Protection of *Nitrosomonas europaea* from Silver-Induced Inhibition

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Introduction

Silver nanoparticle (Ag NP) usage as a biocide in consumer goods (clothing, *etc.*) has increased drastically since 2006 (1). Research has indicated that some leeching may occur when these articles are used or cleaned (2, 3). This leads to Ag NP in wastewater treatment plant (WWTP) influent and WWTP processes. Ammonia oxidizing bacteria (AOB) perform a very important step of WWTP processes, the oxidation of ammonia (NH_3) to nitrite (NO_2^-), and have been shown to be inhibited by a variety of substances, including Ag NP (4).

This presented research studied Ag-NP-induced inhibition of *Nitrosomonas europaea*, a model AOB, in a variety of aquatic chemistries. Five types of silver were used as inhibitors: Ionic silver (Ag^+), 20 nm citrate-stabilized BioPure Ag NP, 20 nm and 80 nm phosphate-stabilized Ag NP and PVP-stabilized 3-5 nm Ag NP (nanoComposix Inc., San Diego, CA). Protection from nitrification inhibition by growth media trace metals were studied as well.

This research examined intracellular potassium (K^+) release in the presence of Ag^+ and Ag NP. K^+ release indicates a destabilization in the integrity of the cell membrane. Additionally Ag NP stability and aggregation were measured in abiotic experiments.

Materials and Methods

Inhibition of *N. europaea* by Ag^+ and Ag NP

To create stable solutions of dilute Ag NP in media with high ionic strength, Ag NP were dispersed in distilled deionized water (DDI H_2O) in test bottles shaken at 250 rpm. After 30 min, a concentrated test media solution was added, to final concentrations of 30 mM HEPES (pH 7.8) and 2.5 mM $(NH_4)_2SO_4$, and shaken again for 30 min. *N. europaea* cells were added to a concentration of ~7 mg/L and shaken at 250 rpm and 25˚C for 3 h. Treatment bottles received varying concentrations of Ag^+, 3-5 nm PVP-stabilized Ag NP, 20 nm citrate-stabilized Ag NP, or 20 nm or 80 nm phosphate-stabilized Ag NP. NO_2^- samples were recorded every 45 min using a colorimetric assay.

Ag NP Dissolution in Test Media

Leeching of Ag^+ from Ag-NP in test media (30 mM HEPES (pH 7.8), and 2.5 mM $(NH_4)_2SO_4$) was measured at varying concentrations of Ag NP. After shaking diluted concentrations of Ag NP for 3 h at 250 rpm, the solutions were filtered using either ultracentrifugation (5kDa pore volume) or a 20 nm syringe filter (for the 80 nm Ag NP) and Ag^+ concentrations were measured both in the unfiltered solution and the filtrate using the ICP-EOS.

K^+ release in the Presence of Ag^+ and Ag NP

K^+ release was measured by shaking control and treatment bottles for 3 h at 250 rpm and then followed by a pelleting of the cells. K^+ in the supernatant and in the pelleted cells (dissolved for 18 h in 3 N HNO_3 was measured using the ICP-EOS.

Stability and Aggregation of Ag NP in Test Media

Ag-NP stability and aggregation were measured with dynamic light scattering (DLS) in the presence of various test medias. Average hydrodynamic radius of Ag-NP in suspension were measured for 1 h.

Results

Nanoparticle Dissolusion

Figure 1

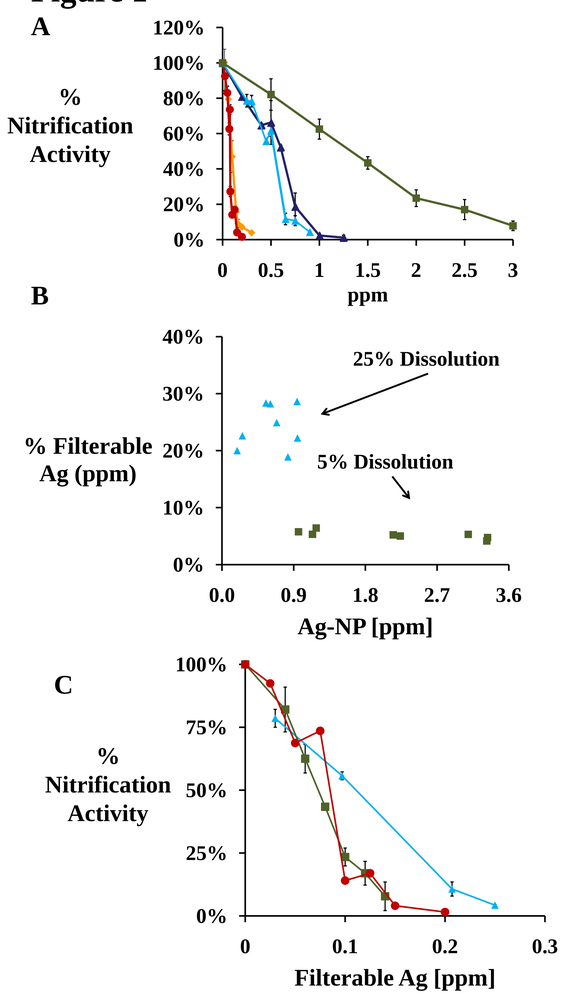

Figure 1. **(A)** *N. europaea* sensitivity to Ag^+ (●), 3-5 nm PVP-stabilized Ag NP (♦), 20 nm citrate-stabilized Ag NP (▲), 20 nm (▲) and 80 nm phosphate-stabilized Ag NP (■). **(B)** % Dissolution of 20 nm (▲) and 80 nm (■) phosphate-stabilized Ag NP. **(C)** *N. europaea* inhibition vs. Ag^+ released from Ag NP.

