Influence of hardness on the bioavailability of silver to a freshwater snail after waterborne exposure to silver nitrate and silver nanoparticles

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Abstract

The release of Ag nanoparticles (AgNPs) into the aquatic environment is likely, but the influence of water chemistry on their impacts and fate remains unclear. Here, we characterize the bioavailability of Ag from AgNO₃ and from AgNPs capped with polyvinylpyrrolidone (PVP AgNP) and thiolated polyethylene glycol (PEG AgNP) in the freshwater snail, Lymnaea stagnalis, after short waterborne exposures. Results showed that water hardness, AgNP capping agents, and metal speciation affected the uptake rate of Ag from AgNPs. Comparison of the results from organisms of similar weight showed that water hardness affected the uptake of Ag from AgNPs, not that from AgNO₃. Transformation (dissolution and aggregation) of the AgNPs was also influenced by water hardness and the capping agent. Bioavailability of Ag from AgNPs was, in turn, correlated to these physical changes. Water hardness increased the aggregation of AgNPs, especially for PEG AgNPs, reducing the bioavailability of Ag from PEG AgNPs to a greater degree than from PVP AgNPs. Higher dissolved Ag concentrations were measured for the PVP AgNPs (15%) compared to PEG AgNPs (3%) in moderately hard water, enhancing Ag bioavailability of the former. Multiple drivers of bioavailability yielded differences in Ag influx between very hard and deionized water where the uptake rate constants ($k_{uw}$, $1 \text{ g}^{-1} \text{d}^{-1} \pm \text{SE}$) varied from 3.1 ± 0.7 to 0.2 ± 0.01 for PEG AgNPs and from 2.3 ± 0.02 to 1.3 ± 0.01 for PVP AgNPs. Modeling bioavailability of Ag from NPs revealed that Ag influx into L. stagnalis comprised uptake from the NPs themselves and from newly dissolved Ag.

Introduction

The use of engineered silver nanoparticles (AgNPs) has become widespread in a diverse range of applications and consumer materials because of their unique physico-chemical properties (Fabrega et al., 2011) and inherent anti-microbial activity (Fabrega et al., 2009). Considering their extensive use, AgNPs will inevitably enter aquatic systems through wastewaters and surface waters (Mueller & Nowack, 2008) and likely transform (Lowry et al., 2012). The increased dispersion of Ag into the environment poses a risk to aquatic biota as Ag is highly toxic to bacteria (Wang & Fisher, 1997), phytoplankton, invertebrates and fish (Erickson et al., 1998, Hiriart-Baer et al., 2006; Hogstrand & Wood, 1998; Karen et al., 1999). It remains unclear whether Ag entering the environment as AgNPs changes that risk (Gao et al., 2012; Navarro et al., 2008).

Metal bioavailability can be defined as the fraction of the total amount of metal present in the environment that is available for uptake by organisms across all possible pathways, including water and food (Luoma & Rainbow, 2005). Metals sorbed to the surface of cells as well as internalized metals are considered bioavailable (Hassler et al., 2004). Both forms are defined as bioaccumulated (Luoma et al., 2014) and either may lead to toxicity. Thus, the risk associated with exposure to AgNPs in the aquatic environment can be addressed by studying bioavailability. Drivers of bioavailability include the organism (i.e. physiology and metabolism), the characteristics of the AgNP including its size and type of capping agent (coating added to increase particle stability), as well as factors in the environment that affect transformation of the AgNPs (Luoma et al., 2014). Here, we ask: what are the roles played by the drivers of bioavailability for AgNPs? In particular, we examine the effects of different capping agents and water chemistry on the uptake rates of Ag from AgNPs by the freshwater snail Lymnaea stagnalis. We show that Ag uptake rates differ among snails of different weight, and therefore interpretations of other influences are restricted to experiments with organisms of similar weight. We exposed snails to two types of capped AgNPs [polyvinylpyrrolidone (PVP) and polyethylene glycol (PEG)] dispersed in synthetic freshwaters that varied up to 300-fold in hardness. In addition to AgNP exposures, we exposed snails to Ag added as AgNO₃ to quantify delivery to snails from Ag ion ($Ag^+$) alone. Calculation of unidirectional uptake and loss...
rates allowed quantification of the influence of physical (aggregation of NPs), chemical (dissolution of NPs) and biological processes on the bioaccumulation of different forms of Ag.

Two of the several types of environmental transformations of NPs are dissolution and aggregation (Lowry et al., 2012). A few studies have examined the effect of water chemistry on aggregation of AgNPs. For example, Römer et al. found that media with high ionic strength (standard OECD media) caused significant aggregation of citrate-capped AgNP (Romer et al., 2011). Dissolution of NPs can also be influenced by water chemistry with implications for bioavailability and toxicity (Croteau et al., 2011b, 2014; Fabrega et al., 2011, Jin et al., 2010). However, there is not yet a consensus about the effects of these processes (Fabrega et al., 2009; Navarro et al., 2008).

