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IMPACTS OF SILVER NANOPARTICLES ON CELLULAR AND TRANSCRIPTIONAL ACTIVITY OF NITROGEN-CYCLING BACTERIA

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Abstract: The widespread use of silver nanoparticles (AgNPs) raises the potential for environmental releases that could impact microbial ecosystem services. In the present study, the authors address how the AgNPs and Ag^+ that they release may impact nitrogen-cycling bacteria. The authors studied the cellular and transcriptional response of the denitrifier *Pseudomonas stutzeri*, the nitrogen fixer *Azotobacter vinelandii*, and the nitrifier *Nitrosomonas europaea* exposed to 35 nm (carbon-coated) AgNPs or to Ag^+ (added as $AgNO_3$). Based on minimum inhibitory concentrations (MICs), Ag^+ was 20 times to 48 times more toxic to the tested strains than AgNPs (including Ag^+ released during exposure). Exposure to sublethal concentrations of AgNPs or Ag^+ (representing 10% of the respective MIC for $AgNO_3$) resulted in no significant effect on the expression of the denitrifying genes *narG*, *napB*, *nirH*, and *norB* in *P. stutzeri* or the nitrogen-fixing genes *nifD*, *nifH*, *vnfD*, and *anfD* in *A. vinelandii*, whereas nitrifying genes (*amoA1* and *amoC2*) in *N. europaea* were upregulated (2.1- to 3.3-fold). This stimulatory effect disappeared at higher silver concentrations (60% of the Ag^+ MIC), and toxicity was exerted at concentrations higher than 60% of the Ag^+ MIC. The MIC for *N. europaea* was 8 times to 24 times lower than for the other strains, indicating higher susceptibility to AgNPs. This was corroborated by the lower half-lethal concentration for *N. europaea* (87 μg/L) compared with *P. stutzeri* (124 μg/L) and *A. vinelandii* (>250 μg/L) when cells were exposed with Ag^+ for 24 h in 1 mM bicarbonate buffer. This suggests that ammonia oxidation would be the most vulnerable nitrogen-cycling process in wastewater treatment plants receiving AgNPs and in agricultural soils amended with biosolids that concentrate them. *Environ Toxicol Chem* 2013;32:1488–1494. © 2013 SETAC

Keywords: Silver nanoparticle Nitrogen cycle Dissolution Aggregation Gene expression

INTRODUCTION

Antimicrobial silver nanoparticles (AgNPs) are the most widely used engineered nanomaterials [1]. They are commonly incorporated into a wide variety of commercial goods (e.g., personal care products, food containers, laundry additives, clothing, paintings, and home appliances [2–4]) and are also used for water treatment, drug and gene delivery, bone prostheses, implantable materials, biosensors, and bioimaging devices [1,5–7]. The widespread use of AgNP-containing products raises the likelihood of accidental or incidental environmental releases that could impact microbial ecosystem services (e.g., biogeochemical cycling). Based on probabilistic material flow analysis in the United States, AgNP concentrations are predicted to be on the order of 0.0001 μg/L in surface waters, 9 μg/L in wastewater treatment plants (WWTPs) (mainly sulfidized forms [8,9]), and 1550 μg/L in sewage sludge [10–12], although higher concentrations should be expected near release points. Whether such concentrations pose a threat to nitrogen cycling in natural or engineered systems (e.g., WWTPs) is an outstanding question of great relevance to ecosystem health and to sustainable nanotechnology.

Several studies have reported inhibition of nitrification upon exposure to 100 μg/L to 1000 μg/L of various types of AgNPs [13–16]. Recent studies have investigated the toxicity of AgNPs with different surface coatings to the nitrifier *Nitrosomonas europaea*, and an increase in heavy metal stress response was observed [15]. Some studies have also reported toxic effects of AgNPs on soil bacteria involved in denitrification and nitrogen fixation [17–19]. Nevertheless, little research has been conducted on how nitrogen-cycling bacteria respond to AgNPs at the molecular level and the resulting impact on the associated metabolic pathways. This underscores the need for more fundamental insight on the relative sensitivity and transcriptomic response of such ecologically important microorganisms to AgNPs and the silver ions (Ag^+) that they release.

