Silver nanoparticles (AgNPs) are the most common materials in nanotechnology-based consumer products globally. Because of the wide application of AgNPs, their potential environmental impact is currently a highly topical focus of concern. Nitrification is one of the processes in the nitrogen cycle most susceptible to AgNPs but the specific effects of AgNPs on nitrification in aquatic environments are not well understood. We report the influence of AgNPs on nitrification and associated nitrous oxide \((N_2O)\) production in estuarine sediments. AgNPs inhibited nitrification rates, which decreased exponentially with increasing AgNP concentrations. The response of nitrifier \(N_2O\) production to AgNPs exhibited low-dose stimulation (<534, 1476, and 2473 \(\mu g\) liter\(^{-1}\) for 10-, 30-, and 100-nm AgNPs, respectively) and high-dose inhibition (hormesis effect). Compared with controls, \(N_2O\) production could be enhanced by >100% at low doses of AgNPs. This result was confirmed by metatrascriptome studies showing up-regulation of nitric oxide reductase (norQ) gene expression in the low-dose treatment. Isotopomer analysis revealed that hydroxylamine oxidation was the main \(N_2O\) production pathway, and its contribution to \(N_2O\) emission was enhanced when exposed to low-dose AgNPs. This study highlights the molecular underpinnings of the effects of AgNPs on nitrification activity and demonstrates that the release of AgNPs into the environment should be controlled because they interfere with nitrifying communities and stimulate \(N_2O\) emission.
is known about the effects of AgNPs on nitrification rates and the associated production of N₂O, which is a greenhouse gas, in aquatic environments. Nitrification is a critical process for the balance of reduced and oxidized nitrogen pools in the environment, linking mineralization to nitrogen loss pathways of denitrification and anaerobic ammonium oxidation (anammox) (24–26). The slow growth of nitrifiers and their high sensitivity to environmental perturbations often result in cell growth inhibition by toxicants, including AgNPs (4). Nitrification is an important pathway for N₂O production in the environment (27, 28). During nitrification, N₂O can be produced either as a by-product of hydroxylamine (NH₂OH) oxidation (NH₂OH → NOH → N₂O₂) or as an end product or intermediate product of nitrite (NO₂⁻) reduction via nitrifier denitrification (29, 30). N₂O has a >300-fold stronger effect on climate warming than carbon dioxide (CO₂) and can react with ozone in the stratosphere (30, 31). Stress from low oxygen can induce nitrifier N₂O production, and we hypothesize that stress from AgNP exposure can also exacerbate nitrifier N₂O production.

Estuaries, especially large-river deltaic systems (for example, the Yangtze Estuary), are dynamic regions where rivers, lands, and oceans interact, and they are among the most productive ecosystems (32). Estuarine ecosystems have long been disturbed by human activities due to their ability to support large human populations (32). In addition to receiving large quantities of AgNPs (19, 20), estuaries have also suffered from a substantial loading of anthropogenic nitrogen (33), which has threatened their overall quality and function (34). Hence, a deeper understanding of how AgNPs affect key nitrogen transformations in estuarine environments is required. Here, we examine the toxicity of PVP-coated AgNPs on the activity of nitrifying communities from the Yangtze estuarine sediments and the effects of AgNPs on associated N₂O production during nitrification. Isotopomer analyses were conducted to identify the pathway of N₂O production most affected by AgNPs. Metatranscriptomes were analyzed to elucidate the metabolic response of nitrifying organisms by tracking the expression of AgNP-responsive genes. This research provides a new view of the AgNP toxicity mechanism affecting nitrification in estuarine environments.

