Impact of sulfidation on the bioavailability and toxicity of silver nanoparticles to *Caenorhabditis elegans*

Daniel L. Starnes¹, Jason M. Unrine^{1,2}, Catherine P. Starnes³, Blanche E. Collin^{1,2}, Emily K. Oostveen¹, Rui Ma^{2,4}, Gregory V. Lowry^{2,4}, Paul M. Bertsch^{1,2,5}, and Olga V. Tsyusko^{1,2,*}

1. Department of Plant and Soil Sciences, University of Kentucky, 1100 South Limestone Street, Lexington, Kentucky 40546, United States

2. Center for Environmental Implications of NanoTechnology (CEINT), P.O. Box 90287, Duke University, Durham, North Carolina 27708-0287, United States

3. Biostatistics, Epidemiology, and Research Design; Center for Clinical and Translational Science, University of Kentucky, Lexington KY 40536, USA

4. Department of Civil & Environmental Engineering, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213, United States

5. Division of Land and Water, CSIRO, Ecosciences Precinct, Brisbane, QLD Australia

*Corresponding Author: e-mail: olga.tsyusko@uky.edu, phone: 859-257-1777

Abstract

Sulfidation is a major transformation product for manufactured silver nanoparticles (Ag-MNPs) in the wastewater treatment process. We studied the dissolution, uptake, and toxicity of Ag-MNP and sulfidized Ag-MNPs (sAg-MNPs) to a model soil organism, *Caenorhabditis elegans*. Our results show that reproduction was the most sensitive endpoint tested for both Ag-MNPs and sAg-MNPs. We also demonstrate that sulfidation not only decreases solubility of Ag-MNP, but also reduces the bioavailability of intact sAg-MNP. The relative contribution of released Ag⁺ compared to intact particles to toxicity was concentration dependent. At lower total Ag concentration, a greater proportion of the toxicity could be explained by dissolved Ag, whereas at higher total Ag concentration, the toxicity appeared to be dominated by particle specific effects.

Keywords: soil, transformation, wastewater treatment, nanotechnology, bioaccumulation

Capsule:

Sulfidation of silver nanoparticles decreases their dissolution and the associated bioavailability and toxicity to *Caenorhabditis elegans*.

Introduction

It is estimated that over 20 tons of silver nanoparticles (Ag-MNPs) are produced annually in the United States (US), with over 300 tons estimated globally, making Ag-MNPs the third most important class of MNPs based on production volume (Gottschalk et al., 2010; Hendren et al., 2011). Ag-MNPs are extensively utilized for their anti-microbial properties in textiles, plastics, and in a variety of health care products (Chen and Schluesener, 2008). During manufacture, use, and product disposal, Ag-MNPs can be released to wastewater streams (Benn and Westerhoff, 2008), where they have been shown to partition largely to sewage sludge during the wastewater treatment process (Kaegi et al., 2011; Schlich et al., 2013b). Nearly 75% of US households are serviced by wastewater treatment plants (WWTPs) and over half of the biosolids generated are land applied as agricultural fertilizer (EPA 1995). Therefore, land application of biosolids is the primary route of Ag-MNP introduction to terrestrial ecosystems (Blaser et al., 2008; Hendren et al., 2013; Whitley et al., 2013). As MNPs enter WWTPs, they are subjected to biogeochemical transformations, collectively referred to as aging. The biogeochemically driven aging processes include MNP interactions with a range of organic and inorganic ligands along a steep redox and often pH gradient, (Levard et al., 2012; Reidy et al., 2013; Wiesner et al., 2009). For Ag-MNPs, the most important transformation is the sulfidation of zerovalent Ag-MNPs to mixed Ag(0)-Ag₂S phases or Ag₂S as indicated by several bench and pilot scale WWTP experiments (Kaegi et al., 2011; Levard et al., 2012; Levard et al., 2011; Whitley et al., 2013). Such transformations of Ag-MNPs can alter their solubility, bioavailability, and toxicity (Kaegi et al., 2013; Levard et al., 2013a; Lombi et al., 2013; Ma et al., 2014). Toxicity of pristine Ag-MNPs has been shown to be driven by both ion- or particle-specific effects (Levard et al., 2012). Xiu and Yang independently reported that toxicity was driven by the ions produced by Ag-MNP

