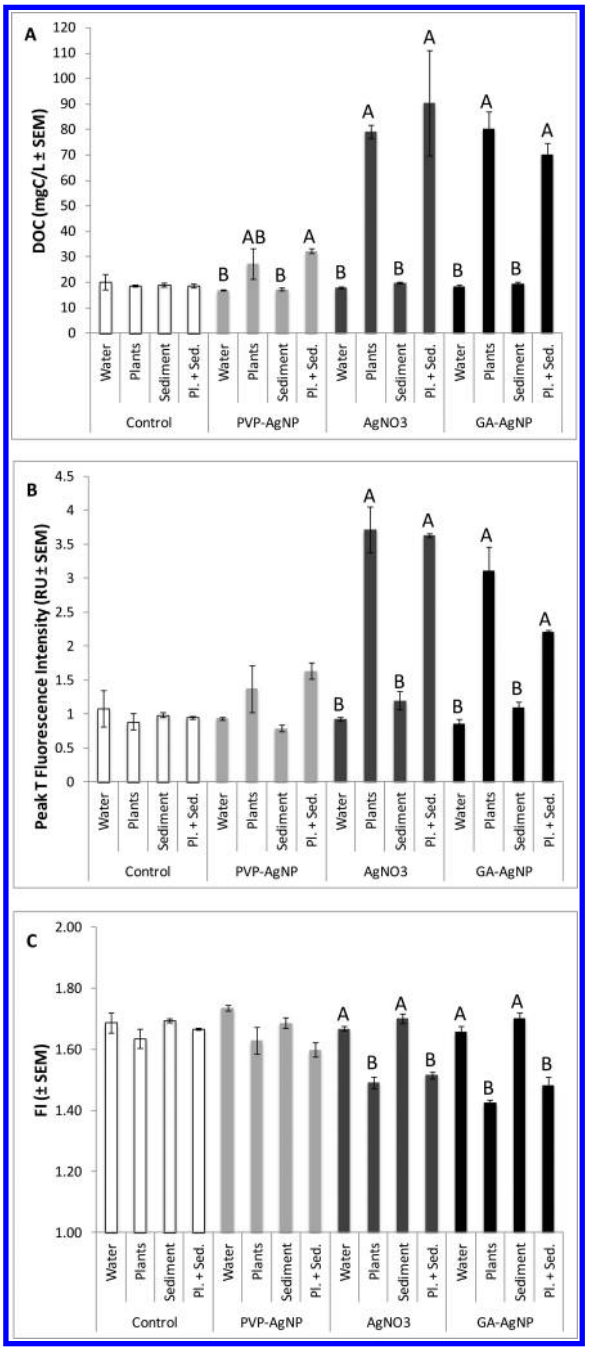

Figure 1. DOM quantity and quality. (A) Total DOC concentration (n = three experimental replicates). (B) Intensity of peak T (Ex/Em 275/340) in Raman Units (RU) (n = three experimental replicates). (C) Fluorescence index (FI) (n = three experimental replicates). Results presented as mean ± SEM. Bars not marked with the same letter represent statistically different matrices within treatment (PVP-AgNP, $AgNO_3$, or GA-AgNP) (ANOVA, Holm-Sidak multiple comparison). Treatments with no letters indicate $p > 0.05$ (ANOVA) within treatment.

DOC results will assume a significant increase in PVP-AgNP-treated matrices with plants compared to those without.