Knowledge of bioaccumulation of Ag in aquatic environments can serve as the initial basis for investigating the bioavailability of AgNPs (Hogstrand & Wood, 1998; Luoma, 2008; Ratte, 1999). However, bioaccumulation is more complex and challenging to define for NPs compared to Ag⁺ given that uptake could occur from the NPs themselves as well as from metal solubilized from the NP (Fabrega et al., 2011; Luoma, 2008; Luoma et al., 2014). The effects of water quality on dissolved Ag bioavailability are well known in contrast to AgNPs, as transformations like aggregation and dissolution add complexities to the assessment of NP bioavailability.

Materials and methods

Exposure media

The effect of water hardness was examined using deionized (DI), soft (SO), moderately hard (MOD), hard (HW) and very hard (VH) water (US EPA, 2002). Media were prepared in ultra-pure water (Millipore) with reagent grade chemicals (JT Baker) (Table S1, Supporting Information).

Model organism

The freshwater snail _L. stagnalis_ served as a model organism. This pulmonate snail has an extensive geographic distribution (Pfleger & Chatfield, 1983). It is an important component of freshwater systems and represents a significant part of the diet of many fish and crayfish (Nystrom & Perez, 1998). This species is a well known in contrast to AgNPs, as transformations like aggregation and dissolution add complexities to the assessment of NP bioavailability.

Nanoparticles

Two different types of coated AgNPs were synthesized, as described by Tejamaya et al. (2012). Briefly, PVP AgNPs were synthesized by reducing silver nitrate with sodium borohydride in the presence of PVP10. The PEG-coated AgNPs were prepared by converting citrate AgNPs by adding thiolated PEG under vigorous stirring. The AgNPs stablized with citrate were originally synthesized by reducing silver nitrate in sodium citrate with sodium borohydride. Synthesized particles were cleaned by ultrafiltration (Amicon 1 kDa cellulose membrane). The particle coatings were both water soluble polymers that stabilized the particles through steric repulsion. Characterization data and TEM images are included in Supporting Information (SI), (Tables S2–S5 and Figures S2–S9). The AgNPs were analyzed by field flow fractionation (FFF) and dynamic light scattering (DLS). Stock solutions of PVP and PEG-SH AgNPs (18 and 8 mg/L, respectively) were kept at 4 °C in the dark. These stock solutions were sonicated prior to making dilutions for experimental treatments.

Study design: waterborne uptake and elimination

Waterborne exposures were conducted to measure the uptake rates of Ag from AgNPs, as well as from Ag added as AgNO₃, into the soft tissues of snails (Croteau et al., 2011b). All vials were cleaned in 20% nitric acid, rinsed in ultra-pure water, and dried in a laminar flow hood. Snails were exposed in 1 l polypropylene containers to Ag concentrations (either AgNPs or AgNO₃) ranging from 1 to 100 nM. This covers the range of concentrations that might be expected in nature (Gottschalk et al., 2009, 2013; Luoma, 2008; Shafer et al., 1998). Exposures were brief (24 h), to minimize the confounding influence of Ag elimination on Ag influxes, allowing quantification of mechanistically important unidirectional influx (Croteau et al., 2004). Ten individual snails were used for each concentration tested.

Separate experiments were also designed to characterize the elimination of Ag following exposure to AgNPs. The total concentration of Ag in snails was measured throughout depuration and used to determine rate constants of loss (as described later). Snails were exposed to 75 nM of either PEG or PVP AgNPs in MOD water for 72 h. Following exposure, snails were fed lettuce and allowed to depurate for 14 days in 150 ml polyethylene containers partially submerged in 201 of MOD water. Ten snails were sampled and frozen at designated time intervals over the 14 days. Water samples were collected from the depuration tank over the course of the experiment and acidified (HNO₃) before analysis for Ag.