(Xiu et al., 2012; Yang et al., 2012). While other have reported Ag-MNP can cause toxic effects greater than their equivalent ionic (metal salt) concentrations (Choi and Hu, 2008; Meyer et al., 2010; Yin et al., 2011). The sulfidation of Ag-MNPs has been shown to reduce the effects of Ag-MNPs attributable to Ag ion release due to their reduced solubility. (Levard et al., 2013a; Levard et al., 2011; Reinsch et al., 2012; Whitley et al., 2013)

Several studies have previously demonstrated that exposure to Ag-MNPs can elicit a range of toxic responses in a variety of organisms including algae, bacteria, and eukaryotic organisms in both aquatic and terrestrial systems (Fabrega et al., 2009; Gorth et al., 2011; Miao et al., 2009; Roh et al., 2009). Impacts of Ag-MNPs on terrestrial organisms, such as earthworms (*Eisenia fetida*, *E. andrei*), have been investigated and include avoidance behavior, decreased reproduction, and changes in expression of several stress response genes linked to oxidative stress (Schlich et al., 2013a; Shoults-Wilson et al., 2011a; Shoults-Wilson et al., 2011b; Tsyusko et al., 2012a).

An emerging tool for assessing the toxicity of MNPs is the use of the model organism, a nematode, *Caenorhabditis elegans*, which is frequently found in decaying plant material associated with soil (Ellegaard-Jensen et al., 2012; Kim et al., 2012; Meyer et al., 2010; Roh et al., 2010; Roh et al., 2009; Tsyusko et al., 2012b; Williams and Dusenbery, 1990; Zhang et al., 2011a). The popularity of *C. elegans* as a test species to evaluate MNP toxicity is driven by its invariable cell fate map, fully mapped genome, the availability of mutants, short generation time, and low maintenance cost (Choi et al., 2014; Handy et al., 2012; Kim et al., 2012; Leung et al., 2008; Ma et al., 2009; Meyer et al., 2010; Roh et al., 2010; Roh et al., 2009; Williams and Dusenbery, 1990; Zhang et al., 2011a). In addition, *C. elegans* can survive and reproduce in a range of media, including those with low ionic strength, which is a more environmentally

representative medium for toxicity testing with Ag-MNPs than e.g. physiological buffer (Choi et al., 2014).

To date, the majority of nanotoxicity studies generally and those utilizing *C. elegans* specifically, have focused on the effects of pristine Ag-MNPs without taking into account the effects of particle aging (Collin et al., 2014). Pristine Ag-MNPs have previously been shown to cause increased mortality, decreased growth, and decreased reproduction, as well as increased oxidative stress in *C. elegans* (Kim et al., 2012; Lim et al., 2012; Meyer et al., 2010; Yang et al., 2012). Levard et al. demonstrated decreases in mortality and impacts to growth of the nematodes exposed to sulfidized Ag-MNP (sAg-MNPs) when compared to pristine Ag-MNPs at a S to Ag ratio as low as 0.019 (Levard et al., 2013a). To our knowledge there are no published studies that demonstrated impacts of sulfidation of Ag-MNPs using other sensitive endpoints in *C. elegans*, such as reproduction or that attempt to quantify differences in Ag bioavailability between Ag-MNPs and sAg-MNPs.

The objectives of this study are to investigate and compare the bioavailability and toxicity of Ag-MNPs and sAg-MNPs to *C. elegans*. Additionally, our aim is to determine if the observed toxicity of Ag-MNPs and sAg-MNPs are exclusively related to the dissolved ion or also include particle-specific effects. We hypothesize that sAg-MNPs will have decreased toxicity and bioavailability compared to pristine Ag-MNPs. We also hypothesize that the ion specific effects of both sulfidized and pristine Ag-MNPs will decrease with increasing exposure concentration based on the limited solubility of these materials, i.e. as the particle to ion ratio increases.

Materials and Methods

Silver Nanoparticle Synthesis and Characterization

Polyvinylpyrrolidone (PVP) coated (pristine) Ag-MNPs were synthesized as previously described (Cheng et al., 2011). These particles were then sulfidated (to produce sAg-MNPs) similarly to Levard et al. (2011) using molar ratio of sulfide as 2:1 S/Ag. More detailed descriptions for sAg-MNPs preparation and particle characterization is provided in SI.