Changes in the DOM pool across environmental matrix and treatment were evaluated via changes in fluorescence index (FI) and changes in the intensity of three dominant fluorescence peaks A, C, and T, which were not correlated to water pH across treatment and matrix combinations. Analysis of DOM fluorescence showed the greatest change in WP and WPS matrix microcosms treated with GA-AgNPs and $AgNO_3$ compared to the controls. The FI decreased in Ag-treated waters, but the magnitude depended on treatment and matrix (Figure 1C). The decrease in FI was most pronounced in WP and WPS matrices treated with GA-AgNPs and $AgNO_3$ relative to controls with a smaller, though nonsignificant, decrease (ANOVA, $p = 0.21$) also observed in WP and WPS matrices treated with PVP-AgNPs. Similar to the pattern in FI in matrices with plants, there was a shift in DOM composition as demonstrated by increases in fluorescence intensities in Ag-treated microcosms at peaks A, C, and T, relative to matrices without plants, with peak T often exhibiting the largest increase. The magnitude of the fluorescence intensity increase varied with the Ag type; WP and WPS matrices treated with GA-AgNPs and $AgNO_3$ exhibited statistically significant increases in peak T intensity relative to W and WS, while WP and WPS matrices treated with PVP-AgNPs exhibited a modest but nonsignificant increase (ANOVA, $p = 0.48$) (Figure 1B). Increases in peaks A and C were also observed in all Ag treatments in WP and WPS matrices (SI Figure S2), but increases were not as large as those for FI and peak T.

Ag Speciation. Silver speciation analysis using X-ray absorption spectroscopy showed partial oxidation of Ag for the suspensions exposed to the microcosm media (Figure 2). Complexation in the first coordination shell of Ag with several ligands was also observed in most cases. PVP-AgNP remained intact to a large extent in all media. LCF of samples with reference materials showed that in all media the majority of Ag in the PVP-AgNPs was zerovalent (92.4−95.5% of total Ag) (SI Tables S1−S8). The oxidized Ag was likely complexed to an inorganic ligand, such as chloride or iodide, or organic matter. $AgNO_3$ in all media was mostly complexed to chloride (69.0−88.2%) (SI Tables S9−S16). The remaining Ag appeared to be complexed to iodide and/or sulfide, but other components not included in our analysis are possible as indicated by the relatively poorer fitting results. In certain cases it was difficult to distinguish between AgCl and AgBr (e.g., SI Table S13) because of the very similar XANES spectra of the two compounds (Figure 2), but chloride is expected to be in far excess of bromide in our media. The most diverse speciation was observed for GA-AgNPs. In water microcosms (W), GA-AgNPs were comprised of mostly zerovalent Ag (92.5% to 97.6%) (SI Tables S17 and S18). In water and sediment media (WS), less Ag remained in the zerovalent state (80.5−85.6%), whereas the rest was most likely complexed to inorganic ligands (SI Tables S19 and S20). The presence of plants (WP) appeared to further enhance Ag oxidation with approximately two-thirds of Ag remaining as zerovalent (67.6−69.2%) and the appearance of a cysteine-like (22.1−23.2%) and oxide (4.6−5.8%) complex (SI Tables S21 and S22). The observed complexation of Ag to a cysteine-like moiety could be a simple Ag-thiol bond, but a contribution from the amine or carboxyl groups is also possible. In the media with both plants and sediment (WPS), approximately half of Ag was zerovalent (52.8−58.8%), while the rest was complexed with thiols (26.3−33.0%) and O or Br (11.0−13.2%) (SI Tables S23 and S24). Thiol containing organic compounds, such as amino acids and proteins are highly reactive toward AgNPs.[15,34] Overall, the XANES data show that PVP-AgNPs remained intact for the most part in all media; $AgNO_3$ mostly formed AgCl complexes that were captured during filtration; and GA-AgNPs were susceptible to oxidation and ligand binding.