We previously investigated the transcriptomic response of nitrogen-cycling bacteria to quantum dots and reported a potential stimulatory effect of sublethal quantum dot concentrations on the N cycle [20,21]. Here, we extend that work to consider interactions of AgNPs and Ag^+ with 3 model N-cycling bacteria: the denitrifier *Pseudomonas stutzeri*, the nitrogen fixer *Azotobacter vinelandii*, and the nitrifier *N. europaea*. These bacteria were chosen because they are relatively well understood at the physiological and genetic levels (Figure 1) [22–24] and are commonly present in both natural and engineered systems. We compare the relative susceptibility of these bacteria to silver and characterize the expression of functional genes involved in nitrogen fixation, nitrification, and denitrification following exposure to sublethal concentrations of AgNPs and Ag^+.

MATERIALS AND METHODS

AgNP preparation and characterization

The AgNPs (35 nm nominal size, with amorphous carbon coating) were purchased from Novacentrix and stored in an

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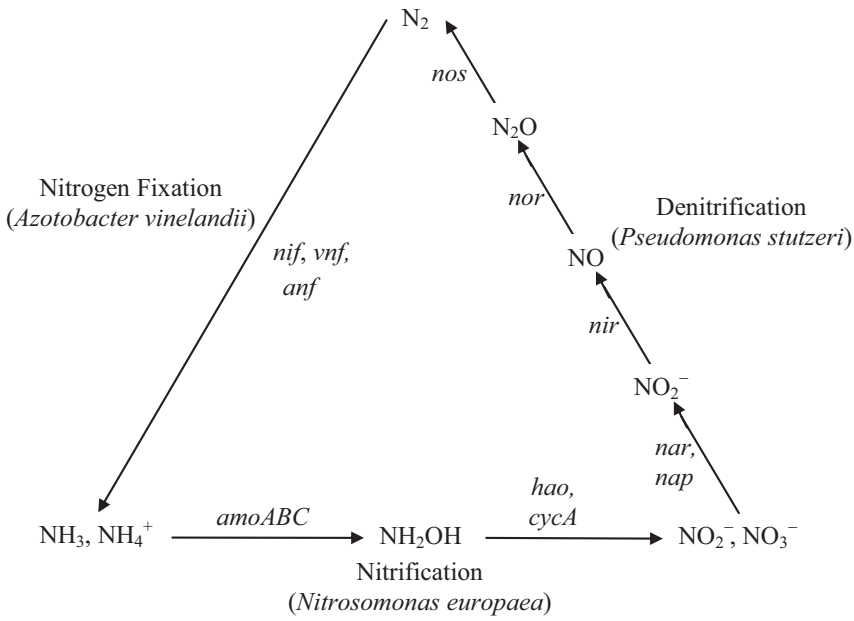

Figure 1. Model nitrogen cycling bacteria and functional genes considered in this study.

anaerobic chamber. A stock solution (5 g/L) was prepared by dispersing AgNPs in MilliQ water. The AgNPs were added as needed to the test media to achieve the desired exposure concentrations. The size distribution and zeta potential were measured by a Zen 3600 Zetasizer Nano (Malvern Instruments). The average hydrodynamic diameter in distilled water was 35.4 ± 5.1 nm, and the zeta potential was −34.10 ± 0.57 mV.

Micro-organisms and chemicals

Bacteria were purchased from the American Type Culture Collection (ATCC). Model bacteria used for transcriptomic analyses of N-cycling processes were *P. stutzeri* (ATCC 17588) for denitrification, *A. vinelandii* (ATCC 13705) for nitrogen fixation, and *N. europaea* (ATCC 19718) for nitrification. The corresponding growth media composition and conditions are described in the Supplemental Data.

Minimum inhibitory concentration measurements

The minimum inhibitory concentration (MIC) is the lowest concentration of an antibacterial agent that inhibits visible growth of a bacterium [25]. The MIC of AgNPs or Ag^+ was measured for each bacterium as described previously [26,27]. Briefly, sterile test tubes containing 1 mL of strain-specific growth medium and serial dilutions of AgNPs or Ag^+ were prepared in triplicate, inoculated with the bacteria, and incubated under aerobic conditions. We incubated *P. stutzeri* overnight at 37 °C, *A. vinelandii* for 2 d at 26 °C, and *N. europaea* for 7 d at 26 °C. The tubes were then visually inspected for turbidity development. Controls (bacteria only) and blanks (broth only) were also prepared in triplicate.