Exposure Medium

The choice of medium can strongly influence the toxicity of Ag-MNPs; therefore, we chose MHRW ($I = 0.00395$) which is a common toxicity testing medium. This medium is low in chloride (0.11 mmol) minimizing precipitation of insoluble AgCl (Tyne et al., 2013; Yang et al., 2012). We used MINTEQ to calculate equilibrium Ag speciation for our exposure solutions in MHRW, and the saturation indices are negative (-2.48, -2.17, and -0.68 for 1, 10, and 100 ppb Ag, respectively) indicating that AgCl(s) does not precipitate. However, the calculations predict that about 8% of the Ag is present as AgCl(aq) ion pairs at each of these three concentrations. We examined the effects of sAg-MNPs in addition to unsulfidated Ag-MNPs. Further, as unsulfidated Ag-MNPs are prone to dissolution and release of free Ag^+ , AgNO_3 (Ag^+) was used as an ion control in all experiments.

Dissolution Experiments

Dissolution was determined by centrifuging 2.0 mL samples in MHRW at 16,800g in polypropylene microcentrifuge tubes for 3 hours after 1 and 24 hour incubation at 20°C in darkness. Stoke's law predicts that this sedimented Ag-MNPs larger than 1 nm in diameter. AgNO_3 controls (at 10 $\mu\text{g Ag L}^{-1}$) were also used to account for any ion loss during centrifugation. We measured dissolution of Ag-MNPs and sAg-MNPs at two concentrations of

1.5 and 0.05 mg Ag L⁻¹. Immediately after centrifugation, the top 1 mL of supernatant was removed and adjusted to 0.12 M HCl. One mL of non-centrifuged samples was digested in 9 mL of concentrated HNO₃ and 3 mL of concentrated HCl, heated in a microwave to 100°C, held at that temperature for 10 min, and diluted to final acid concentration of 0.45 M HNO₃/0.012 M HCl for analysis. Samples were analyzed for Ag using inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7500cx, Santa Clara, CA) with duplicate digestions, matrix spikes, cross calibration verification, and reagent blanks.

Nematode exposure and toxicity experiments

The toxicity protocols employed represent modifications of previously established *C. elegans* toxicity testing methods (Williams and Dusenbery, 1988, 1990) and those previously used in our lab (Tsyusko et al., 2012b) with modifications stated below.

Wild type N2 Bristol strain of *C. elegans* was obtained from the Caenorhabditis Genetics Center and were age-synchronized using NaClO/NaOH solution (Williams and Dusenbery, 1988). Mortality with feeding, growth, and reproduction experiments were conducted in the presence of bacterial food, *Escherichia coli* (OP50 strain), was added (OD₆₀₀ = 1) at the rate of 10 µL mL⁻¹ of exposure solution. Exposure solutions for growth and reproduction experiments were replaced after 24 h and fresh bacterial food was added.

Mortality. For mortality testing, larval L3 stage nematodes were exposed to varying concentrations of Ag-MNPs, sAg-MNPs, and AgNO₃ in MHRW with and without bacterial food for 24 h in a 24 well polycarbonate tissue culture plate. At least five concentrations were used for every treatment. Unfed nematodes were exposed to Ag-MNPs (50-100 µg Ag L⁻¹), sAg-MNPs (250-1500 µg Ag L⁻¹), and AgNO₃ (2.5-10 µg Ag L⁻¹); fed nematodes were exposed to

130 Ag-MNP (1000-4500 $\mu\text{g Ag L}^{-1}$), sAg-MNP (2000-10,000 $\mu\text{g Ag L}^{-1}$), and AgNO_3 (25-85 $\mu\text{g Ag}$
 131 L^{-1}). Exposures without feeding were conducted to differentiate between mortality due to
 132 dissolution and mortality due to intact particles, where dissolution and subsequent binding of
 133 Ag^+ ions to microbial cells would have complicated interpretation (Levard et al., 2014).
 134 Dissolution rates after 24 hours at each concentration of Ag-MNP and sAg-MNP were
 135 determined using the methods described above. The calculated amount of Ag^+ present in the
 136 solution at every concentration were then compared to the mortality concentration-response
 137 curve for Ag^+ to identify the level of mortality caused by that concentration of Ag^+ . Experiments
 138 with feeding were conducted to establish ranges for growth and reproduction experiments.
 139 Feeding is necessary to prevent dauer formation at early stages of larval development in growth
 140 and reproduction assays. Each treatment had 4 replicates per concentration with 10 (± 1)
 141 nematodes in each well and all concentrations were tested in two independent experiments.