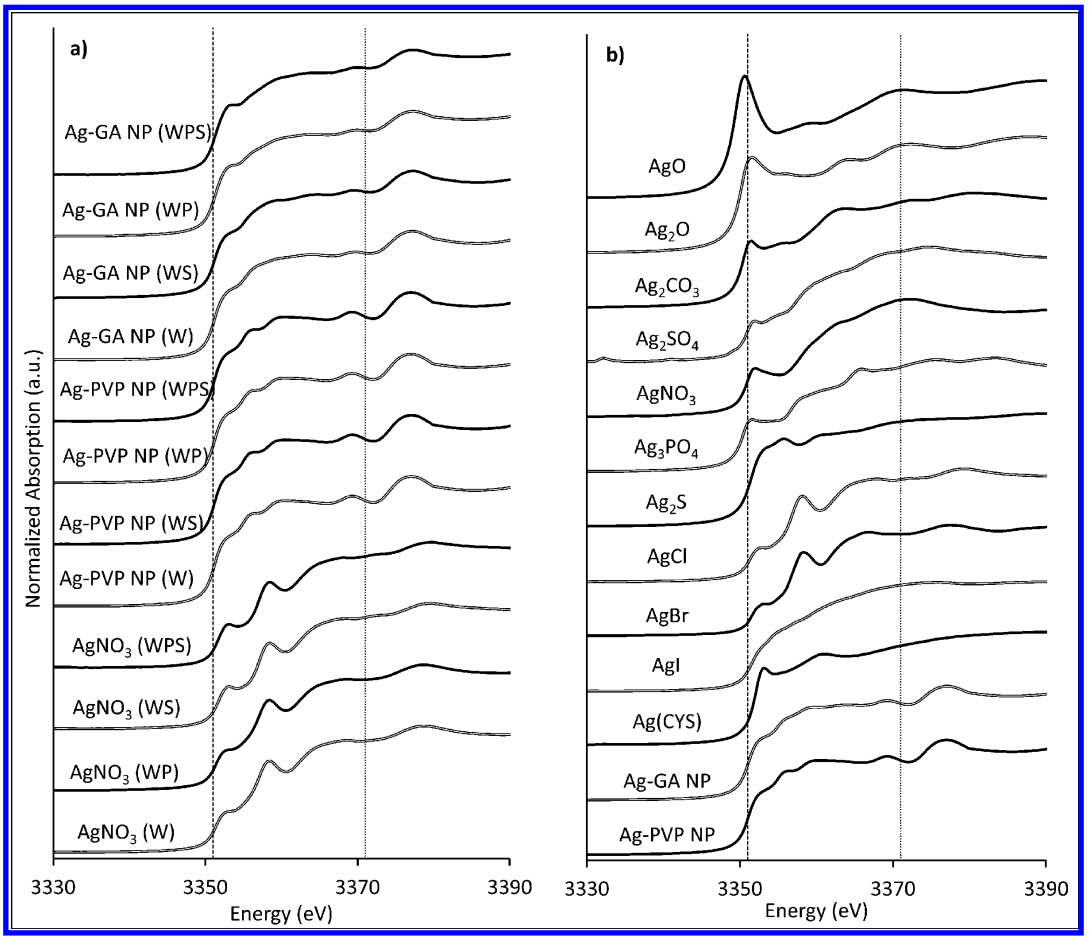

Figure 2. (a): Silver L3-edge XANES spectra of PVP-Ag NP and GA-Ag NP suspensions and $AgNO_3$ in microcosm matrices, water only (W), water + sediment (WS), water + plants (WP), and water + plants + sediment (WPS). (b): Silver L3-edge XANES spectra of reference compounds AgO, Ag_2O, Ag_2CO_3, Ag_2SO_4, $AgNO_3$, Ag_3PO_4, Ag_2S, AgCl, AgBr, AgI, Ag(CYS), and the stock PVP- and GA-AgNPs. The adsorption energy and fitting range are indicated by dashed vertical lines.

Acute Toxicity. With the exception of PVP-AgNP treatments for zebrafish, all treatments were diluted for zebrafish (1:1) and *Daphnia* (1:9). No significant differences in mortality for zebrafish were seen between matrices within the PVP-AgNP treatment (ANOVA, $p = 0.057$) due to high variability between microcosms (Figure 3A). However, the WP and WPS matrices had lower mortality for zebrafish (8% ± 5% and 10% ± 4% mortality) when compared to the W matrix (74% ± 16%). The strong trends seen in this data support a protective role of plants against PVP-AgNP toxicity and discussion will continue on under this assumption. For *Daphnia,* only the WP matrix (20% ± 10%) was significantly different from the W (83% ± 17%) matrix, whereas neither the WS (80% ± 12%) nor WPS (33% ± 12%) were significantly different from the W matrix (ANOVA, $p = 0.016$) (Figure 3C). Again, the strong trends in this data support the assumption that plants, whether alone or in combination with sediment, are protective against PVP-AgNP toxicity and discussion will continue on under this assumption.