Metal digestion and analysis

After exposures, snails were collected, rinsed thoroughly in DI water, and frozen. After thawing, soft tissue was removed and placed on acid-cleaned Teflon sheeting. Tissues were dried at 40 °C for at least 48 h, weighed, placed in 5 ml Teflon vials, and 300 µl of concentrated nitric acid was added (either ulti x or double-distilled). Samples were digested at room temperature for five days, and then 120 µl of hydrogen peroxide (Ultrax Grade II) was added. After 24 h, samples were diluted to volume with ultrapure water. Germanium (internal standard) was added to all samples and calibration standards to account for instrument drift and changes in sensitivity. Standard reference materials (DOLT-3, National Research Council, Canada) and procedural blanks were digested with samples from each experiment. Tissue and water samples were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) (Croteau et al., 2011b). Recovery of standard reference material averaged 98 ± 3% for 13 sample runs. Blanks were below the method detection limit (MDL) of 0.002 µg/l (0.019 nM) (Glaser et al., 1981).
Centrifugal ultrafiltration

The dissolution of the AgNPs was examined using centrifugal ultrafiltration (Millipore Amicon Ultra-43 K, Millipore, Darmstadt, Germany) through a 3 kDa molecular weight membrane (Navarro et al., 2008). AgNPs were diluted in both DI and MOD water to achieve a total Ag concentration of 10 μg/l and incubated for 24 h to mimic the experimental conditions. Solutions (3 ml) were centrifuged for 40 min at 7500g (Sorvall RC 5 C Plus, DuPont, Wilmington, DE). The filtrate was acidified and analyzed by ICP-MS to determine the dissolved Ag concentration. The procedure was repeated with AgNO₃ (stock of AgNO₃ 10 μg/l).

Speciation and BLM modeling

To consider the effect of speciation on bioavailability, the Ag⁺ concentration was estimated using MINERQL+ (version 4.6; Environmental Research Software, Hallowell, ME). We estimated Ag⁺ concentration in the PVP and PEG AgNP exposures using the percentage of dissolved Ag. The amount of Ag bound to the biotic ligand (Ag–BL) under different conditions was also estimated using a standard application of the biotic ligand model (BLM) version 2.2.3 (Hydroqual, Inc., Mahwah, NJ, 2007). The concentrations of Ag measured from the media were used as input into the BLM. Data were collected using a parameter file run in Ag speciation-mode (Hydroqual, Inc., Mahwah, NJ, 2007), which is not species-specific.

Calculation of Ag uptake rate constants

The influx of Ag from water into L. stagnalis (Ag influx) is the product of a rate constant of uptake from solution (k uw in nmol g⁻¹ d⁻¹ per nmol l⁻¹, or βg/d), and the waterborne concentration of Ag ([Ag] w in nmol/l) in Equation (1).

\[ \text{Ag influx} = k_{uw} \times [\text{Ag}]_w \] (1)

The term k uw is determined from the slope of the linear portion of the relationship between Ag uptake rate into L. stagnalis and the total Ag exposure concentration.

Equation (2) (Croteau et al., 2014) was used to delineate the contribution of AgNP uptake from that of Ag⁺ uptake to the overall Ag influx into L. stagnalis. Briefly, the Ag influx from the dissolved portion was calculated using the first term in Equation (2), where \( k_{uw}^{Ag^+} \) is the rate constant of uptake for Ag⁺ and [Ag]ₐw is the concentration of newly solubilized Ag from the NPs.

\[ \text{Ag influx} = \frac{k_{uw}^{Ag^+} \times [\text{Ag}]_{aw}}{\text{Contribution of dissolved Ag}} + \frac{k_{uw}^{AgNP} \times [\text{AgNPs}]}{\text{Contribution of dissolved AgNPs}} \] (2)

The [Ag]ₐw was calculated as the product of the percentage of dissolved Ag, determined by centrifugal ultrafiltration, and the measured total Ag concentration in the exposure. Changes in dissolution with concentration (Hadioui et al., 2013) were not considered. The rate constant of uptake of AgNPs (k uwNP) was calculated from the slope of the linear relationship between uptake rate of Ag from the AgNPs themselves and the AgNP exposure concentration. The contribution of uptake from AgNPs (second term in Equation (2)) was calculated as the difference between the total influx of Ag (Ag influx) and the predicted contribution of influx from dissolved Ag (first term in Equation (2)). The exposure concentration of NPs ([AgNP]) was calculated as the difference of total Ag and [Ag]ₐw.

Calculation of elimination rate constants

The elimination rate constant (k e in d⁻¹) was determined by fitting the proportional loss data to an exponential decay equation (Equations S2 and S3 in SI) (Croteau et al., 2004, 2011b). Growth rate constants were also quantified to characterize loss of Ag by growth dilution (Equation (S1) in SI).

Biodynamic model

The biodynamic model describes metal accumulation in terms of uptake and elimination (Luoma & Rainbow, 2005). It predicts the steady-state concentration of metal accumulated after waterborne exposure by:

\[ [M]_{ss} = \frac{k_{uw} \times [\text{Ag}]_{water}}{k_e + k_f} \] (3)

where the steady-state concentration of metal in the organism ([M]ₜ in nmol g⁻¹) is a function of the rate constant of uptake from water, k uw, the Ag concentration in water ([Ag] water nmol l⁻¹), the physiological elimination (k e, d⁻¹) and body growth dilution (k f, d⁻¹).