142 *Growth.* Eggs were hatched in the exposure solutions made with MHRW with OP50 food source
 143 ($10 \mu\text{L mL}^{-1}$). Nematodes were exposed in 15 mL centrifuge tubes with 4 mL of the exposure
 144 medium; Ag-MNPs (500-3000 $\mu\text{g Ag L}^{-1}$), sAg-MNP (3000-9000 $\mu\text{g Ag L}^{-1}$), and AgNO_3 (6.25-
 145 30 $\mu\text{g Ag L}^{-1}$) in MHRW. Nematode body length was measured after 48 hours. Nematodes were
 146 allowed to settle for 30 min and at least 30 nematodes were removed from exposures and treated
 147 with sodium azide (3 μL of 170 mM, to straighten them) and imaged using a light microscope.
 148 Measurements were then made using NIS-Elements BR 3.0 image processing software (Nikon).
 149 Each concentration was tested in two independent experiments.

150 *Reproduction.* Eggs were hatched on K-agar plates with OP50 bacterial lawns (Williams and
 151 Dusenbery, 1988) and were placed into the exposure solutions. For each exposure, six L1

nematodes were exposed to at least five concentrations per treatment of Ag-MNPs (250-1000 $\mu\text{g Ag L}^{-1}$), sAg-MNPs (1000-5000 $\mu\text{g Ag L}^{-1}$), and AgNO_3 (5-25 $\mu\text{g Ag L}^{-1}$) in MHRW. After 50 h of exposure, individual nematodes were transferred to K-agar plates for egg laying. Adult worms were transferred to fresh K-Agar plates every 24 h for 72 h. Plates were incubated for 24 h to allow hatching, then stained with rose bengal (0.5 mg L^{-1}), heated to 50°C for 55 min, then fully hatched juveniles were counted. Each exposure was tested in two independent experiments.

Particle-specific effects versus Ion-specific effects

To determine the effect of Ag^+ produced by the Ag-MNPs and sAg-MNPs, particle free supernatants were generated after 24 h incubation and prepared as described above in the dissolution experiment section. Nematodes exposed to particle free supernatants were compared to nematodes exposed to whole suspensions that had been incubated for 24 h. Concentrations above the LC_{50} were selected for all treatments, and exposure conditions were the same as in the experiments for mortality without feeding.

Synchrotron x-ray fluorescence microscopy

Distribution of Ag was examined in L4 stage *C. elegans* exposed to Ag^+ (10 $\mu\text{g L}^{-1}$), Ag-MNPs (100 $\mu\text{g Ag L}^{-1}$), and sAg-MNPs (3000 $\mu\text{g Ag L}^{-1}$) for 4 h. These concentrations were selected because relatively high tissue concentrations are required for detection by X-ray fluorescence and because they resulted in similar mortality after 4 h of exposure (~ 30%). Approximately 100-150 nematodes were collected, washed twice with MHRW, and preserved in 20% ethanol prior to X-ray analysis. Scanning X-ray fluorescence microscopic measurements were performed at sectors 13 and 20 at the Advanced Photon Source, Argonne National Laboratory (Lemont, IL, USA). Additional details on synchrotron methods are provided in SI.

Statistical Methods

For growth, reproduction, and mortality, means and standard errors were calculated per treatment and concentration. Probit analysis using the PROBIT procedure in SAS v9.3 (SAS Institute, Cary, NC, USA) was used to determine the LC₁₀ and LC₅₀ values, presented at 95% confidence intervals. To test whether the observed mortality was due to particle specific effects or simply due to the release of Ag⁺ into solution, student's t-tests were used to compare the mortality of nematodes exposed to particle free supernatants and those exposed to the whole suspensions. Student's t-tests with Dunnett's multiple comparison corrections were applied to test whether each concentration within a treatment was significantly different from control for all experiments. Homogeneity of variance and normality of errors assumptions were checked using Q-Q plots and studentized residual plots. EC₁₀ and, when appropriate, EC₅₀ values were calculated from parameters determined using linear regression. SAS v9.3 was used for all statistical analyses and SigmaPlot 12.3 (Systat, San Jose, CA, USA) was used to generate all figures.

Results

Characterization and Dissolution Measurements

We observed that the zeta potential of the Ag-MNPs decreased with sulfidation from -6.1 mV for Ag-MNPs to -28.1 mV for sAg-MNP. However, the size of the MNPs changed less than 10% with sulfidation when measured by TEM, 58.3 ± 12.9 nm (mean $\pm$ standard deviation) for Ag-MNPs and 64.5 ± 19.4 nm for sAg-MNPs. A similar change was observed in the z-average hydrodynamic diameter, for Ag-MNPs 79.6 ± 0.5 and 88.5 ± 0.5 for sAg-MNP. The TEM images (SI1) and DLS particle size distributions (intensity and volume weighted) in MHRW (SI2 and SI3) corresponding to the data described above are provided in the supplemental information