RESULTS AND DISCUSSION

Effect of AgNPs on nitrification rate

The toxicity of AgNPs to the activity of nitrifying communities from the intertidal sediments of the Yangtze Estuary was examined via a sediment slurry incubation experiment (fig. S1). Three different sizes (10, 30, and 100 nm) of PVP-coated AgNPs were chosen. The toxicity of Ag⁺ (in the form of AgNO₃) was also investigated to compare its toxicity with that of AgNPs. Within 30 hours of acute exposure, the nitrification inhibition level increased gradually for the first 12 hours (fig. S2) and then remained relatively stable, probably due to nearly saturated silver toxicity. Thus, dose-response curves were constructed by plotting the measured nitrification inhibition level against AgNP exposure concentrations over 12 hours (Fig. 1). Nitrification inhibition increased exponentially as the AgNP dose increased (P < 0.01). On the basis of the dose-response curves, we determined AgNP concentrations that produced 10% (EC₁₀ or effective concentration) and 50% (EC₅₀) reductions in the nitrification rate relative to the control. The toxicity of AgNPs to nitrification activity was size-dependent, with EC₁₀ values of 32, 145, and 502 μg liter⁻¹ and EC₅₀ values of 421, 1775, and 6020 μg liter⁻¹ for the 10-, 30-, and 100-nm AgNPs, respectively (Fig. 1). The increased toxicity of the smaller AgNPs was perhaps caused by increased contact between cell membranes and the surface of the smaller particles or indirectly caused by the increased dissolution of Ag⁺ from the smaller AgNPs due to their greater surface area–to–volume ratio (35).

The mechanisms by which AgNPs exert their toxicity are debatable (36). Recently, the direct particle-specific effect of AgNPs was ruled out because no toxicity was observed when they were synthesized and tested under anaerobic conditions where Ag⁺ release was precluded (36). AgNP morphological properties—such as particle size, shape, and capping agent—might still be indirect effectors influencing Ag dissolution (22, 36). This point is confirmed in this study, wherein AgNP toxicity to nitrification correlated significantly with dissolved Ag⁺ concentration (P < 0.01) (fig. S3 and table S1). Dissolved Ag⁺ exerted toxicity via interaction with the thiol groups of the cysteine residues in respiratory chain enzymes, such as NADH (reduced form of NAD⁺) dehydrogenase, which decoupled respiration and adenosine 5′-triphosphate (ATP) synthesis (22, 37). In addition, Ag⁺ may bind with transport proteins, leading to proton leakage and proton motive force collapse (37, 38).

AgNO₃ (as a source of dissolved Ag⁺) exhibited a greater inhibitory effect on nitrification than AgNPs, with EC₁₀ and EC₅₀ values of 1 and 11 μg liter⁻¹, respectively (Fig. 1). However, the Ag⁺ dissolved from AgNPs was more toxic than the same concentration of Ag⁺ present as AgNO₃ (Fig. 1 and fig. S3). This result may indicate that AgNPs deliver Ag⁺ more effectively to nitrifiers because they are less susceptible to binding and reduced bioavailability by common natural ligands, such as chloride, sulfide, thiosulfate, and dissolved organic carbon, compared with AgNO₃ (36, 39). During the AgNP inhibition experiment, no NO₂⁻ accumulation (always below the detection limit) was detected in the sediment slurries, supporting the idea that AgNPs inhibit ammonia (NH₃) oxidation more than NO₂⁻ oxidation (4). The PVP capping agent (less than 0.5% coating on AgNPs by weight) may contribute to the toxicity of AgNPs. However, on the basis of the independent inhibition experiment conducted for PVP, less than a 1.5% reduction in the nitrification rate, accounting for only 0 to 5% of the AgNP toxicity, resulted from exposure to PVP (0.05 to 15 μg liter⁻¹) for 12 hours (fig. S4).

Fig. 1. Percentage reduction of nitrification rate in AgNP or Ag⁺ treatments compared to the no-silver control (incubation time = 12 hours; n = 3). EC₁₀ and EC₅₀ represent the concentrations that produced a 10 or 50% reduction in nitrification rate relative to the control, respectively. Nonlinear fitted curves (ExpDec1) and equations are given (P < 0.01).
Effect of AgNPs on N₂O production

The increasing release and atmospheric accumulation of the powerful greenhouse gas N₂O have caused concern during this era of rapid environmental change (27, 34). N₂O production during nitrification was affected by AgNP exposure (figs. S5 and S6). For 10-nm AgNPs, although concentrations below 0.1 µg liter⁻¹ apparently did not affect the N₂O emission (Student’s t test, P > 0.05), those between 1 and 10 µg liter⁻¹ stimulated N₂O emission by up to 17.6% during the 30-hour incubation (Student’s t test, P < 0.05). When exposed to higher concentrations (100 to 500 µg liter⁻¹), an initial inhibition (up to 23.5%) of N₂O emission occurred (Student’s t test, P < 0.05) but switched to stimulation (up to 60.8%) after 12 hours of incubation (Student’s t test, P < 0.05). However, when concentrations were increased to 1000 to 2000 µg liter⁻¹, inhibition of up to 90.0% was always observed (Student’s t test, P < 0.01). Similar results were recorded for the effects of increasing dosages of 30- and 100-nm AgNPs and Ag⁺ (provided as AgNO₃) on nitrifier N₂O production (fig. S6). Thus, nitrifying communities respond rapidly to AgNPs or Ag⁺ exposure, probably by regulating gene transcription and adjusting metabolic pathways. One important response was the regulation of the by-product N₂O emission.

