Influence of Water Hardness on Silver Ion and Silver Nanoparticle Fate and Toxicity Toward *Nitrosomonas europaea*

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Abstract

This study investigated the influence of water hardness (Mg^{2+} and Ca^{2+}) on the fate and toxicity of 20 nm citrate silver nanoparticles (AgNPs) and Ag^+ toward *Nitrosomonas europaea*, a model ammonia-oxidizing bacterium. Nitrification inhibition of *N. europaea* by 1 ppm AgNPs and 0.5 ppm Ag^+ was reduced from 80% and 83%, respectively, in the absence of Mg^{2+} to 2% and 33%, respectively, in the presence of $730 \mu\text{M Mg}^{2+}$. Introduction of Mg^{2+} resulted in the rapid aggregation of the AgNP suspensions and reduced the 3 h Ag^+ dissolution rates from 30%, in the absence of Mg^{2+} , to 9%, in the presence of $730 \mu\text{M Mg}^{2+}$. Reduced AgNP dissolution rates resulted in decreased concentrations of silver that were found adsorbed to *N. europaea* cells. Increasing AgNP concentrations in the presence of Mg^{2+} increased the observed inhibition of nitrification, but was always less than what was observed in the absence of Mg^{2+} . The presence of Mg^{2+} also reduced the adsorption of Ag^+ to cells, possibly due to multiple mechanisms, including a reduction in the negative surface charge of the *N. europaea* membrane and a competition between Mg^{2+} and Ag^+ for membrane binding sites and transport into the cells. Ca^{2+} demonstrated similar protection mechanisms, as Ag^+ toxicity was reduced and AgNP suspensions aggregated and decreased their dissolution rates. These results indicate that the toxicity of Ag^+ and AgNPs to nitrifying bacteria in wastewater treatment would be less pronounced in systems with hard water.

Key words: adsorption; magnesium; *Nitrosomonas europaea*; silver ions; silver nanoparticles; toxicity

Introduction

SILVER NANOPARTICLES (AgNPs) are the most widely used type of nanoparticle in consumer goods, and they are found in more than 50% of nanoparticle-containing products (Woodrow Wilson International Center for Scholars and the Pew Charitable Trust, 2012). Silver in a nanoparticle form retains the anti-microbial characteristics of Ag^+ and has been used in medical equipment and household goods, including odor-reducing garments, textiles, and sanitizing equipment (dishwashers, washing machines, etc.) (Benn and Westerhoff, 2008; Wijnhoven *et al.*, 2009).

AgNPs in these applications are released either inadvertently (through the washing of the textiles) or intentionally (as used in sanitizing appliances) into commercial and residential wastewater systems and may bioaccumulate in wastewater treatment plant (WWTP) biosolids (Benn and Westerhoff, 2008; Blaser *et al.*, 2008; USEPA, 2009; Benn *et al.*, 2010). In addition, small but significant fractions of the

AgNPs are expected to leave the WWTP through the effluent and enter into the receiving bodies of water (Kaegi *et al.*, 2011). Therefore, AgNPs have the potential to interfere with critical biological functions, such as the nitrification process performed by ammonia-oxidizing bacteria (AOB), in both WWTPs and the natural environment (Choi and Hu, 2009; Giao *et al.*, 2012).

Nitrosomonas europaea, a model AOB, has been used in studies investigating the toxicity of numerous pollutants on AOB, including AgNPs (Arp *et al.*, 2002; Radniecki and Lauchnor, 2011; Radniecki *et al.*, 2011). The relative toxicity of AgNPs toward nitrifying bacteria depended on the size and surface chemistries of the AgNPs, the presence of silver ligands, and the ionic strength of the aquatic environment (Maramba-Jones and Hoek, 2010; Radniecki *et al.*, 2011; Arnaout and Gunsch, 2012; Kostigen Mumper *et al.*, 2013; Yuan *et al.*, 2013). All of these factors influence the rate of release of Ag^+ from AgNPs, which has been shown to be a primary toxicity mechanism of AgNPs toward nitrifying bacteria (Radniecki *et al.*, 2011; Arnaout and Gunsch, 2012).