(SI). For both Ag-MNPs and sAg-MNPs the dissolved Ag, expressed as a % of the total, was greater at for the 50 $\mu\text{g L}^{-1}$ Ag-MNP concentration compared to the 1500 $\mu\text{g L}^{-1}$ Ag-MNP concentration, with a more pronounced difference at 24 h compared to 3 h. For Ag-MNP at 3 h the percent dissolved Ag was found to be 3.5% and 1.25% for 50 and 1500 $\mu\text{g L}^{-1}$, respectively, and at 24 h was found to be 5.8 and 2.9 % for 50 and 1500 $\mu\text{g L}^{-1}$, respectively (**Fig 1**).

Figure 1. The percent dissolution of pristine silver manufactured nanoparticles (Ag-MNP) and artificially aged silver manufactured nanoparticles (sAg-MNP) at 3 and 24 hours at two concentrations (50 and 1500 $\mu\text{g L}^{-1}$) in moderately hard reconstituted water (Main) and sAg-MNP (Insert) at full scale. Error bars represent the minimum and maximum total Ag concentration.

For the sAg-MNP the dissolved Ag at 3 h was found to be 0.05% and 0.03% for 50 and 1500 $\mu\text{g L}^{-1}$, respectively, and was found to be 0.43% and 0.23% at 24 h for 50 and 1500 $\mu\text{g L}^{-1}$, respectively. (**Fig. 1**). The dissolution of sAg-MNP at 24 h was found to be reduced by 13-fold and 12-fold at 50 $\mu\text{g L}^{-1}$ and 1500 $\mu\text{g L}^{-1}$, respectively, compared to Ag-MNPs.

Toxicity and Bioavailability

Reproduction was found to be the most sensitive endpoint tested while growth was the least sensitive. For all endpoints tested, sAg-MNP required at least a 2-fold higher concentration to achieve equivalent levels of toxicity as compared to Ag-MNPs. *Mortality*. Mortality in the absence of food for sAg-MNP never exceeded 40%.

Figure 2. Mortality of *Caenorhabditis elegans* after 24 hours without feeding exposure to Ag⁺, pristine silver manufactured nanoparticles (Ag-MNP), and artificially aged manufactured nanoparticles (sAg-MNP) in moderately hard reconstituted water. Red portions of Ag-MNP and sAg-MNP bars represent the amount of mortality that can be attributed to their dissolution. Data are presented as mean percent mortality with error bars indicating standard error of the mean. Mortality at all concentrations was found to be significantly greater than control ($p < 0.001$), except for Ag⁺ 2.5 µg L⁻¹, Ag-MNP 50 µg L⁻¹, and sAg-MNP 250 µg L⁻¹.

The LC_{10s} with corresponding 95% confidence intervals (given in parentheses) were 5.1 (4.35, 5.70) µg L⁻¹, 51.7 (47.75, 54.84) µg L⁻¹, and 315.5 (48.09, 515.07) µg L⁻¹, for Ag⁺, Ag-MNPs, and sAg-MNPs, respectively. The LC₅₀ with corresponding 95% confidence intervals (given in

parentheses) were 7.5 (7.01, 7.89) $\mu\text{g L}^{-1}$ 72.5 (70.19, 74.90) $\mu\text{g L}^{-1}$ and 4612 (2166.00, 130428.00) $\mu\text{g L}^{-1}$, for Ag^+ , Ag-MNPs, and sAg-MNPs respectively (Fig. 2).

The concentrations at which *C. elegans* exhibited equivalent mortality were 4 to 30 fold higher in the experiment with feeding than without feeding at the LC_{10} . The LC_{10} (95% CI) for Ag^+ , Ag-MNPs, and sAg-MNPs were 24.8 (12.83, 33.94) $\mu\text{g L}^{-1}$, 2002 $\mu\text{g L}^{-1}$, and 4106 $\mu\text{g L}^{-1}$, respectively, for fed organisms. The LC_{50} (95% CI) for Ag^+ was 54.8 (43.31, 63.40) $\mu\text{g L}^{-1}$ and for Ag-MNP was 3119 (2976, 3262) $\mu\text{g L}^{-1}$.

Figure 3. Mortality of *Caenorhabditis elegans* after 24 hours in the presences of bacterial food (*Escherichia coli* strain OP50) when exposed to Ag^+ , pristine silver manufactured nanoparticles (Ag-MNP), and artificially aged manufactured nanoparticles (sAg-MNP) in moderately hard reconstituted water. Data are presented as mean percent mortality with error bars indicating standard error of the mean. * Indicate treatments found to be significantly different than control ($p < 0.001$).