Dose-response curves were constructed between Ag exposure concentration and N₂O emission over 12 hours (Fig. 2). Although nitrification rates decreased exponentially as the AgNP or Ag⁺ dose increased (P < 0.01) (Fig. 1), the dose-response relationship between Ag concentration and N₂O emission showed both low-dose stimulation and high-dose inhibition (that is, hormesis (40)) (Fig. 2 and fig. S7). N₂O emission increased with the increasing Ag concentrations, reaching maximum increases of 71.8, 70.9, 97.2, and 125.3% under Ag concentrations of 5.2 µg liter⁻¹ for Ag⁺, and 257, 713, and 1173 µg liter⁻¹ for 10-, 30-, and 100-nm AgNPs, respectively, based on the constructed curves (Fig. 2). The stimulation of N₂O production due to Ag addition might be a stress response, as is observed under low-O₂ conditions (41). However, with increasing Ag concentrations, the degree to which N₂O emission was stimulated was initially reduced, after which the emission of N₂O became inhibited compared with the no-silver control, showing that the damage from Ag to nitrifying cells increased until it was strong enough to counteract the stimulated N₂O production. This observed hermetic effect of AgNPs on N₂O production during nitrification has not been reported previously. This finding is globally significant as environmental inputs of AgNPs have been increasing exponentially due to their rising usage and disposal levels worldwide (20, 42, 43).

Although low dissolved oxygen (DO) conditions (<3 mg liter⁻¹) enhance N₂O emissions, mainly through the nitrifier denitrification (41), the DO concentration in this study ranged from 6.3 to 9.1 mg liter⁻¹ (fig. S8). The DO concentration was significantly lower in the no-silver control (6.3 to 6.5 mg liter⁻¹) than in the AgNP-treated samples where N₂O production was greatly enhanced (Student’s t test, P < 0.01). Therefore, the simulated N₂O emission due to the AgNP treatments was likely not caused by DO stress. High NO₃⁻ concentrations can promote nitrifier denitrification and N₂O production (44). However, NO₃⁻ concentrations remained below the detection limit during the incubation, indicating that NO₃⁻ stress did not enhance N₂O production. The possibility that the PVP capping agent might contribute to enhanced N₂O emission was excluded, as the impact of PVP (0.05 to 15 µg liter⁻¹) on N₂O emission remained at only 0.1 to 1.2% of inhibition (fig. S4).

Identification of key N₂O production pathways

As shown here, increasing AgNP concentrations in estuarine environments can enhance N₂O emissions. However, the mechanisms by which AgNPs stimulate N₂O production are still unclear. N₂O isotopomer analysis is a powerful tool to distinguish whether N₂O originates from NH₂OH oxidation or NO₂⁻ reduction (30). It is based on the intramolecular distribution of ¹⁵N in the central position (¹⁵N¹⁴N¹⁶O) and the end position (¹⁴N¹⁵N¹⁶O) of asymmetric N₂O molecules (45). The ¹⁵N-site preference (SP) is defined as the difference in the bulk nitrogen isotope ratios of N₂O between δ¹⁵N¹⁴N and δ¹⁵N¹⁶O, where ¹⁵N¹⁴N and ¹⁵N¹⁶O represent the ¹⁵N/¹⁴N ratios at the central (α) and end (β) sites of the nitrogen atoms, respectively (45). Because N₂O produced through NH₂OH oxidation and NO₂⁻ reduction have different SP values [33% (per mil) for NH₂OH oxidation and 0% for NO₂⁻ reduction (46, 47)], analyzing the SP enables the identification of the sources of N₂O produced during these two respective processes.