Membrane transport characteristics

Metal influx can also be described in terms of membrane transport processes when influxes reach saturation. The maximum number of transport sites (B max in nmol g⁻¹) was calculated (Croteau et al., 2011b) and summarized in the SI section (Equation (S4)).

Statistical analysis

The normality and equality of variance were tested by the Shapiro–Wilks and Levene tests, respectively. The effect of water hardness on Ag uptake rates was determined by the analysis of covariance (ANCOVA), (SYSTAT software, San Jose, CA). The differences between k uwNP were tested by ANCOVA. Significant differences were evaluated at p ≤ 0.05.

Results

Influence of body weight

Two separate experiments, with snails of average weights 3.7 ± 1.0 and 7.5 ± 1.5 mg, showed that the smaller snails took up Ag from AgNO₃ at a faster rate than larger snails (Figure 1A); but the differences were not statistically significant. The effect of weight on uptake rates was more evident when individuals were compared at one exposure concentration (20 nM; Figure 1B). The relationship was statistically significant. It was driven by the fastest uptake rates in snails that weighed less than 3 mg. To avoid biases among experiments, comparisons of effects among treatments were restricted to experiments, where mean weight was between 3 and 6 mg, a range over which there was no detectable trend.

Silver speciation and uptake of Ag added as AgNO₃

In order to quantify uptake rates from dissolved Ag from the AgNP exposures, speciation and uptake rates from AgNO₃ were characterized under different water quality conditions. The proportion of Ag⁺ in the experimental media decreased as hardness increased (Table S6). For example, in VH water at pH 8, approximately 65% of the Ag was Ag⁺ and 33% was AgCl₀, while 93% of the total Ag was Ag⁺ and 7% was AgCl₀, in SO water at pH 6.5. As Ag⁺ is an important driver of bioavailability (Campbell, 1995), we assumed that bioavailability of Ag would decrease with increasing water hardness. However, uptake rates of Ag into L. stagnalis were similar among experiments conducted in SO and HW (Figure S10, k uwNP ~3.01 g⁻¹ d⁻¹). Plotting influxes of Ag against Ag–BL or Ag⁺ concentrations did not result in a detectable change in the relationships (Figure 2A and B).
as Ag–BL and Ag\(^+\) concentrations were significantly correlated (\(R = 0.997\) and \(R = 0.999\) for SO and HW, respectively; Figure 2C).

Uptake rates of Ag from solution demonstrated saturation kinetics at each water hardness at high exposure concentrations (Figure S10). The binding site capacity, \(B_{\text{max}}\), of the Ag influx curve against the Ag\(^+\) concentrations was 63 ± 4 nmol/g, which corresponded to a half saturation constant (\(K_{\text{metal}}\)) of 14 ± 2 nmol of Ag\(^+\) (Figure 2A). Similarly, the \(B_{\text{max}}\) and corresponding \(K_{\text{metal}}\) for the relationship between Ag influx and BL–Ag were 65 ± 5 nmol/g and 2.3 ± 0.5 nmol/g of BL–Ag, respectively (Figure 2B).

The effect of competition on BL–Ag by major cations was revealed by comparing predicted Ag–BL and the estimated Ag\(^+\) (Figure 2C). For example, slightly less Ag–BL was estimated for HW compared to SO at the same Ag\(^+\) concentration. The slopes (nmol/g per nmol/l, or l/g ± SE) for the relationships between BL–Ag and Ag\(^+\) were 0.14 ± 0.005 and 0.12 ± 0.003 for the SO, and HW experiments, respectively. The slopes were significantly different (ANCOVA \(F = 40.2\) and \(p < 0.0001\)).

**Characterization of AgNPs**

The \(z\)-average hydrodynamic size for the PVP AgNPs (4 ppm) and PEG AgNPs (2.4 ppm) diluted in MOD were approximately 34 ± 2 nm and 44 ± 1 nm, respectively, and their zeta potentials were \(-9.3 ± 0.6\) and \(-10 ± 1\) mV, respectively, as measured by DLS. The hydrodynamic sizes measured by FFF were 30.4 ± 0.7 and 44.7 ± 1.9 nm for PVP and PEG AgNPs, respectively. The core size was similar at 11.3 ± 2.6 and 12.5 ± 3.6 for PVP and PEG AgNPs, respectively. Although the hydrodynamic size of PVP AgNPs were slightly smaller than that of PEG AgNPs, we assumed that the effects on bioavailability should be minimal as DLS can overestimate size (Romer et al., 2011). Both were monodispersed (Baalousha & Lead, 2013) in DI and MOD water, as evidenced by TEM, FFF and UV–Vis data (SI Tables S2–S5 and Figures S2–S9). The PVP AgNPs in MOD water showed little aggregation, as observed on TEM images after 24 h (Figures S3–S5). This was supported by FFF, DLS and UV–Vis results. The PVP AgNPs were slightly more stable in DI water compared to MOD water as indicated by UV–Vis data (SI Figure S2). A 6% loss of UV absorption signal after 24 h was observed for PVP AgNPs diluted in MOD water, compared to the minimal loss of signal for PVP NPs (1.4%) diluted in DI water. In contrast, greater aggregation was observed in TEM images for PEG AgNPs (SI Figure S9). The loss of UV signal for PEG AgNPs in DI water was similar to PVP AgNPs (6.6%), but the PEG AgNPs were much less stable than PVP AgNPs in MOD water (57% loss of UV signal; SI Figure S6). Thus, PEG AgNPs aggregated more readily than PVP AgNPs in MOD water, and aggregation of PEG AgNP was affected more by hardness.