Nematodes exposed to 10,000 $\mu\text{g L}^{-1}$, the highest concentration of sAg-MNP tested, only resulted in an observed mortality of 20% (Fig. 3).

Particle versus Ion Specific mortality. Except for the 50 $\mu\text{g L}^{-1}$ Ag-MNP treatment, the mortality observed at all other concentrations cannot be fully explained by dissolution and release of Ag^+ for both Ag-MNPs and sAg-MNPs (**Fig. 2**). The proportion of mortality attributable to ion release from MNPs into the exposure medium as measured by the dissolution experiments over 24 h, decreased with increasing exposure concentration for both Ag-MNPs and sAg-MNPs. The mortalities for the nematodes exposed to each particle free supernatants of Ag-MNPs and sAg-MNPs were less than 10% and were not significantly different from that in controls, while mortality in the whole suspensions were at 78% for Ag-MNPs and 62% for sAg-MNPs (**Fig. 4**). When the nematodes were exposed to supernatants of Ag^+ the mortality was 85%, similar to the 88% mortality observed in the whole (uncentrifuged) Ag^+ solution ($p = 0.2554$). The mortality estimates for the Figures 2 and 4 were derived from two independent experiments, which explain slight variation in responses at the same concentrations.

Figure 4. Mortality of *Caenorhabditis elegans* after 24 hours without feeding when exposed to particle free supernatants (Supernatant) versus whole solutions of Ag^+ , pristine silver manufactured nanoparticles (Ag-MNP), and artificially aged manufactured nanoparticles (sAg-MNP) (Whole Solution) in moderately hard reconstituted water. Data are presented as mean percent mortality with error bars indicating standard error of the mean. * Indicate treatments found to be significantly different than control ($p < 0.001$).

Growth. Nematodes exposed to Ag^+ , Ag-MNPs, and sAg-MNPs all exhibited significantly shorter body lengths compared to control, regardless of concentration ($P < 0.001$ for all comparisons) (**Fig. 5**). The greatest decrease from the control in mean body length was observed in Ag^+ exposed nematodes, followed by Ag-MNP exposed nematodes, with the smallest decrease in response to sAg-MNP exposed nematodes. These significant decreases were observed at every concentration tested versus controls ($p < 0.001$) The EC_{10} for Ag^+ , Ag-MNPs and sAg-MNPs were $6.6 \mu\text{g L}^{-1}$, $1118 \mu\text{g L}^{-1}$, and $5946 \mu\text{g L}^{-1}$, respectively.

Figure 5. Observed mean body length of *Caenorhabditis elegans* measured after 48 hours from L1 stage exposed to Ag^+ , pristine silver manufactured nanoparticles (Ag-MNP), and artificially aged manufactured nanoparticles (sAg-MNP) in moderately hard reconstituted water in the presence of bacterial food (*Escherichia coli* strain OP50). Data are presented as mean percent mortality with error bars indicating standard error of the mean. All concentrations were found to be significantly different than control ($p < 0.001$).

Reproduction. Nematodes exposed to Ag^+ , Ag-MNPs, and sAg-MNPs exhibited significant decrease in number of offspring per adult compared to control, regardless of concentration ($p < 0.001$ for all comparisons) (**Fig. 6**) Nematodes exposed to sAg-MNPs had a significantly higher

average number of offspring per adult than those exposed to Ag^+ ($p = 0.015$) or Ag-MNPs ($p = 0.004$), regardless of concentration. The EC_{10} for Ag^+ was $3.1 \mu\text{g L}^{-1}$ and EC_{10} for Ag-MNPs and sAg-MNPs were $113.1 \mu\text{g L}^{-1}$ and $802.4 \mu\text{g L}^{-1}$, respectively. The EC_{50} for Ag^+ was $15.3 \mu\text{g L}^{-1}$, for Ag-MNPs was $566 \mu\text{g L}^{-1}$, and for the sAg-MNPs it was $4011 \mu\text{g L}^{-1}$.

Figure 6. Mean total number of offspring per adult nematode *Caenorhabditis elegans* after 48 hours from L1 stage exposed to Ag^+ , pristine silver manufactured nanoparticles (Ag-MNP), and artificially aged silver manufactured nanoparticles (sAg-MNPs) in moderately hard reconstituted water in the presence of bacterial food (*Escherichia coli* strain OP50). Data are presented as mean percent mortality with error bars indicating standard error of the mean. * Indicate treatments found to be significantly different than control ($p \leq 0.001$).