Release of Ag^+ from AgNPs and equilibrium speciation modeling

To quantify the released Ag^+ concentration from the MIC of AgNPs, the supernatant of different broths was separated by ultracentrifugation (11 500 × *g* for 3.5 h) [21,28] after 1 d of suspension in sterile *P. stutzeri* broth, 2 d for *A. vinelandii* broth, and 7 d for *N. europaea* broth. The total dissolved Ag^+ concentrations were measured by inductively coupled plasma-mass spectrometry using an Elan 9000 instrument (Perkin-Elmer). Speciation modeling was constructed with Visual MINTEQ, version 3.0, to assess the likelihood of metal precipitation and estimate the chemical species prevailing at equilibrium in solution. The Stockholm humic model was used to assess potential carbon source–metal interactions in the microbial broth with poorly defined organic substrates (i.e., *P. stutzeri* and *A. vinelandii* media) [29].

Measurement of reactive oxygen species

Intracellular reactive oxygen species (ROS) production was investigated as a potential cellular response to AgNPs. For an opaque 96-well plate, 200 μL of *P. stutzeri*, *A. vinelandii*, or *N. europaea* suspension (OD_{600} = 0.001–0.005) was transferred to each well, after being washed twice and resuspended in phosphate-buffered saline buffer [30]. Blank controls were amended with the same volume of phosphate-buffered saline buffer and treated in the same way. Bacteria were then exposed to AgNPs (50 μg/L and 500 μg/L) for 1 h, and a positive control was treated with H_2O_2 (100 μM). One microliter of dichlorodihydrofluorescein diacetate (H_2DCFDA, 4 mM in dimethyl sulfoxide) was subsequently added to each well, and fluorescence was measured with an Infinite M1000 fluorometer (Tecan Systems) at an excitation wavelength of 495 nm and an emission wavelength of 525 nm after 30-min incubation. Negative controls with AgNPs and H_2DCFDA alone (no cells) were prepared to ensure that H_2DCFDA did not yield false-positives, and AgNP fluorescence background was subtracted from the signals [31]. Bacterial controls without AgNPs were also prepared to provide a baseline for signals from different treatment samples. All samples were replicated at least 3 times.

Effect of AgNPs on gene expression

For the transcriptomic analysis, bacteria were exposed to sublethal concentrations of AgNPs or $AgNO_3$. The total silver concentration of either AgNPs or Ag^+ selected for each bacterium was 10% of the corresponding $AgNO_3$ MIC (i.e., 2.5 μg/L for *N. europaea*, 20 μg/L for *P. stutzeri*, and 25 μg/L for *A. vinelandii*). Furthermore, gene expression was investigated at 60% of the Ag^+ MIC (15 μg/L) for *N. europaea*. The

housekeeping gene *gapA*, encoding D-glyceraldehyde-3-phosphate dehydrogenase, was chosen as an internal standard for each bacterium [32]; and its expression was consistent among the control and the different treatments. Gene expression was quantified using reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) as described previously [20]. For *P. stutzeri*, expression of the superoxide dismutase gene *sodB*, the membrane nitrate reductase gene *narG*, the periplasmic nitrate reductase gene *napB*, the nitrite reductase gene *nirH*, the NO reductase gene *norB*, and the metal efflux gene *czcC* was quantified. For *A. vinelandii*, transcriptional levels of the nitrogenase genes *nifD*, *nifH*, *vnfD*, and *anfD*; the metal transcriptional regulatory gene *cadR*; and the superoxide dismutase gene *sodA* were determined. We also quantified expression of the ammonia monooxygenase genes *amoA1*, *amoB2*, and *amoC2*, which catalyze the oxidation of ammonia (NH_3) to hydroxylamine (NH_2OH) [33,34], as well as the hydroxylamine oxidoreductase gene *hao2* and the superoxide dismutase gene *sodB* in *N. europaea* [35]. Bacteria were collected at mid-log phase for RNA extraction. Bacteria were centrifuged at 2300 × g for 10 min and resuspended in RNAprotect Bacteria Reagent (Qiagen). Cells were treated with 3000 mg/L lysozyme in Tris/ethylenediamine tetraacetic acid buffer for 10 min, and RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Concentrations of RNA were determined by Nanodrop ND-1000 (Nanodrop Products). The cDNA was synthesized overnight at 42 °C by RT-PCR of RNA (2–5 μg) using random primers, RNaseOUT, dNTPs, and Superscript II reverse transcriptase (Invitrogen). Purification of cDNA was performed with a QIAquick PCR Purification Kit (Qiagen) using the manufacturer's instructions. The primers were designed using PrimerQuest (http://www.idtdna.com/Scitools/Applications/Primerquest/ Default.aspx), and their sequences are listed in Supplemental Data, Table S1. Quantitative PCR was performed using a 7500 real-time PCR system from Applied Biosystems in 15 μL of reaction mixture composed of 1 ng cDNA, SYBR Green Master Mix (7.5 μL), 0.3 μM of each primer, and water. The C_t values (cycle threshold) were calculated with SDS 1.3.1 (Applied Biosystems), and the $2^{-\Delta\Delta CT}$ method was used to determine relative gene expression [36]. All treatments were run in triplicate, and each sample was prepared in triplicate during PCR test.