Mortality of zebrafish in the GA-AgNP treatment for WP (19% ± 4%), WPS (6% ± 4%), and WS (35% ± 9%) matrices were all significantly lower than in the W matrix (81% ± 13%) (Figure 3B). For *Daphnia*, no significant differences in mortality were seen between matrices within the GA-AgNP treatment (ANOVA, $p = 0.073$) (Figure 3C). However, trends in the data strongly suggest that the WPS matrix (57% ± 18%) was protective against Ag toxicity compared to W (100%), and neither WP (87% ± 13%) nor WS (100%) matrices were different from W.

Mortality of zebrafish when exposed to $AgNO_3$, was significantly lower in the WP (67% ± 10%) matrix compared to the W (100%) matrix, while WPS (83% ± 8%), WS (100%), and W (100%) matrices were not significantly different from each other (Figure 3B). For *Daphnia*, none of the matrices were significantly different from each other (ANOVA, $p = 0.13$) (Figure 3C). While a slight reduction in mortality was seen in the WPS (77% ± 15) matrix, the result was not significantly different from W (100%). Again, the trends seen in this data support that plants, whether alone or in combination with sediment, can be protective against Ag toxicity and discussion will continue on under this assumption.

Correlates of Toxicity. To determine the extent to which AgNP fractionation and speciation related to toxicity, we correlated these factors with % mortality by treatment (Table 1). The strongest predictor of toxicity within Ag treatment was total concentration of unfiltered Ag (data available in Unrine et al.)[19] The concentration of Ag correlated strongly with percent mortality for both *Daphnia* and zebrafish regardless of environmental matrix within a given form of Ag (Figure 4), yielding environmentally mediated dose−response curves. For zebrafish, this pattern was strongest for unfiltered GA-AgNP and PVP-AgNP ($r = 0.89$ and $r = 0.79$, respectively; Table 1),