Bioavailability. From μ -XRF Ag fluorescence intensity maps of *C. elegans* exposed to Ag^+ , Ag-MNPs, and sAg-MNP at approximately LC_{30} concentrations (estimated after 4 hrs of exposure), lower intensities of Ag were observed in sAg-MNP treatment than Ag^+ or Ag-MNP treatments. Nematodes exposed to Ag^+ and Ag-MNPs showed similar spatial distributions and emission intensities for Ag (**Fig. 7**). Tissue concentrations were too low to collect x-ray absorption spectroscopic data to determine speciation.

Figure 7. Ag K α 1 fluorescence of *Caenorhabditis elegans* specimens (stage L4) that were exposed to LC₃₀ (at 4 hr) for Ag⁺ (Top), pristine silver manufactured nanoparticles (Middle), and artificially aged silver manufactured nanoparticles (Lower) in moderately hard reconstituted water in the absence of bacterial food, as determined using a synchrotron-based X-ray fluorescence microprobe. Color bar indicates normalized Ag K α 1 fluorescence intensity. The data are normalized to the incident beam ion chamber reading, so they are normalized with respect to each other.

Discussion

Importantly, our results demonstrate that it is not just the decrease in solubility of Ag₂S relative to Ag (0) that accounts for the observed reductions in toxicity, but also a decrease in the uptake of intact sAg-MNPs resulting in decreased particle-specific effects of sAg-MNPs compared to pristine Ag(0)-MNPs (*vide infra*). Whether the observed toxicological effects of Ag-MNPs are due to particle-specific effects, or simply due to the release of Ag⁺ into solution

has been a matter of debate since the first reports of Ag MNP toxicity (Xiu et al., 2012; Yang et al., 2012; Yin et al., 2011). Using multiple experimental approaches, we demonstrate that undissolved particles in the exposure media cause toxicity. Although it is likely that dissolution of the particles following uptake elicits toxicity through the release of ions, we consider this route of cellular uptake and tissue specific delivery to be particle-specific (Tsyusko et al., 2012a). It is also possible that presence of the nematodes or food can promote dissolution of Ag-MNPs. If, the released dissolved Ag is immediately bound to ligands in the nematodes and the food, the Ag-MNPs would release more dissolved Ag to maintain the equilibrium between dissolved and particulate Ag. This could lead to an underestimation of the effects due to dissolved ions. From previously published data, there appears to be an emerging agreement that the toxic effects of Ag-MNPs are due in part to the release of Ag^+ and in part from particle effects during exposure (Ellegaard-Jensen et al., 2012; Kim et al., 2012; Meyer et al., 2010). The results of this study also support this emerging paradigm. Recently, Croteau et al. suggested that bioaccumulation due to the release of ions is concentration dependent in snails (Croteau et al., 2014). However, we believe that this is among the first studies to clearly demonstrate that the relative importance of dissolution and release of Ag^+ in the exposure media for toxicity is concentration dependent. Therefore, it is likely that some previous conflicting reports concerning the importance of dissolution may be due to differences in exposure concentration (Xiu et al., 2012; Yang et al., 2012). Although we observed that sulfidation of Ag-MNPs greatly reduces the amount of Ag^+ released and associated toxicity at any of the concentrations tested, a large proportion of the effects of sAg-MNPs observed in this study were particle specific. The reduction in the release of Ag^+ as a result of sulfidation has been reported (Levard et al., 2013a; Levard et al., 2012; Levard et al., 2011; Liu et al., 2011); however, we believe this to be the first

report demonstrating particle specific effects of sAg-MNPs. The role of sAg-MNPs was not simply a continuing source of dissolved ions to be scavenged by ligands in the nematodes, since the XRF imaging indicated that at LC₃₀ for mortality, there are different amounts of Ag accumulated from each treatment indicating that the toxicity was not associated with equivalent Ag body burdens as for the AgNO₃ or Ag-MNP treatments.