The isotopomer analysis showed that SP in the no-silver control was 29.3‰ at 12 hours, indicating that approximately 89% of the released N₂O was produced via NH₂OH oxidation, whereas only approximately 11% was attributed to NO₂⁻ reduction (Fig. 3, A and B), assuming that each process is linearly proportional to SP (47). When exposed to AgNPs (100 µg liter⁻¹, 10 nm; 500 µg liter⁻¹, 30 nm; or 1000 µg liter⁻¹, 100 nm), under which N₂O production was enhanced by 43.0, 84.1, and 121.5%, the SP values increased to 30.0, 31.5, and 35.6‰, respectively, at 12 hours (Student’s t test, P < 0.05) (Fig. 3A). In addition, SP increased to 32.2‰ when exposed to Ag⁺ (5 µg liter⁻¹) (Student’s t test, P < 0.01), exhibiting a 73.9% N₂O enhancement. Under low-dose AgNP exposure, δ¹⁵N increased, whereas δ¹⁵N reduced simultaneously decreased (Fig. 3, C and D), thus resulting in higher SP. The increased SP indicated that low-dose AgNPs stimulated NH₂OH oxidation, which contributed to more than 90% of N₂O emission or even became the sole pathway, whereas the contribution of NO₂⁻ reduction was minor or nil (Fig. 3B).

The inhibition mechanism of high AgNP concentrations on N₂O production during nitrification was investigated. When exposed to AgNPs (2000 µg liter⁻¹ 10 nm, 3000 µg liter⁻¹ 30 nm, and 100 nm) or Ag⁺ (50 µg liter⁻¹), wherein N₂O production decreased by 89.9, 48.3, 19.0, and 59.2%, respectively, SP decreased to 17.4, 24.2, 26.9, and 23.3‰, respectively, at 12 hours (Student’s t test, P < 0.01) (Fig. 3A). These results imply that, although NH₂OH oxidation remained
the main contributor of N\textsubscript{2}O, its contribution was reduced under high-dose Ag exposure, which was reduced to approximately 53% when the N\textsubscript{2}O emission was inhibited by 89.9% (Fig. 3B). Therefore, N\textsubscript{2}O reduction was reported to increase SP, and the effect of NO\textsubscript{2}\textsuperscript{−} reduction might be underestimated when N\textsubscript{2}O reduction is intense (48). However, no significant increase in the \(^{18}\text{O}\) of N\textsubscript{2}O was detected during the incubation (it remained approximately 20‰, Student’s \(t\) test, \(P > 0.05\)), showing that N\textsubscript{2}O reduction to N\textsubscript{2} was not important (47). This study is the first to explore and distinguish the effect of AgNPs on different N\textsubscript{2}O production pathways of nitrifying communities.

Although NO\textsubscript{2}\textsuperscript{−} reduction was not the main contributor to N\textsubscript{2}O production in this study, N\textsubscript{2}O isotopomer analysis does not distinguish the relative contributions of nitrifier denitrification and heterotrophic denitrification (30). When the headspace O\textsubscript{2} concentration is between 0.5 and 3% (v/v), at least 50% of the total N\textsubscript{2}O production generally derives from the NH\textsubscript{3} oxidation pathway rather than heterotrophic denitrification (41). In addition, when the dissolved O\textsubscript{2} concentration is ca. 0.06 mg liter\(^{-1}\), the N\textsubscript{2}O production by denitrification is completely inhibited (49). Therefore, considering that the dissolved O\textsubscript{2} concentration remained above 6.3 mg liter\(^{-1}\), NO\textsubscript{2}\textsuperscript{−} reduction via heterotrophic denitrifiers is likely to be minor (fig. S8). This conclusion was supported further by the metatranscriptomic analyses (see below).

**Transcriptional response to AgNP exposure**

AgNP toxicity may cause intracellular metabolic disturbances. To explore the impacts of AgNPs on nitrogen metabolic pathways in nitrifying communities, we conducted metatranscriptomic analyses.