Presence of divalent cations (e.g., Mg^{2+} or Ca^{2+}) can induce AgNP aggregation, which can result in reduced AgNP dissolution and potentially limit the ability of AgNPs to adsorb onto microbial biomass (Jin *et al.*, 2010; Zhang and

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Oyanedel-Craver, 2012). The concentrations of Mg^{2+} and Ca^{2+} can vary widely from 0.2–0.9 mM and 0.4–1.7 mM, respectively, for pristine surface waters to approximately 10 mM in groundwaters and wastewater impacted surface waters (Smith *et al.*, 1987; Awadallah *et al.*, 1996; DeSimone *et al.*, 1997; van Langerak and Hamelers, 1997; Hammes *et al.*, 2003). Thus, the presence of divalent cations may play a critical role in the expected fate, transport, and toxicity of AgNPs in both natural and engineered aquatic environments.

While recent studies have focused on the influence of divalent cations on AgNP stability and toxicity, few studies have examined how divalent cations influence the fate and toxicity of dissolved Ag^+ , a major source of AgNP toxicity. This study investigated the effects of Mg^{2+} and Ca^{2+} on Ag^+ and AgNP toxicity toward *N. europaea*, including their influence on AgNP stability and dissolution rates. This work also demonstrates that Mg^{2+} and Ca^{2+} can lower Ag^+ toxicity by preventing the adsorption of Ag^+ by *N. europaea* cells. Thus, we propose that Mg^{2+} and Ca^{2+} can reduce AgNP toxicity through multiple mechanisms: (1) The destabilization of AgNPs resulting in a decrease in Ag^+ dissolution rates and (2) The prevention of Ag^+ from binding to *N. europaea* cells.

Materials and Methods

N. europaea culturing methods

N. europaea ATCC 19718 cells were grown in minimal growth medium in batch reactors as previously described (Radniecki and Lauchnor, 2011). After 3 d, cells reached mid-exponential growth ($OD_{600} \sim 0.070$) and were harvested via centrifugation. The cells were washed in a 30 mM HEPES buffer (pH 7.8) to remove the presence of trace metals. The cells were suspended in a 30 mM HEPES buffer (pH 7.8) and were maintained at 4°C until they were used for experimentation.

AgNP and Ag^+ inhibition studies

Previously developed methods were used to create dispersed AgNP suspensions in test media (Radniecki *et al.*, 2011). In short, aliquots from 20 nm Citrate BioPure™ silver nanoparticles (nanoComposix, Inc., San Diego, CA; 1,000 ppm) or silver nitrate ($AgNO_3$ -10 ppm) stock solutions were placed into batch bioreactors containing deionized (DI) water and were dispersed via shaking. After 15 min of shaking, ammonium sulfate $[(NH_4)_2SO_4]$ and HEPES buffer (pH 7.8) were added to final concentrations of 2.5 mM and 30 mM, respectively. At this time, magnesium sulfate ($MgSO_4$) was also added to Mg^{2+} -containing batch bioreactors to a final concentration ranging from 0 to 730 μM , and calcium nitrate $[Ca(NO_3)_2]$ was added to Ca^{2+} -containing batch bioreactors to a final concentration ranging from 0 to 730 μM . The batch bioreactors were shaken for an additional 30 min before *N. europaea* cells were added to a final concentration of 5 mg protein L^{-1} ($OD_{600} \approx 0.072$). All conditions were run in triplicate batch bioreactors.

The batch bioreactors were shaken in the dark at 250 rpm for 3-h at 30°C. To monitor *N. europaea* nitrification activity, nitrite production was measured at 45 min intervals throughout the 3-h experiment, via colorimetric assay, as previously described (Hageman and Hucklesby, 1971; Radniecki *et al.*,

2011). The nitrite production rates in both treatment and control bioreactors were normalized to their respective cell densities. The nitrite production rates for all control conditions tested were $1.61 \pm 0.07 \mu mol NO_2^- / mg \text{ protein} \cdot min$. Percent nitrification activity refers to the nitrite production rates of bioreactors containing AgNP or Ag^+ compared with the nitrite production rates of control bioreactors not containing AgNP or Ag^+ .

Abiotic AgNPs stability studies

The aggregation state of 20 nm citrate AgNP suspensions, created as described earlier, in the presence and absence of either Mg^{2+} or Ca^{2+} was quantified using time-resolved dynamic light scattering (TR-DLS) by measuring the z-average diameter at 15 s intervals for 60 min with a 90Plus particle size analyzer (Brookhaven Instruments, Holtsville, NY). Due to the interference of their large size, *N. europaea* cells were not present. To minimize background noise, the DDI water and HEPES buffer (pH 7.8)/ $(NH_4)_2SO_4$ solution were filtered through a 20 nm syringe filter (Whatman International Ltd., Maidstone, England) to remove small ambient particles before the AgNP were added to a final concentration of 1 ppm.