Meyer et al. (2010) demonstrated that *C. elegans* are able to internalize Ag-MNPs with different coatings and smaller size than the Ag-MNPs we tested and also our results are in agreement with other paper from the same group where sulfidation significantly decreased toxicity (Levard et al. 2013a) , but the effects on reproduction and differences in bioavailability were not investigated by the authors(Meyer et al., 2010). The lowered particle specific toxicity of sAg-MNPs compared to Ag-MNPs can be in part explained by their decreased bioavailability, as was observed from the synchrotron based X-ray fluorescence images. The Ag concentrations in the nematodes exposed to the sAg-MNPs at LC₃₀ were below the XRF detection limit. In contrast, internal Ag concentrations in nematodes treated with AgNO₃ and Ag-MNPs were readily detectable suggesting that the particle specific toxicity elicited by sAg-MNPs may not involve uptake. A less likely alternative hypothesis is that it is caused by very low tissue concentrations of sAg-MNPs that were below the XRF detection limit. The toxicity is also not explained by soluble impurities, coatings, or other decomposition products released from the sAg-MNPs since particle free supernatants caused minimal mortality. One possible mechanism by which sAg-MNPs could cause an effect in the absence of uptake is through cuticle damage, since the cuticle serves as a primary defense of the nematode against environmental stressors (Cox et al., 1981; Handy et al., 2012; Kim et al., 2012).

Although there have been several investigations into the effects that the exposure medium has on the toxicity of Ag-MNPs, little attention has been paid to the presences/absence of a food source on toxicity (Yang et al., 2014). We expected there to be a decrease in the toxicity in the presence of bacterial food as the nematodes being fed should have increased resistance to the stress caused by Ag-MNP exposure and because partitioning of particles and the released Ag ions to bacteria could also be anticipated to reduce toxicity. Our results show that the addition of bacterial food decreased the toxicity in contrast to Ellegaard-Jensen et al., (2012) who found an opposite effect. They speculated that bacteria represent a dietary exposure route via trophic transfer. Higher ionic strength media have been shown to decrease toxicity of Ag-MNPs (Levard et al., 2013b; Tyne et al., 2013; Yang et al., 2012). We used a different exposure medium (MHRW) than Ellegaard-Jensen et al., (2012) who utilized K-meduma, which has approximately a 3-fold higher ionic strength than MHRW. Additionally, the study of Ellegaard-Jensen et al., (2012) used 28 nm Ag-MNPs that were also coated with PVP but were approximately three times smaller than the Ag-MNPs used in our study. The solubility (Ma et al., 2012) and dissolution rate of Ag MNPs increases with decreasing size (Zhang et al., 2011b). These differences may explain in part the discrepancy between the studies.

We are unaware of any other investigations that have examined both the bioavailability and toxicity of Ag-MNPs and sAg-MNPs for three ecologically relevant endpoints in *C. elegans*. Our experimental approach also allowed us to tease out the relative importance of particle specific versus ion specific effects for Ag-MNPs and sAg-MNPs. Our results combined with those of Levard et al. (2013) demonstrate that aging of Ag-MNPs in the presence of sulfide reduces toxicity as measured by both mortality and growth (Levard et al., 2013a). The results of Levard et al., (2013a) demonstrate that sAg-MNPs at 1,500 $\mu\text{g Ag L}^{-1}$ caused less than 5 percent

mortality and an EC₅₀ for growth was over 10,000 µg Ag L⁻¹ in *C. elegans* using particles similar to sAg-MNPs we employed. However, the impact of sAg-MNPs on reproduction in *C. elegans*, which is the most sensitive endpoint we examined, has not been previously reported.

Overall, these results demonstrate greatly reduced toxicity of sAg-MNPs as compared to Ag-MNPs, suggesting that transformation via the WWTP would likely result in a greatly reduced toxicity of Ag-MNPs. This indicates that hazards of Ag-MNP exposure are likely greater in applications where Ag-MNPs are introduced directly to soil rather than through biosolids application. For example, Ag-MNPs are being researched for use as pesticides or as pesticide delivery vehicles (Marimuthu et al., 2011; Santhoshkumar et al., 2011). However, Ag-MNPs that are introduced as unsulfidized materials are quickly sulfidated in reducing environments such as freshwater sediment (Lowry et al., 2012). The recognition that the relative importance of particle specific effects are concentration dependent may also bring some clarity to observed inconsistencies in the literature. Finally, it is apparent from this study, that sAg-MNPs may elicit toxicity independent of Ag⁺ ion release. Whitley et al. (2013) observed that sAg-MNPs were present in pore water of soils amended with Ag-MNP spiked biosolids. Although particle specific toxicity of sAg-MNPs is not great in *C. elegans*, it would be prudent to test the toxicity of sAg-MNPs in other receptor species and in food chain exposures where significant dissolution could occur in the gastrointestinal systems of certain ecoreceptors.

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