RESULTS AND DISCUSSION

Susceptibility of nitrogen-cycling bacteria to Ag^+ and AgNPs

In all tested bacteria, Ag^+ was more toxic than AgNPs, with MIC values 20-fold to 48-fold lower (Table 1). *Nitrosomonas europaea* was significantly more susceptible to silver (8-fold to 24-fold lower MICs) than *P. stutzeri* and *A. vinelandii* (Table 1 and Supplemental Data, Figure S1). We recognize that differences in the composition of the growth media used in the MIC assays (to meet the nutritional and physiological requirements of different bacteria) could result in differences in AgNP aggregation and precipitation and in Ag^+ release. This can confound the interpretation of microbial toxicity data. Nevertheless, conducting growth assays under the same conditions would have introduced significant bias because (heterotrophic) denitrifying bacteria and nitrogen-fixing bacteria will not grow in media optimized to autotrophic nitrifying bacteria and vice versa. Therefore, additional experiments were conducted to discern the relative susceptibility of the model bacteria to silver by exposing resting cells to Ag^+ for 24 h in 1 mM bicarbonate buffer without growth substrates, as described in the Supplemental Data. This experiment corroborated the higher susceptibility of *N. europaea* under equal exposure conditions, which exhibited a smaller half-lethal concentration for Ag^+ (87 μg/L) than *P. stutzeri* (124 μg/L) and *A. vinelandii* (>250 μg/L; Supplemental Data, Figure S2).

Nitrifiers have also been reported to be relatively susceptible to ZnO NPs and organic xenobiotics [37–39]. Whether the high sensitivity of nitrifiers to potential chemical stressors is due to their relatively low margin for energy harvesting associated with their chemolithoautotrophic metabolism or to a physiologic limitation to withstand such stress remains to be determined [40,41]. Regardless of the underlying mechanisms, the relatively high susceptibility of nitriers to AgNPs has important implications for water quality. Hindrance of ammonium oxidation would enhance nitrogen retention in soil (because cationic ammonium adsorbs to a greater extent onto negatively charged soil surfaces than anionic nitrite and nitrate) and decrease water pollution through agricultural drainage [42]. However, nitrification inhibition by AgNPs would hinder biological nitrogen removal in WWTPs [14].

Microbial transcriptional response to AgNPs

Exposure to a low, sublethal concentration of Ag^+ or AgNPs (25 μg L^{-1} for *A. vinelandii* and 20 μg L^{-1} for *P. stutzeri*, representing 10% of their respective $AgNO_3$ MIC, as total silver) had no significant effect on the expression of denitrifying genes or nitrogen-fixing genes, probably due to their higher tolerance to silver. Although the monitored metal-resistance genes *czcC* in *P. stutzeri* and *cadR* in *A. vinelandii* were not overexpressed, these strains may have exhibited other resistance mechanisms. For example, *P. stutzeri* often contains plasmids encoding silver resistance [43] and has also been reported to crystallize Ag^+ (50 mM) into intracellular particles of reduced toxicity [44], whereas *A. vinelandii* produces extracellular polymeric substances that restrict uptake of metal ions and immobilize them [45].