Incubation of AgNPs also resulted in more dissolved Ag in DI water than in MOD water. For example, 3 ± 0.5 SE % of the total Ag dissolved from the PEG AgNPs incubated in MOD over 24 h compared to 14 ± 4 SE% in DI water. Dissolved concentrations of Ag were also greater for the PVP AgNPs than for the PEG AgNPs in both MOD (15 ± 3 SE % dissolved Ag) and DI water (40 ± 4 SE % dissolved Ag). The recovery of Ag from AgNO\(_3\) was 97 ± 5% SE.

**Uptake of Ag from AgNPs: influence of water hardness**

Uptake rates of Ag into *L. stagnalis* after exposures to PEG AgNPs and PVP AgNPs increased linearly through the lower
range of exposures (Figure S11), similar to uptake rates of Ag added as AgNO₃ (Figure S10 and Figure 2A). In contrast to AgNO₃, however, the influx of Ag from both PEG and PVP AgNPs varied with water hardness (Figure S12). The $k_{uw}$s for the PEG AgNPs declined from 3.1 ± 0.7 to 0.2 ± 0.006 l/g/d as hardness increased, with much of the difference occurring with the first addition of major ions (Table 1). The $k_{uw}$s for the PVP AgNPs decreased to a lesser extent with increasing hardness, i.e. from 2.3 ± 0.02 in DI to 1.3 ± 0.01 in HW. At a similar total Ag concentration, the uptake rates of Ag from both PEG and PVP NPs were slower than uptake rates of Ag from AgNO₃ in almost all waters (Table 1; Figure S11).

In DI water, the highest uptake rate for Ag from exposure to PEG AgNPs was 60 nmol g⁻¹ d⁻¹ (Figure S12). In HW, it decreased to 26 nmol g⁻¹ d⁻¹. Presumably, this could reflect a reduction in binding capacity with increasing water hardness caused by competition with major ions for transport sites, change in particle size due to aggregation, or both. Unlike PEG AgNPs, the maximum Ag uptake rates into L. stagnalis were similar following exposure to PVP AgNPs in different water hardness.

Increased hardness shifted the differences in $k_{uw}$s between the two types of capping agents (Figure 3). For example, in DI water, the $k_{uw}$ for PEG AgNPs exceeds that for PVP AgNPs (3.1 versus 2.31 g⁻¹ d⁻¹), whereas the opposite trend was observed in SO, HW and VHW (Table 1). In DI water, the $k_{uw}$ for PEG AgNPs was similar to that of Ag⁺ in the AgNO₃ exposure.

**Uptake of Ag from AgNPs: influence of dissolved Ag and aggregation**

To isolate the influence of dissolved Ag on the Ag uptake rates during the AgNP exposures, we estimated dissolved Ag concentrations at each exposure concentration using the percentage of dissolved Ag determined in MOD water for each AgNP. The corresponding Ag⁺ concentrations were then estimated using MINEQL+. As shown in Figure 4, Ag uptake rates from AgNP exposures exceeded Ag uptake rates from AgNO₃ exposures when plotted as a function of the actual Ag⁺ concentrations in each treatment. For example, at 3 nM of Ag⁺, uptake rates of Ag from PVP and PEG AgNPs in SO and HW waters was 3-fold greater than Ag uptake rates from AgNO₃.