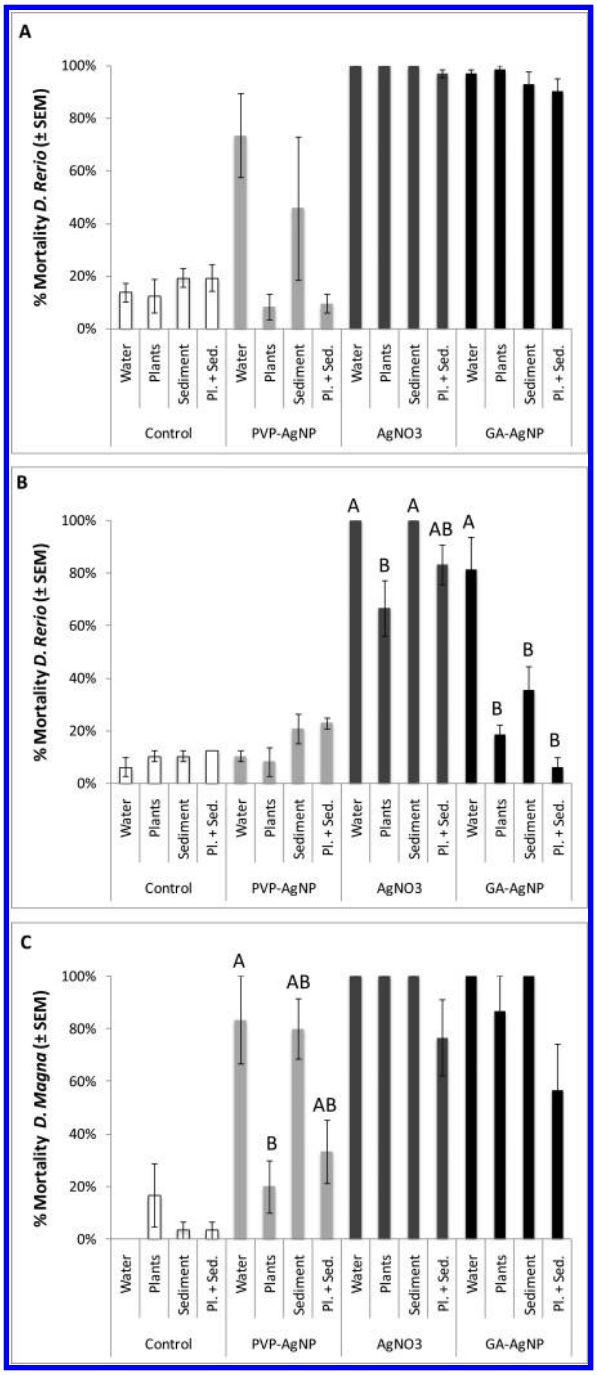

Figure 3. Toxicity of microcosm samples by treatment and matrix. (A) Zebrafish embryo mortality at 48 h post dosing using undiluted water samples from each microcosm (n = three experimental replicates, 24 embryos/sample). (B) Zebrafish embryo mortality at 48 h post dosing with water samples from each microcosm diluted 2× using EPA MHW (n = three experimental replicates, 16 embryos/sample. (C) Daphnia neonate mortality at 48 h post dosing with water samples from each microcosm diluted 10× using EPA MHW (n = three experimental replicates, five neonates/sample). Results presented as mean ± SEM. Bars not marked with the same letter represent statistically different matrices within treatment (PVP-AgNP, $AgNO_3$, or GA-AgNP) (ANOVA, Holm-Sidak multiple comparison). Treatments with no letters indicate $p > 0.05$ (ANOVA) within treatment.

whereas the dose–response curves for $AgNO_3$ were much steeper, nonlinear, and had 100% mortality at much lower concentrations than the AgNPs. The same pattern held true for

Table 1. Pearson's r Correlations between Different Measures of Silver and Mortality of Zebrafish and *Daphnia*

	GA-AgNPs		PVP-AgNPs	
silver	zebrafish (MHW)	*Daphnia*	zebrafish (undiluted)	*Daphnia*
unfiltered	0.89[c]	0.63[a]	0.79[b]	0.84[c]
filtered (0.7 μm)	0.79[b]	0.72[b]	−0.29	−0.51
ultrafiltered 3 kDa	−0.37	0.06	0.15	0.05
Ag^0	−0.37	0.65[a]	−0.35	−0.40
primary particles	0.77[b]	0.53	−0.70[a]	−0.83[c]

[a] $p < 0.05$. [b] $p < 0.01$. [c] $p < 0.001$.

Daphnia (r = 0.63, r = 0.84). The concentration of GA-AgNPs passing through a 0.7 μm filter also correlated to toxicity of both zebrafish (r = 0.79) and *Daphnia* (r = 0.72), while the concentration of PVP-AgNPs after filtration (0.7-μm) did not. In addition, for GA-AgNPs the percent of Ag present as