Abiotic AgNPs dissolution studies

The dissolution of 20 nm citrate AgNP suspensions, created as described earlier, in the presence and absence of either Mg^{2+} or Ca^{2+} was quantified using ultra-filtration and inductively coupled plasma optical emission spectroscopy (ICP-OES) as previously described (Radniecki *et al.*, 2011). In short, abiotic batch reactors were shaken at 250 rpm in the dark at 30°C for 3 h, at which time the total silver [both dissolved silver (Ag^+) and AgNP] concentrations were measured via ICP-OES using standard methods (APHA, 1998). After 3 h, 15 mL of the test solution was filtered through a centrifugal ultra-filtration device with a 5 kDa molecular-weight cutoff (Amicon Ultra-15; Millipore Co., Billerica, MA) for 30 min at 5,000 g per manufacturer's instruction. The Ag^+ content of the filtrate was measured via ICP-OES and may include the sum of numerous silver chemical species [e.g., Ag^+ , $AgSO_4$, $AgNH_3^+$, or $Ag(NH_3)_2^+$]. All conditions were tested in triplicate abiotic batch reactors.

To determine the amount of Ag^+ that may be retained by the filter, 0.5 to 2 mM $AgNO_3$ solutions were filtered using centrifugal ultra-filtration as described earlier. On average, $10\% \pm 0.6\%$ of the $AgNO_3$ was retained by the filter for all concentrations tested.

Ag^+ and AgNP adsorption studies

The adsorption of either Ag^+ or 20 nm citrate AgNP suspensions to *N. europaea* cells was measured on completion of the 3 h inhibition assays described earlier. Cells were harvested from the batch bioreactors via centrifugation for 20 min at 9,000 rpm (8,324 g). The resulting cell pellet was suspended in 500 μL of DI water and digested overnight at 60°C in 3 mL of an 11.2 N nitric acid and 12% phosphoric acid solution. The digested pellets were diluted twofold in DI water before Ag analysis was conducted via ICP-OES, as described above.

To determine the effect of centrifugation on the AgNP suspensions, abiotic control batch reactors were run for 3 h, as

described above. The abiotic control batch reactors contained 2 ppm AgNPs, 30 mM HEPES buffer (pH 7.8), and an absence of both *N. europaea* cells and (NH₄)₂SO₄. (NH₄)₂SO₄ is a known accelerator of AgNP dissolution and was removed to limit AgNP dissolution to nondetectable rates over the course of a 3-h experiment (Kostigen Mumper *et al.*, 2013). The contents of the abiotic control batch reactors were placed into a centrifuge tube and centrifuged at 9,000 rpm (8,324 g) for 20 min, after which the supernatant was placed into a fresh centrifuge tube.

One milliliter of HEPES buffer (pH 7.8) was added to the centrifuge tube and vigorously shaken and pipetted to dislodge any pellet that may have formed during the centrifugation processes. This is the same procedure that was applied to dislodge the *N. europaea* cell pellet which formed during the biotic tests before placing the cell pellet in the acid digestion mixture. The 1 mL of HEPES buffer (pH 7.8) was then placed into a fresh centrifuge tube.

The initial AgNP concentration of the abiotic control batch reactors along with the AgNP concentration of the supernatant and “cell pellet” was measured via scanning UV-vis spectroscopy from 300 to 1,100 nm, as previously described (Kostigen Mumper *et al.*, 2013; Ostermeyer *et al.*, 2013).

Results

Effect of centrifugation on the AgNP suspensions

Results of the abiotic control batch reactors indicated that 42% ± 5% of the AgNPs pelleted out of solution during the centrifugation process. However, the vast majority of the AgNPs that pelleted out of solution remained immobilized on the plastic centrifuge tube, as only 7% ± 4% of the AgNPs were transferred from the “cell pellet” into a fresh centrifuge tube (Supplementary Fig. S1). The immobilization of the AgNPs to the plastic centrifuge tube is further confirmed by the poor mass balance observed by the AgNP biological tests compared with the excellent mass balance observed during the Ag⁺ biological tests (Supplementary Fig. S2).