We used Equation (2) to calculate uptake rate constants ($k_{NP_{uw}}$) for AgNPs alone. All of the $k_{NP_{uw}}$s were significantly lower than their respective $k_{uw}$ with the exception of PEG AgNPs in DI water, as both $k_{NP_{uw}}$ and $k_{uw}$ were similar (Table 1). The $k_{NP_{uw}}$s for both PEG AgNPs and PVP AgNPs declined with water hardness. The uptake rate constants specific to the NPs, $k_{NP_{uw}}$, were calculated only when dissolution was measured.
Correspondingly, we used Equation (2) to compare the relative contributions of newly solubilized Ag and AgNPs to the total uptake of Ag. Rates of uptake of Ag from concentrations of 25 nM AgNPs were used, as this was the highest concentration in the linear range of uptake and closest to the concentration at which dissolved Ag was determined. Uptake of Ag from PVP and PEG AgNPs was attributed largely to the NPs as opposed to dissolved Ag, except for PVP AgNPs in DI water (Figure 5). Even where dissolved Ag was high (40% in PVP DI), uptake of Ag for NPs was still important (45%).

Elimination rate constants

The elimination rate constant \( k_{ew} \) for PVP AgNP was 0.04 ± 0.01 (SE), which means that, in the absence of Ag in the media, half of the snail’s accumulated Ag (from both AgNPs and newly solubilized Ag) was lost every 17.3 days. The \( k_{ew} \) for PEG AgNP was not detectable (SI Figures S13 and S14). However, body growth dilution was significant (\( k_g \) of 0.06 ± 0.01 d\(^{-1}\) for 0–4 days and 0.03 ± 0.004 d\(^{-1}\) for 4–14 days).

Discussion

Biological influence: weight of experimental snails

Croteau et al. (2014) observed higher \( k_{uw} \) values in smaller snails (2–3 mg) compared to larger snails (10 mg) for uptake of Ag\(^+\) and Ag from AgNPs coated with citrate. In order to avoid this bias when evaluating effects of water quality and capping agent, we omitted results from one AgNP treatment, where the mean weight of snails was greater than 6 mg (PVP AgNP, MOD; Table 1). We also considered this confounding influence when interpreting two treatments with AgNO\(_3\) (HW and SO). Effects of size (weight or length) on metal bioaccumulation by mollusks are well known (Strong & Luoma, 1981; Wang & Fisher, 1997). It is now clear that similar effects might be expected with AgNPs. The intended use of animals in a narrow size range may prove challenging in

Table 1. Rate constants of uptake (\( k_{uw} \)) and average snail weights for experiments with AgNO\(_3\) and AgNPs.

| Experiments with AgNO\(_3\) | Hard | Hard | Soft |
|----------------------------|------|------|------|
| \( k_{uw} \) (l g\(^{-1}\) d\(^{-1}\)) | 3.5 ± 0.61 | 3 ± 0.4 | 3 ± 0.3 |
| Dry wt (mg) | 3.7 ± 1.0 | 7.5 ± 1.5 | 8.1 ± 2.4 |
| Free Ag\(^+\) (%) | 78 | 78 | 93 |

| Experiments with AgNPs PEG-SH | V. Hard | Hard | MOD | Soft | DI |
|-------------------------------|--------|------|-----|------|----|
| \( k_{uw} \) (l g\(^{-1}\) d\(^{-1}\)) | 0.2 ± 0.006 | 0.4 ± 0.02 | 0.5 ± 0.02 | 0.6 ± 0.03 | 3.1 ± 0.7 |
| Dissolution (%) | 3 | 3 | 3 | 14 | 14 |
| \( k_{np}^{uw} \) (l g\(^{-1}\) d\(^{-1}\)) | 5.9 ± 1.5 | 3.9 ± 1 | 3.3 ± 1 | 3.7 ± 1 | 4 ± 1 |
| Dry wt (mg) | 1.3 ± 0.01 | 1.8 ± 0.06 | 0.7\(^{a}\) ± 0.01 | 2.1 ± 0.2 | 2.3 ± 0.02 |
| Dissolution (%) | 15 | 15 | 15 | 40 | 40 |
| \( k_{np}^{uw} \) (l g\(^{-1}\) d\(^{-1}\)) | 3.2 ± 0.6 | 5.2 ± 1.5 | 8.1\(^{a}\) ± 2.5 | 3.2 ± 1.1 | 2.6 ± 0.6 |
| Dry wt (mg) | 1.3 ± 0.01 | 1.8 ± 0.06 | 0.7\(^{a}\) ± 0.01 | 2.1 ± 0.2 | 2.3 ± 0.02 |

The standard error is given for \( k_{uw} \)s and weights. All \( k_{uw} \)s for PEG AgNP experiments are significantly different from each other except for moderately hard (MOD) and soft water experiments. All \( k_{uw} \)s for PVP AgNP experiments are significantly different from each other, except for soft and DI water. The \( k_{np}^{uw} \)s, uptake rate constant of the NPs themselves, were calculated for experiments with dissolution measurements.

\(^{a}\)The lower \( k_{uw} \) reported for the PVP AgNPs in MOD water may not be compared given the larger sized snails used in the experiment.
experimental studies. If so, experimental design should include a way to counter size as a potential confounding influence on results.