Intracellular Potassium Release

Figure 2

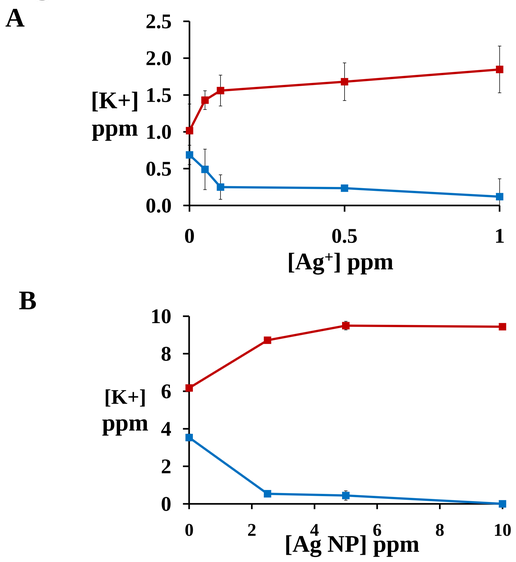

Figure 2. **(A)** K^+ release in *N.* europaea vs. [Ag^+] **(B)** K^+ release vs [Ag NP]. ■ = intracellular K^+, and ■ = extraceullular K^+.

Trace Metal-Induced Protection

Figure 3

Trace Metals	Treatment: Ag+ (0.1ppm)	Treatment: 3-5 nm Ag NP (0.14ppm)	Treatment: 20 nm Ag NP(0.75ppm)
No trace metals	13	16	19
All trace metals	100	100	100
730 µM $MgSO_4$	100	76	98
200 µM $MgSO_4$	65	78	65
730 µM $CaCl_2$	100	74	90
200 µM $CaCl_2$	63	35	88
400 µM NaCl	52	33	49
1460 µM NaCl	82	35	69
9.9 µM $FeSO_4$	45	56	55
0.65 µM $CuSO_4$	39	16	45

Figure 3. % Activity of treated cells vs. controls (30mM HEPES (pH 7.8), and 2.5mM $(NH_4)_2SO_4$, and added aquatic constituents).

Ag NP Aggregation

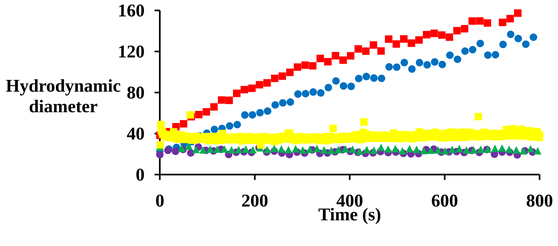

Figure 4. Average hydrodynamic diameter of: Ag-NP in DDI H_2O (▲), in the presence of 2.5 mM $(NH_4)_2SO_4$ and 30 mM HEPES (pH 7.8), 9.9 µM FeSO4, and 0.65 µM CuSO4 (■), 730 µM MgSO4 (■), 730 µM $CaCl_2$ (●), and 200 µM $CaCl_2$ (●).

Conclusions

1. Smaller Ag NP are more inhibitory than larger Ag NP, and may be just as inhibitory as Ag^+ (Figure 1A). This inhibition largely comes from Ag^+ that have leeched from the Ag NPs (Figures 1B & 1C).
2. *N. europaea* cell membranes can be destabilized by exposure to Ag^+ and Ag NP, as shown by intracellular K^+ release (Figure 2A & 2B).
3. *N. europaea* can be protected from Ag^+ and Ag NP-induced inhibition to an extent by various aquatic constituents, most reliably by Mg^{2+} and Ca^{2+} (Figure 3).
4. Mg^{2+} and Ca^{2+}-induced protection from Ag NP occurs in two ways:
   1. Mg^{2+} and Ca^{2+} induces aggregation of Ag NP in the presence of Mg^{2+} and Ca^{2+} (Figure 4) and thus reduces the amount of Ag^+ released due to a larger surface area to volume ratio
   2. Mg^{2+} and Ca^{2+} also protected *N. europaea* from the released Ag^+ througha hypothesized competition with Ag^+ for passage into the cell and for placement into Ag^+/Mg^{2+}/Ca^{2+} binding sites on various cellular components (*e.g.* proteins, fatty acids, *etc.*).

References

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