Given the high density of AgNPs compared with the density of bacterial cells, our measurements indicated that the AgNPs fell out of solution first, followed by *N. europaea* cells with adsorbed Ag⁺/AgNPs followed by *N. europaea* cells not containing adsorbed Ag⁺/AgNPs. Therefore, during centrifugation, about 40% of the AgNPs not attached to *N. europaea* cells pelleted out of solution first, became immobilized against the plastic centrifuge tube, and were not transferred over with the *N. europaea* cell pellets into the acid digestion vessel of the total silver assay. Thus, our measurements of the Ag⁺/AgNPs associated with cells were not significantly biased by the precipitation of nonadsorbed AgNPs.

Mg²⁺-induced reduction of AgNP toxicity

N. europaea cells exposed to 1 ppm 20 nm citrate AgNPs showed a nonlinear increase in nitrification activity with increasing Mg²⁺ concentrations (Fig. 1). At 0 μM Mg²⁺, exposure to 1 ppm AgNPs reduced nitrification activity to 20% ± 9%; while the addition of 730 μM Mg²⁺ increased nitrification activity to 98% ± 5% of the nonexposed control cells. When 730 μM Mg²⁺ was present in the abiotic reactors, the AgNPs rapidly aggregated and the 3-h dissolution rate decreased from 30% to 9% (Fig. 2). This reduction in AgNP

FIG. 1. Percent nitrification activity of *N. europaea* after 3 h exposure to test media containing 1 ppm 20 nm citrate AgNPs (●) concentrations of Mg²⁺ ranging from 0 to 730 μM (0–17.7 ppm). Error bars represent 95% confidence intervals.

dissolution can help explain how Mg²⁺ protects *N. europaea* cells from AgNP toxicity. However, the protection provided by 730 μM Mg²⁺ was overcome by increasing the AgNP concentrations to 2 and 4 ppm, which resulted in a reduction of nitrification activities to 30% ± 5% and 14% ± 2%, respectively (Fig. 3A).

When Mg²⁺ was absent from the test medium, *N. europaea* cells accumulated higher total silver concentrations as the AgNP concentrations increased (Fig. 3B). When exposed to 0.5 ppm AgNPs, *N. europaea* cells accumulated 7 ± 2 μg of Ag/mg of protein; while when the AgNP concentration was increased to 4 ppm, 46 ± 8 μg of Ag/mg of protein accumulated. Interestingly, when 730 μM Mg²⁺ was present in the test medium, *N. europaea* cells did not accumulate additional total silver when AgNP concentrations increased from 2 to 4 ppm. Rather, the cell-associated silver concentrations were 8 ± 3 and 7 ± 0.5 mg of Ag/mg of protein, respectively (Fig. 3B). However, the overall trend showed that nitrification activity tracked strongly with the amount of total silver associated with the cells for all AgNP concentrations and conditions tested (Fig. 3).

Mg²⁺-induced reduction of Ag⁺ toxicity

N. europaea cells exposed to 0.5 ppm Ag⁺ showed a nonlinear increase in nitrification activity with increasing

FIG. 2. Average diameter of 20 nm AgNPs in test media containing 0 μM Mg²⁺ (○) and 730 μM (17.7 ppm) Mg²⁺ (●). Percent dissolution rates were calculated after 3 h of exposure to the test media.

FIG. 3. Percent nitrification activity of *N. europaea* cells (A) and the amount of adsorbed silver to *N. europaea* cells (B) after 3 h exposures to various concentrations of 20 nm citrate AgNPs in test media containing either 0 μM (0 ppm) Mg^{2+} ($\circ$) or 730 μM (17.7 ppm) Mg^{2+} ($\bullet$). Error bars represent 95% confidence intervals.

Mg^{2+} concentrations (Fig. 4A). At 0 μM Mg^{2+} , exposure to 0.5 ppm Ag^+ reduced nitrification activity to 17%; while the addition of 730 μM Mg^{2+} increased nitrification activity to 67%. The increase in nitrification activity correlated with a decrease in total silver accumulated on the cells (Fig. 4B). At 0 μM Mg^{2+} , *N. europaea* cells accumulated silver to a concentration of 9 ± 1 μg of Ag/mg of protein, while the addition of 730 μM Mg^{2+} decreased the accumulation of silver to 1.5 ± 1.6 μg of Ag/mg of protein.