Chemical speciation modeling: effects of hardness on uptake of Ag

Water hardness had a marginal influence on the uptake of Ag from AgNO₃ by L. stagnalis despite differences in speciation. Others have shown that hardness cations have less influence on bioavailability of Ag compared to parameters such as Cl⁻ or DOM (Bianchini & Wood, 2008; Niyogi & Wood, 2004). Besides Ag⁺, MINEQL suggested the remaining significant silver species, AgCl⁻, was present in increasing concentrations in SO and HW, at 7% and 21%, respectively. In contrast, Ag⁺ concentrations declined with increasing major ion concentrations. Some chloro complexes of Ag are bioavailable (Hogstrand & Wood, 1998; Niyogi & Wood, 2004). If so, the increase in uptake driven by the chloro complex may have balanced the decrease in Ag⁺ as hardness increased, resulting in no detectable net effect of hardness on bioavailability of Ag from AgNO₃.

Uptake rates from AgNO₃ in L. stagnalis were proportional to Ag⁺ and Ag–BL concentrations. The latter is similar to the relationship of toxicity and BL–Ag in other species (Niyogi & Wood, 2004). There was also a small, but significant, increase in the amount of BL–Ag with decreasing hardness (Figure 2C). This probably reflected a small influence of major ions on association of Ag with the biotic ligand.

On the other hand, uptake rates of Ag by L. stagnalis exposed to AgNPs were not simply a function of Ag⁺ or BL–Ag (Figure 4). Three-fold faster influxes of Ag were observed at similar Ag⁺ concentrations for the PVP AgNP and PEG AgNP compared to AgNO₃ exposures (Figure 4). Additionally, BL–Ag did not predict that water hardness would have an effect on Ag uptake rates from AgNP exposures (results of BLM modeling with AgNPs not shown). Models that successfully predict influences of water quality on Ag bioavailability or toxicity will not be good predictors of bioavailability from AgNPs, at least partly because they cannot account for influential particle transformations and uptake directly from particles.

Ag uptake from exposures to AgNO₃ versus AgNPs

Uptake of Ag added as AgNO₃ was faster than that of Ag from AgNPs at the same total Ag concentration in the media.
Therefore, at the same total Ag concentration, more bioavailable Ag is present in AgNO₃ exposures than in AgNP exposures. However, uptake of Ag in the AgNP experiments was greater than in the AgNO₃ experiments at the same free Ag⁺ concentrations (Figure 4). Similarly, (Navarro et al., 2008) showed toxicity in excess of that explained by Ag⁺ in algal cells. The “excess” uptake in the AgNP experiments with *L. stagnalis* seems likely attributable to uptake of the AgNPs themselves. If so, the $k_{uw}$ derived from total Ag concentrations in the AgNP experiments is composed of two sub-$k_{uw}$s: one describing uptake of newly dissolved Ag and one describing a nanoparticle-specific uptake ($k_{NP}^{uw}$) (e.g. Equation (2)). Uptake of particulate Ag was slower than uptake of dissolved Ag at the same concentration as evidenced by $k_{NP}^{uw}$s that were slower than $k_{uw}$s for Ag⁺. Quantification of the relative importance of the two components of uptake showed that the AgNP-specific component is always important and is the dominant driver of uptake in most cases presented here. This is likely because AgNP concentrations exceed the newly dissolved Ag concentrations in all cases. Only where the highest dissolved Ag was measured (40%), the NP-specific uptake was less important than uptake from the dissolved fraction of Ag (PVP AgNPs in DI, Figure 5). Croteau et al. recently found that the majority of uptake from citrate capped AgNPs was from the NP portion (approximately 80%) in *L. stagnalis* at concentrations in the range studied here (above 0.1 µg/l or approximately 1 nM) (Croteau et al., 2014). In general, these results support that simultaneous, multiple pathways of bioaccumulation from NPs should be considered in mechanistic exposure and toxicity models (Khan et al., 2014).

**Water hardness: influences on uptake of Ag from AgNPs**

The synthesis of most nanoparticles includes adding a capping agent to increase their stability by either charge repulsion or steric forces (El Badawy et al., 2010; Kvitek et al., 2008). These coatings can also influence AgNP transformations under different environmental conditions. In the present study, similar sized PEG and PVP-coated AgNPs showed different percentages of dissolved Ag and responded differently, in terms of aggregation, to changes in major ion concentrations. Major ion concentrations affect the surface characteristics of AgNPs and thereby influence their aggregation and dissolution in the aqueous environment (Tejamaya et al., 2012), even if the AgNPs are sterically stabilized (as in the present study) (El Badawy et al., 2010). Jin et al. reported that Mg²⁺ and especially Ca²⁺ were more effective at inducing aggregation of uncapped AgNPs compared to other ions (e.g. Na⁺, Cl⁻, K⁺, SO₄²⁻ and HCO₃⁻) (Jin et al., 2010). Teyamaja et al. (2012) reported greater agglomeration of PEG AgNPs compared to PVP AgNPs in OECD *Daphnia* media, similar to the results reported here and consistent with the recognized role of PVP as one of the most effective dispersing agents for AgNPs.