In *N. europaea*, a similar relative dose of AgNPs or Ag^+ (2.5 μg/L, also representing 10% of $AgNO_3$ MIC) upregulated

Table 1. Minimum inhibitory concentrations (MICs) and average aggregate sizes of AgNPs in various microbial growth media

Species	AgNPs		Ag^+ MIC (μg/L)	Ionic strength (μS/cm) and organic carbon source (g/L)
	MIC (μg/L)	Average Ag aggregate size (nm)		
Pseudomonas stutzeri (denitrification)	4000	459 ± 21[a]	200	1192.5 ± 3.5, peptone (5) beef extract (3)
Azotobacter vinelandii (nitrogen fixation)	12 000	826 ± 76[a]	250	1019.5 ± 0.7, sucrose (20) yeast extract (3)
Nitrosomonas europaea (nitrification)	500	458 ± 148[a]	25	5515.0 ± 21.2, no organic carbon source

[a]Significant increase compared with the original size in distilled water ($p < 0.05$).

the ammonia monooxygenase genes *amoA1* (2.7- to 3.5-fold) and *amoC2* (1.9- to 2.5-fold; Figure 2C). Low silver doses (5 μg/L) were also reported to increase microbial viability [46], and sublethal levels of other nanomaterials, such as QDs, ZnO, and TiO_2, have also exerted stimulatory effects [20,47,48]. Whether this stimulation is a response to increase respiration rates and obtain more energy to repair damage or overcome stress remains to be determined. Nevertheless, a stimulatory effect was not observed at 60% of the Ag^+ MIC (15 μg/L) (Supplemental Data, Figure S3). Higher levels of AgNPs (1000 μg/L) are known to repress ammonia mono-oxygenase and hydroxylamine oxidoreductase in *N. europaea* [49]. Furthermore, silver concentrations on the order of 300 μg/L were reported to inhibit nitrification in WWTPs [13]. Such potential inhibition is a concern not only for wastewater treatment efficiency but also for the fertility of soils amended with WWTP biosolids, which have been reported to contain up to 856 mg/L of total silver [50].