Similar to AgNPs, increasing the Ag^+ concentration resulted in a decrease in nitrification activity in the presence and absence of 730 μM Mg^{2+} (Fig. 5A). In cell exposures to Ag^+ in medium containing 730 μM Mg^{2+} , the nitrification activity was consistently 20–22% higher compared with cell exposures to Ag^+ in medium not containing Mg^{2+} across all Ag^+ concentrations tested. The increased protection is due to a decrease in Ag^+ adsorbed in the *N. europaea* cells, which was 67–78% lower in exposures containing Mg^{2+} compared with those lacking the Mg^{2+} (Fig. 5B). It is interesting to note the similarities in the results obtained with the AgNP exposures and the Ag^+ exposures, indicating similar inhibition processes.

*Ca*²⁺-induced reduction of Ag^+ and AgNP toxicity

In the presence of 200 μM Ca^{2+} , 20 nm citrate AgNPs aggregated slightly and the 3-h dissolution rate decreased from $30\% \pm 1.6\%$ to $19\% \pm 2\%$ (Fig. 6A). This decrease in dissolution corresponded with a decrease in toxicity with

FIG. 4. Percent nitrification activity ($\blacktriangle$) of *N. europaea* cells (A) and amount of adsorbed silver ($\triangle$) to *N. europaea* cells (B) after 3 h exposures to 0.5 ppm Ag^+ and concentrations of Mg^{2+} ranging from 0 to 730 μM (0–17.7 ppm). Error bars represent 95% confidence intervals.

FIG. 5. Percent nitrification activity of *N. europaea* cells (A) and amount of adsorbed silver to *N. europaea* cells (B) after 3 h exposures to various concentrations of Ag^+ in test media containing either 0 μM Mg^{2+} ($\square$) or 730 μM (17.7 ppm) Mg^{2+} ($\blacksquare$). Error bars represent 95% confidence intervals.

FIG. 6. (A) Average diameter of 20 nm AgNPs in test media containing 200 μM (8 ppm) Ca^{2+} (Δ) and 730 μM (29.2 ppm) Ca^{2+} ($\blacktriangle$). Percent dissolution rates were calculated after 3 h of exposure to the test media. (B) Percent nitrification activity of *N. europaea* cells after 3 h exposures to 0.1 ppm Ag^+ ($\blacksquare$) or 1 ppm 20 nm AgNPs ($\square$) and 0 μM (0 ppm), 200 μM (8 ppm), or 730 μM (29.2 ppm) Ca^{2+} . Error bars represent 95% confidence intervals.

nitrification activity rising from $20\% \pm 9\%$ to $82\% \pm 11\%$ (Fig. 6B).

AgNPs exposed to 730 μM Ca^{2+} rapidly aggregated and the 3-h dissolution rate drastically decreased from $30\% \pm 1.6\%$ to $2\% \pm 1\%$ (Fig. 6A). This extreme decrease in AgNP dissolution corresponded with a complete absence of toxicity with nitrification rates measured at $100\% \pm 5\%$ (Fig. 6B).

Similar to Mg^{2+} , increased Ca^{2+} concentrations resulted in increased nitrification rates in *N. europaea* cells exposed to Ag^+ . The nitrification activity increased from $13.8\% \pm 1\%$ when exposed to 0.1 ppm Ag^+ , to $70.8\% \pm 14.6\%$ when 200 μM Ca^{2+} was present, and to $94.9\% \pm 8.3\%$ when 730 μM Ca^{2+} was present (Fig. 6B).

Discussion

This work demonstrates that Mg^{2+} and Ca^{2+} have a protective effect on *N. europaea* cells exposed to AgNPs with increasing concentrations of these divalent cations, resulting in increased protection (Figs. 1, 3A and 6B). Similar trends have been observed in other gram-negative bacteria exposed to AgNPs and varying concentrations of Mg^{2+} and Ca^{2+} (Marambio-Jones and Hoek, 2010; Zhang and Oyanedel-Craver, 2012; Zhang *et al.*, 2012).