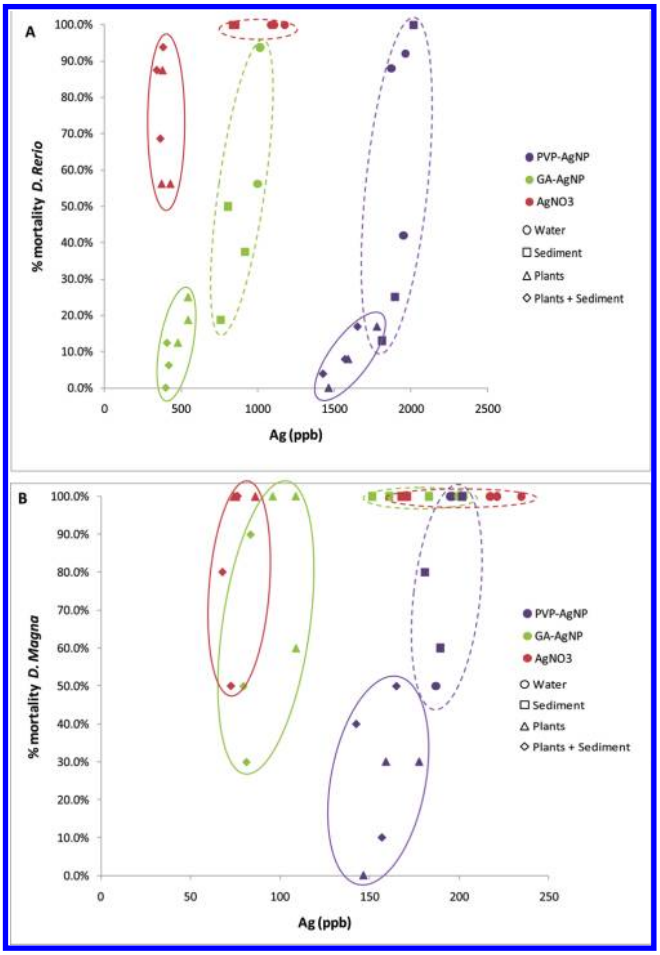

Figure 4. Total Ag measured in the water column vs toxicity. Different colors (purple, green, red) represent treatment while different shapes represent matrix. Plants only and plants + sediment matrices are grouped by solid circles and water only and sediment only are grouped by dashed circles in order to denote the effect of matrix on Ag concentration and toxicity. (A) Zebrafish embryo toxicity at 48 h post dosing using undiluted water samples from each microcosm for GA-AgNP and $AgNO_3$. Data for PVP-AgNP is reported for 2× diluted water samples from each microcosm. (B) Daphnia neonate toxicity at 48 h post dosing with water samples from each microcosm diluted 10× using EPA MHW.

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primary, unaggregated particles as measured by AF4-ICP-MS correlated positively with toxicity for both zebrafish and *Daphnia* (r = 0.77, r = 0.53). The same held true for percent of Ag present as Ag^0 as measured by XANES (r = 0.88, r = 0.65).

DISCUSSION

The nature of the environmental matrix in this experiment had a clear effect on the acute toxicity of Ag to both vertebrate and invertebrate aquatic organisms, with the strongest effect being a reduction in toxicity following exposures of Ag in the microcosms containing plants. Sediment-only matrices (WS) appeared to be correlated to reduced toxicity in some cases (e.g., GA-AgNP in zebrafish), and WPS appeared to have a greater effect than plants alone (WP). However, the contribution of sediment to protection was minor in comparison to that of plants. This result is not surprising, since—across Ag forms—microcosms with plants, and to some extent those with sediment alone (WS), had lower Ag concentrations than those with only water (W).[19]

The presence of plants and sediment could have reduced Ag concentrations in the water column in several different ways. First, the plants themselves could have directly sorbed dissolved Ag or AgNPs, thus decreasing Ag concentrations in water. Plants have been shown to sorb and take up Ag added in either dissolved or NP form, though the mechanism and form is not known.[35] The results from the companion study suggest that GA-AgNPs were primarily removed from the water column through dissolution and subsequent binding of Ag ions to submerged surfaces such as the plants, sediment and/or the container walls.[19] On the other hand, plants did not appear to increase the dissolution of PVP-AgNPs, but rather stabilized them as primary particles and little oxidation of these particles was apparent from our XANES measurements. This suggests that removal of PVP-AgNPs from the water column was through direct binding of particles. The presence of DOM has been shown to reduce toxicity to aquatic organisms for both dissolved Ag and AgNP.[14,36,37] The increase of DOC seen in the WP and WPS matrices for all Ag treatments may have occurred as a toxic response to the Ag by the plants, causing them to release cellular contents as the plants died. This is supported by the strong increase in peak T fluorescence intensity in WP and WPS matrices, which has in some cases been attributed to organic matter derived from fresh plant material.[28,29] Alternatively, the increased DOC could be from an exudate released from the plants as a detoxification mechanism.[19] A large fraction of the Ag was observed to be bound to a DOM fraction with a molecular weight of approximately 30 kDa in the $AgNO_3$ and GA-AgNP treatments. The narrow range of molecular weight of the Ag-DOM fraction observed in the AF4-ICP-MS measurements strongly suggests that the DOM fraction was an exudate rather than cellular contents, which would have had a broad range of molecular weights.