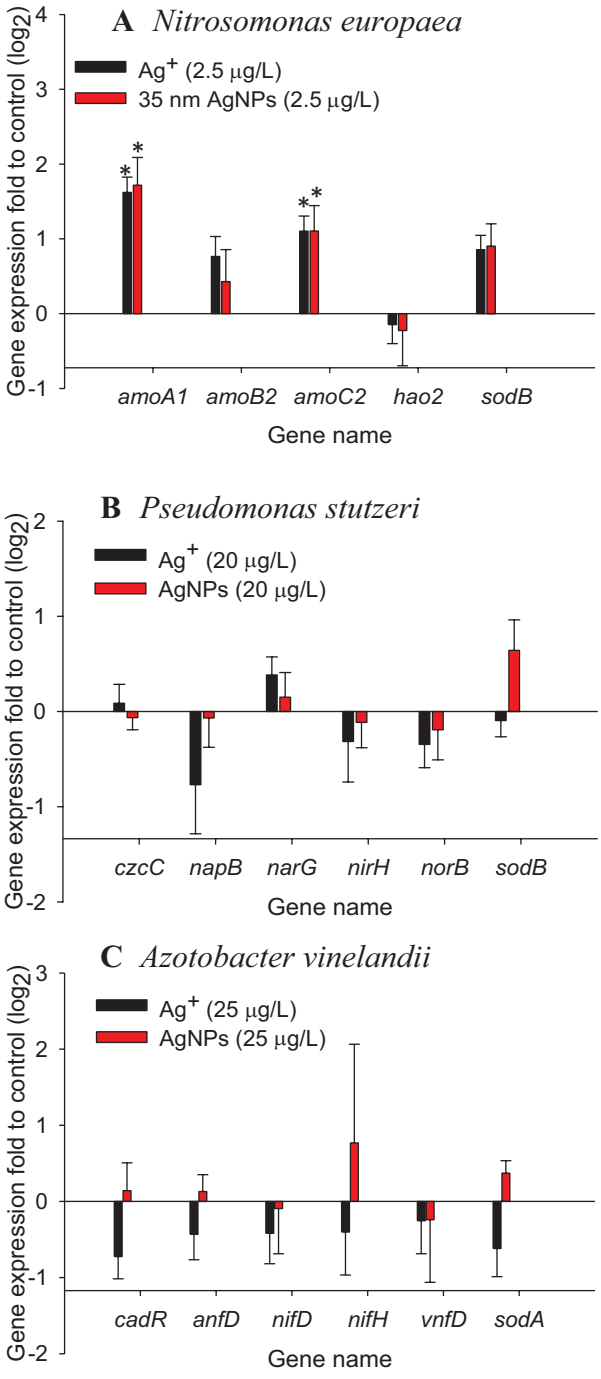

Figure 2. Expression of nitrogen cycling and stress response genes during exposure to silver nanoparticles (AgNPs) or Ag^+ (at 10% of the minimum inhibitory concentration for Ag^+). Asterisk indicates significant upregulation compared with housekeeping gene *gapA* ($p < 0.05$). Error bars represent ± 1 standard deviation from the mean of triplicate measurements. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

Release of Ag^+ from AgNPs

The Ag^+ released from AgNPs was recently demonstrated to be the critical effector of antibacterial activity [46], with AgNPs offering a potentially more effective means of Ag^+ delivery to cells when Ag^+ bioavailability is hindered by complexation or precipitation with common ligands (e.g., chloride, sulfide, phosphate, and organic matter) [51]. Therefore, the Ag^+ released during the toxicity assays was monitored and its equilibrium speciation modeled (Table 2). The highest dissolution (18.9%) of AgNPs was observed in *N. europaea* broth (4.7–6.3 times higher than the other 2 broths), and AgNP dissolution in *N. europaea* broth gradually increased with exposure time (Supplemental Data, Figure S4). The longer exposure time (7 d compared with 2 d for *A. vinelandii* and 1 d for *P. stutzeri*) and relatively high ionic strength in *N. europaea* likely contributed to higher Ag^+ release [52,53].

Based on Visual MINTEQ simulations [54,55], most of the released Ag^+ ions would form complexes with organic matter in the bacteria media, and Ag precipitation was unlikely (Table 2). For example, $Ag(NH_3)_3^+$ would prevail in *N. europaea* broth, whereas most Ag^+ ions (81–88%) would combine with organic matter in *P. stutzeri* and *A. vinelandii* broths. No Ag^+ was detected in *P. stutzeri* and *A. vinelandii* broth after filtration with 10-kDa filters (EDM Millipore). This suggests that Ag^+ was complexed by organic matter with a molecular weight of 10 kDa or higher and corroborates the tendency for Ag^+ to associate with organic matter as predicted by speciation modeling (Table 2). The bioavailability and toxicity of released Ag^+ decrease after it combines with ligands or organic matter [51,56]. Yet, MIC data indicated that Ag^+ still exerted higher toxicity than equivalent AgNP concentrations, probably due to slow dissolution.

Aggregation of AgNPs in different types of broth was assessed since smaller aggregates offer higher surface areas and result in faster dissolution [57]. Aggregation of AgNPs was observed in all 3 types of broth (Figure S5), and dynamic light-scattering analysis showed that the aggregate sizes ranged from 458 nm to 826 nm (Table 1). Although low concentrations of organic matter (e.g., 100 mg/L) have been reported to stabilize AgNPs by steric or electrostatic interaction [56], both organic matter (varying from 8000–23 000 mg/L) and inorganic salts in the *P. stutzeri* and *A. vinelandii* media were separately found to promote AgNP aggregation in these experiments (Supplemental Data, Table S2). The smallest AgNP aggregates (458 nm) were observed in *N. europaea* broth (which lacks organic matter), even though this medium contained the highest ionic strength. The ionic strength of the solution is known to contribute to AgNP aggregation [58].