Aggregation can, but does not always, affect bioavailability (Croteau et al., 2011a; Tejamaya et al., 2012). Lee and Ranville, for example, found that water hardness increased aggregation of gold nanoparticles (AuNP), but, unlike our study, uptake of AuNPs in *Daphnia magna* was not affected by aggregation (Lee & Ranville, 2012). However, Gao et al. observed increased aggregation of AgNPs and decreased toxicity in *C. dubia* with increasing ionic strength (Gao et al., 2009). Consistent with the above, increasing major ion concentrations caused aggregation of
PEG AgNPs in the present study. Aggregation of PVP AgNPs was minimal or at least much less than aggregation of PEG AgNPs in MOD water. Three lines of evidence suggested this aggregation reduced the bioavailability of Ag from AgNPs to *L. stagnalis*, in contrast to the *Daphnia* studies. First, rate constants of uptake from both types of AgNPs declined with hardness, coincident with aggregation. Second, the rate constants of uptake from PEG AgNPs declined more with increased hardness than from PVP AgNPs, coincident with greater aggregation of the former compared to the latter. Third, in DI water, where aggregation was low, rate constants of uptake ($k_{uw}$ and $k_{uw}^*$) were higher for PEG AgNPs than for PVP AgNPs. But with the addition of major ions, the ranking was reversed, again coincident with the greater aggregation of PEG AgNPs.

The increased dispersion of NPs in diluted media with lower ionic strength may facilitate the dissolution of the particles and release of Ag⁺ (Li & Lenhart, 2012). Greater dispersion may also be the reason that PVP AgNP was more susceptible to dissolution than PEG AgNPs in all waters. Greater dissolved Ag appeared to contribute to the higher rate constant of uptake ($k_{uw}$) observed for PVP AgNPs than for PEG AgNPs in SO, MOD and HW. When the components of uptake were separated, 25% of uptake from PVP AgNPs was attributable to dissolved Ag in MOD water and 55% was attributable to dissolved Ag in DI water. The percentages from dissolved Ag were much smaller with PEG AgNPs (18% for MOD and 14% for DI). In most natural waters, PVP AgNPs might appear inherently more bioavailable than PEG AgNPs. But, this enhanced bioavailability should be recognized as the outcome of transformations that occur in both types of particles as a result of major ion interactions that are probably common in natural waters.

Elimination

The type of capping agent also influenced the rates of elimination of Ag following AgNP exposures. Although the rate constant of uptake of Ag from PEG AgNPs was lower than that from PVP AgNPs for most hardness levels, the potential for accumulation is greater for the PEG AgNPs because of differences in the elimination rate constant (calculation in SI). The difference in rate constants of loss between the capping agents is not likely caused by the greater uptake of Ag⁺ in the PVP AgNP exposures, because the $k_{uw}$ for the PVP AgNPs (0.04 ± 0.01 d⁻¹) was also greater than that for Ag⁺ (0.004 ± 0.013 d⁻¹) (Croteau et al., 2011b), although the two experiments employed snails of different average weight (i.e. 3 mg versus 15 mg, for the PVP and AgNO₃ experiments, respectively).

Conclusion

Failure to consider the interplay of drivers of bioavailability, like those studied here, may be one reason that conflicting results are common in the literature on environmental implications of nanomaterials. The present study shows that bioaccumulation from AgNPs is not just a function of total Ag concentration, but also influenced by transformations that change bioavailability of Ag from the AgNPs. Both newly dissolved Ag and direct uptake of AgNPs contribute to bioaccumulation, with the relative contribution of the two depending upon NP transformations. In the regulatory context, there is discussion of whether traditional approaches to metal risk assessment will be adequate to assess environmental risks from metal-based nanomaterials. Metal risk assessment does not account for particle-specific processes like aggregation and dissolution or particle-specific avenues of uptake. Advanced metal bioavailability models, if driven by speciation (like the BLM), cannot account for such effects and would not accurately capture the degree of risk and the factors affecting that risk from AgNPs.

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Declaration of interest

There is no additional information to disclose.

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**Supplementary material available online**

Supplementary Table S1–S6, Supplementary Figures S1–S14 and Supplementary Equations S1–S4