For PVP-AgNPs, AF4-ICP-MS data showed that, while particles were highly aggregated in W and WS matrices, mostly primary particles were present in the WP and WPS matrices.[19] This increase in stabilization was also likely due to the increase in DOC. The presence of DOM has been shown to stabilize nanoparticles in water.[17] A decrease in aggregation is generally considered to increase toxicity of AgNPs to aquatic organisms due to increased bioavailability when the NPs are present in the water column as opposed to removed from the water column by sedimentation.[10,38] While this could apply to the current study, the increase in DOC that is contributing to increased stability could also be coating the nanoparticle and thereby preventing release of Ag^+, or complexing released Ag^+ and forming less bioavailable and/or less toxic Ag complexes.[37] Particle-specific toxicity could also be altered, decreasing the reactivity of the particles by passivating them or decreasing their affinity for biological surfaces through changes in charge or hydrophobicity. XANES data showed little difference in PVP-AgNP speciation across all matrices, suggesting that the surface modifications of PVP-AgNPs that changed their stability and toxicity did not involve the formation of covalent bonds between the plant DOM and the PVP-AgNPs.

In general, GA-AgNPs exhibited greater overall stability against aggregation than PVP-AgNPs and were mostly present as primary particles.[19] However, in the WP and WPS matrices, a lower concentration of primary particles and an increase in the presence of Ag bound to DOM suggests greater susceptibility to oxidation and subsequent binding by DOM. The greater stability of GA-AgNPs could be contributing to the increased toxicity of GA-AgNPs compared to PVP-AgNPs in the W matrices, but the GA NPs also had greater solubility, so increased Ag^+ concentrations were present. However, as stated previously, increased stability does not necessarily result in decreased toxicity. Unlike PVP-AgNPs, speciation differed dramatically among the matrices. Without plants present, the majority of GA-AgNPs was present as the intact particle with a small percentage oxidized as AgCl. However, the presence of plants appeared to contribute to increased oxidation of Ag, with almost 25% of the Ag present as an oxidized form resembling Ag-cysteine. This complex is thought to be representative of Ag bound to organic matter, which agrees with the presence of Ag-DOM complexes seen in the AF4-ICP-MS data.[19] Binding of Ag as Ag-DOM and AgCl have been shown to lead to decreased toxicity in aquatic organisms.[39] The toxicity of GA-AgNPs was correlated to this decrease in the percentage of Ag present as the intact particle; however, since the treatments with fewer intact particles (matrices containing plants) also contained less total Ag, this correlation cannot be separated from the overall decrease in Ag concentration. Similarly, the WP and WPS matrices had a greater percentage of Ag bound to DOM.[19] This pattern also holds true for the amount of particles present as primary particles as measured by AF4-ICP-MS.

For $AgNO_3$ treated microcosms, particulate bound Ag was present mostly as AgCl across all matrices. However, in the presence of plants, more Ag was present as Ag bound to thiol-like ligands while in the absence of plants more Ag was present as Ag_2S. These data agree with the AF4-ICP-MS data, which found the presence of Ag-DOM complexes in microcosms with plants. Overall, mortality rates in $AgNO_3$ treatments were much higher than those in the AgNP treatments. However, the slight decreases in mortality seen in matrices containing plants could, again, be due to the complexation of Ag by DOM or the overall decrease in Ag concentration seen in these microcosms.

The presence of aquatic plants reduced toxicity of both AgNPs and $AgNO_3$. While reductions in the overall water column concentrations are the clearest explanation, we also observed differences in the aggregation state and dissolution rate of the particles, which were coating dependent. Further, a significant proportion of Ag in the $AgNO_3$ and GA-AgNP treatment was shown to be bound to an organic substance released from the plants.[19] Taken together with the results from the companion paper, we have demonstrated for the first time

that Ag either in ionic or nanoparticulate form likely stimulates the release of exudates from aquatic plants that ameliorate the toxicity of AgNPs or ions to aquatic animals. Interestingly, reductions in water column concentrations due to organic matter released from the plants occurred through distinct mechanisms depending on particle coating.[19] This is among the first studies to suggest that not only abiotic but biotic interactions within aquatic communities can modify the concentration, speciation, and toxicity of AgNPs. This should be taken into account when attempting to predict ecological consequences of AgNPs in aquatic ecosystems based on laboratory assays conducted in simplified exposure media with a single species.

■ ASSOCIATED CONTENT

Supporting Information

Additional information, including extended XANES and EEMs data and methods and TEM of particles. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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