One possible mechanism for the observed protection from AgNP toxicity is the Mg^{2+} - or Ca^{2+} -induced aggregation of the AgNPs, which resulted in significant decreases in the dissolution of Ag^+ (Figs. 2 and 6A) and a decrease in silver associated with the *N. europaea* cells (Fig. 3B). Several studies have previously shown that Mg^{2+} can neutralize the stabilizing electrostatic double layer of AgNPs, which results in aggregation of the AgNPs. The aggregation leads to a reduction in AgNP dissolution rates due to a smaller surface area-to-volume ratio (Jin *et al.*, 2010; Marambio-Jones and Hoek, 2010; Huynh and Chen, 2011; Zhang and Oyanedel-Craver, 2012; Zhang *et al.*, 2012). This reduction in dissolution rates is critical, as the dissolution of Ag^+ has been shown to be responsible for the majority of the inhibition observed in *N. europaea* cells exposed to AgNPs (Radniecki *et al.*, 2011; Arnaout and Gunsch, 2012; Kostigen Mumper *et al.*, 2013).

However, even in a highly aggregated state, AgNPs can still be toxic at high concentrations (≥ 2 ppm), though less toxic than their nonaggregated counterparts at the same concentration (Fig. 3A). It should be noted that AgNP concentrations ≥ 2 ppm are not likely to occur in most natural environments outside of an accidental spill site.

Similar to Mg^{2+} , previous research has demonstrated that Ca^{2+} can induce aggregation of AgNPs through a reduction in the electrostatic double layer, and this aggregation results in a decrease in Ag^+ dissolution as the surface area-to-volume ratio decreases (Huynh and Chen, 2011; Zhang and Oyanedel-Craver, 2012). This was observed in AgNP suspensions exposed to 730 μM Ca^{2+} as they rapidly aggregated, decreasing their dissolution rates by 93% compared with nonaggregated AgNPs (Fig. 6A). This decrease in dissolution eliminated their observed toxicity toward *N. europaea* (Fig. 6B).

Toxicity of the AgNPs was also greatly reduced, by 77% (from 80% inhibition to 18% inhibition), when 200 μM Ca^{2+} was present (Fig. 6B). However, dissolution rates only decreased by 37% (from 30% to 19%) on exposure to 200 μM Ca^{2+} (Fig. 6A). Thus, the observed protection from AgNP-induced inhibition afforded by 200 μM Ca^{2+} cannot be solely explained by a decrease in AgNP dissolution rates.

To investigate what may be causing the observed decrease in toxicity of the AgNPs in the presence of 200 μM Ca^{2+} , the protective potential of Ca^{2+} against Ag^+ toxicity was explored. Similar to the AgNP experiments, increasing concentrations of Ca^{2+} resulted in increased protection from Ag^+ toxicity (Fig. 6B). Since the Ca^{2+} was added as $\text{Ca}(\text{NO}_3)_2$, equilibrium models do not predict a difference in silver speciation between media containing Ag^+ without $\text{Ca}(\text{NO}_3)_2$ compared with media containing Ag^+ with $\text{Ca}(\text{NO}_3)_2$ (Supplementary Table S1). Thus, it is unlikely that the protection observed is related to potential changes in silver speciation.

Further studies were conducted to determine whether Mg^{2+} has a similar protective effect against Ag^+ toxicity. Surprisingly, results from these studies demonstrated that the presence of Mg^{2+} can greatly reduce Ag^+ toxicity toward *N. europaea* with higher Mg^{2+} concentrations, resulting in lower toxicity levels (Fig. 4A). The Mg^{2+} were added to the system, as MgSO_4 and equilibrium models do not predict a difference in silver speciation between media containing Ag^+ with MgSO_4 and media containing Ag^+ without MgSO_4 (Supplementary Table S1). Therefore, similar to the results

with Ca^{2+} protection, the observed protection against Ag^+ toxicity is not likely to be due to changes in silver speciation.

However, the protection mechanism of Mg^{2+} against Ag^+ toxicity becomes more evident when the amount of silver adsorbed to the *N. europaea* cells is measured. Increased Mg^{2+} concentrations led to decreased accumulated silver adsorbed to the *N. europaea* cells with the addition of $730\ \mu\text{M}$ Mg^{2+} resulting in an 83% reduction in adsorbed silver compared with *N. europaea* cells exposed to Ag^+ without Mg^{2+} present (Fig. 4B). It can, therefore, be concluded that this lack of silver accumulation is responsible for the observed protection of Mg^{2+} against Ag^+ toxicity. These results are consistent with our original observation that AgNP toxicity toward *N. europaea* is correlated with the silver adsorbed to the cells (Radniecki *et al.*, 2011).