Assessment of ROS induction as toxicity mechanism

Induction of ROS is commonly reported to be a toxicity mechanism for nanoparticle exposure [13]. We previously observed ROS induction in nitrogen-cycling bacteria exposed to

Table 2. Measured (released) Ag^+ concentrations and simulated equilibrium speciation in different broths amended with silver nanoparticles (AgNPs)

Medium and AgNP concentration (μg/L)		Extent of AgNP dissolution (%) and released Ag^+ concentrations (μg/L)	Relative abundance of dissolved species (%) and their concentrations (μg/L)						
			Ag^+-DOM	$AgCl_2^-$	$AgNO_3$	$Ag(NH_3)_3^+$	$AgNH_3^+$	Ag^+	AgCl (aq)
Pseudomonas stutzeri broth	4000	4.0% (159.8 ± 26.7)	88.0% (140.6)	—	0.1% (0.2)	—	—	11.9% (19.0)	—
Azotobacter vinelandii broth	12 000	3.0% (360.2 ± 29.1)	80.7% (290.7)	3.8% (13.7)	—	—	—	3.1% (11.2)	12.3% (44.3)
Nitrosomonas europaea broth	500	18.9% (94.5 ± 2.4)	—	—	—	98.1% (92.7)	1.7% (1.6)	0.1% (0.1)	0.1% (0.1)

quantum dots [20]. However, no ROS induction was detected when these bacteria were exposed to AgNPs (up to 500 μg/L for 1 h) under aerobic conditions (Figure 3). To validate the test, H_2O_2 (100 μM) was used as a positive control. Oxidative stress and ROS damage can be mitigated by some enzymes (e.g., superoxide dismutase coded by *sod*) that are induced by H_2O_2 and intracellular ROS [59,60]. Yet, no upregulation of *sod* was observed, consistent with the lack of ROS detection (Figure 2). Although ROS induction by AgNPs and Ag^+ has been reported [61,62], ROS concentrations were not measured in these studies. Nitrification inhibition by AgNPs was also claimed to correlate with intracellular ROS [13], but it is uncertain whether the ROS was produced by nitrifying bacteria or other micro-organisms in the same culture. Our fluorescence (ROS) and transcriptomic analyses rule out ROS induction as a mechanism for acute toxicity of AgNPs to N-cycling bacteria.

IMPLICATIONS AND CONCLUSIONS

The widespread use, high release potential, and strong antimicrobial activity of AgNPs underscore their potential to impact microbial ecosystem services. The present study suggests that nitrification may be the most vulnerable microbial process of the nitrogen cycle, although mild stimulatory effects might also result upon exposure to a narrow range of sublethal AgNP concentrations. Adverse effects to N cycling could be exerted in systems where AgNPs accumulate at milligram-per-liter levels, such as in WWTPs that serve as common sinks for urban releases or in agricultural soils amended with associated biosolids. If AgNP concentrations reach inhibitory levels, a decrease in nitrification activity would likely occur before other nitrogen-cycling processes are impacted. This would hinder nitrogen removal and treatment efficiency in WWTPs and possibly hinder beneficial microbial–plant interactions in agricultural fields. Overall, localized impacts on nitrogen cycling could affect soil fertility, water quality, and ecosystem productivity, which underscores the importance to mitigate and intercept incidental and accidental releases of AgNPs and their associated silver ions.

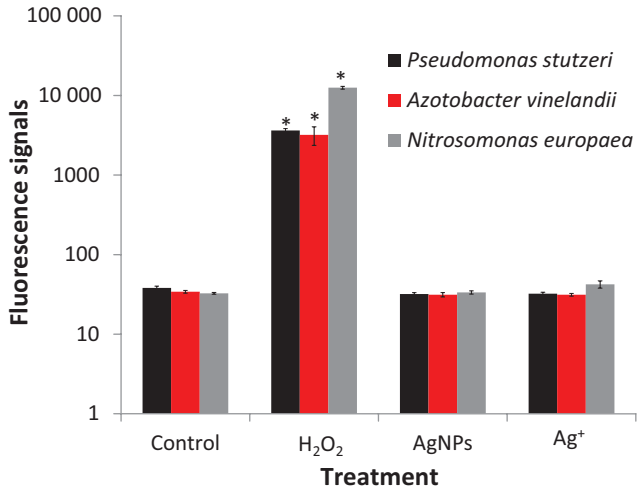

Figure 3. Intracellular reactive oxygen specie (ROS) generation in *Pseudomonas stutzeri*, *Azotobacter vinelandii*, and *Nitrosomonas europaea* after exposure to 500 μg/L silver nanoparticles (AgNPs) and Ag^+. We used H_2O_2 (100 μM) as a positive control. Asterisks indicate significant ROS production compared to unexposed control samples ($p < 0.05$). [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

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SUPPLEMENTAL DATA

Table S1 and S2.
Figure S1 to S5. (427 KB DOC).

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