One possible explanation of the decrease of Ag^+ accumulation in the presence of Mg^{2+} is the reduction of the bacterial membranes' negative surface charge. Gram-negative bacteria have a negative net surface charge due to the negatively charged functional groups of the lipopolysaccharide (LPS) layer (Costerton *et al.*, 1974). Mg^{2+} acts to stabilize bacterial membranes through the bridging of the negative charges of LPS functional groups, including phosphates, phosphatidic acid, and 2-keto-3-deoxyoctulosonic acid (Schindler and Osborn, 1979; Rivera *et al.*, 1988; Amro *et al.*, 2000). Mg^{2+} will also bind to other membrane constituents, including inorganic phosphates and teichoic acids (Heptinstall *et al.*, 1970; Naumann *et al.*, 1989; van Veen, 1997). Thus, the presence of Mg^{2+} has been shown to reduce the net surface charge of gram-negative bacteria (De Kerchove and Elimelech, 2008; Zhang and Oyanedel-Craver, 2012; Zhang *et al.*, 2012). This lower surface charge may reduce Ag^+ interactions with *N. europaea* by decreasing the electrostatic attraction between the positively charged Ag^+ and the negatively charged *N. europaea*.

In addition to reducing attractive electrostatic forces, excess Mg^{2+} may reduce Ag^+ ability to bind to *N. europaea* cells by competing with Ag^+ for these binding spots on these membrane constituents. Excess Mg^{2+} may also reduce Ag^+ ability to enter into the *N. europaea* cells through Mg^{2+} transporting proteins, as Ag^+ have been shown to be capable of entering into cells through Mg^{2+} transporter proteins (Nies, 1999). Previous studies have demonstrated that increased Mg^{2+} will out compete other heavy metals, including Cd^{2+} and Zn^{2+} , for entrance into *N. europaea* cells through these transporters (Radniecki *et al.*, 2009a, 2009b). Thus, higher concentrations of Mg^{2+} may reduce Ag^+ transport through these proteins.

Conclusion

Mg^{2+} and Ca^{2+} are the primary constituents of water hardness and may play a critical role in the fate, transport, and toxicity of AgNPs in both engineered and natural water systems. While it has been previously established that divalent cations can decrease AgNP toxicity by inducing aggregation and decreasing the rate of Ag^+ release, the effect of divalent cations on the toxicity of the released Ag^+ has not been as thoroughly characterized.

The work presented here is among the first to demonstrate that Mg^{2+} and Ca^{2+} utilize multiple mechanisms to protect *N. europaea* from AgNPs and Ag^+ . At relatively high concentrations (e.g., $730\ \mu\text{M}$), both Mg^{2+} and Ca^{2+} induced rapid

aggregation of the AgNP suspensions, which led to highly reduced dissolution rates, which accounted for the vast majority of the protection observed. However, at lower Ca^{2+} concentrations (e.g., $200\ \mu\text{M}$), the AgNP suspensions only slightly aggregated, which led to a moderate decrease in dissolution rates and could not account for the high level of protection observed. This suggested that the Ca^{2+} are not only decreasing the dissolution rates of the AgNPs but also reducing the toxicity of the dissolved Ag^+ which are released.

Both Ca^{2+} and Mg^{2+} were shown to be able to protect *N. europaea* from Ag^+ toxicity. Mg^{2+} , specifically, was shown to reduce Ag^+ adsorption to the *N. europaea* cells. This reduction in silver accumulation could account for the observed decrease Ag^+ toxicity. Therefore, these studies suggest that Mg^{2+} and Ca^{2+} can have an important impact on the ultimate fate and toxicity of the dissolved Ag^+ from AgNPs and should be carefully considered during AgNP toxicity testing.

Based on the results of this study, it can be expected that in hard water environments, the toxicity of AgNPs and dissolved Ag^+ would be significantly muted compared with soft water environments. In addition, in hard water environments lacking stabilizing silver ligands (e.g., natural organic matter), AgNPs would be expected to aggregate and begin to fall out of solution. This may have important implications in determining the fate and toxicity of AgNPs in WWTPs, where AgNPs may precipitate out of solution in primary clarifiers, or attach to microbial biomasses in the secondary clarifier, thus limiting their release from the WWTP through the effluent.

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Author Disclosure Statement

No competing financial interests exist for the authors of this work.

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