Although NO\textsubscript{2}\textsuperscript{−} reduction was not the main contributor to N\textsubscript{2}O production in this study, N\textsubscript{2}O isotopomer analysis does not distinguish the relative contributions of nitrifier denitrification and heterotrophic denitrification (30). When the headspace O\textsubscript{2} concentration is between 0.5 and 3% (v/v), at least 50% of the total N\textsubscript{2}O production generally derives from the NH\textsubscript{3} oxidation pathway rather than heterotrophic denitrification (41). In addition, when the dissolved O\textsubscript{2} concentration is ca. 0.06 mg liter\(^{-1}\), the N\textsubscript{2}O production by denitrification is completely inhibited (49). Therefore, considering that the dissolved O\textsubscript{2} concentration remained above 6.3 mg liter\(^{-1}\), NO\textsubscript{2}\textsuperscript{−} reduction via heterotrophic denitrifiers is likely to be minor (fig. S8). This conclusion was supported further by the metatranscriptomic analyses (see below).
hormetic stimulation (80%) on N₂O production (Student’s t test, P < 0.01) were observed. Metatranscriptome studies showed that community compositions were not significantly affected by AgNP exposure during the 12-hour incubation period, and nitrifiers accounted for 51.7 and 47.7% of the total organisms in the AgNP-treated and no-silver control samples, respectively (fig. S11).

Metatranscriptomic analyses indicated that the expression of known nitrification-associated genes varied between the AgNP-exposure and control groups (Fig. 4, A and B). The expression of the potentially active subunit of the ammonia monoxygenase gene (amoA) was down-regulated by 1.4-fold when exposed to 500 µg liter⁻¹ of 30-nm AgNPs for 12 hours, consistent with the nitrification inhibition (25%). Real-time quantitative polymerase chain reaction (qPCR) based on RNA samples also showed that amoA expression was down-regulated under AgNP exposure (1.5-fold; Student’s t test, P < 0.01) (table S3). In addition, expression of amoB [recently suggested as a catalytic subunit (53)] decreased by 1.2-fold (Student’s t test, P < 0.05), whereas the transcript level of amoC remained unchanged (Student’s t test, P > 0.05), after exposure to AgNPs (Fig. 4, A and B, and table S3). Posttranscriptional disturbance might occur, because ammonia monoxygenase (amo) uses a copper metal center. Dissolved Ag⁺ from AgNPs may interfere with enzyme activity by substituting copper with Ag⁺ or inducing the production of chelators, both of which could deactivate amo (22, 54). The amo transcripts of the recently discovered comammox bacteria were also discovered (fig. S13), which exhibit up to 99% similarity with the amoABC sequences of comammox Nitrospira (55, 56). However, these genes only showed less than 80% similarity with those of known AOB (fig. S13), and their expression accounted for approximately 45 and 60% of amo transcripts in the AgNP treatment and no-silver control samples, respectively. These results suggest that comammox bacteria are present, possibly playing an important role in NH₃ oxidation in estuarine sediments.

AgNPs exhibited variable impacts on the expression of genes in the hydroxylamine oxidase (hao) gene cluster (haoAB-cycAB) (Fig. 4, A and B). The transcript level of haoA (encoding hao) and cycA (encoding electron transfer protein cytochrome c₅₅₅₅) remained unchanged (Student’s t test, P > 0.05), whereas expressions of haoB (encoding the putative membrane anchor protein haoB) and cycB (encoding quinone reducing cytochrome c₅₅₅₅) were down-regulated by 1.4- and 1.6-fold (Student’s t test, P < 0.05), respectively (Fig. 4, A and B, and table S3). The cytochrome c₅₅₅₅ is believed to function as the physiological electron acceptor of hao, and then, electrons from cytochrome c₅₅₅₅ may traverse the membrane-anchored tetraheme cytochrome c₅₅₅₅ to ubiquinone and then to amo, during which ATP is synthesized (Fig. 4A) (57). Cytochrome c₅₅₅₅ can also function as an enzyme with significant nitric oxide (NO) reductase activity (57). However, this function was not confirmed at the transcriptional level, as cycA expression remained unchanged (Student’s t test, P > 0.05) when N₂O production was significantly enhanced (75.1%). The expressions of nitrite oxidoreductase genes nxrA and nxrB were not affected by 30-nm AgNP (500 µg liter⁻¹) exposure (Fig. 4, A and B), consistent with the qPCR results (Student’s t test, P > 0.05) (table S3).

When exposed to AgNPs, the expression of the nitrite reductase (NO-forming, nirK) gene in nitrifiers was up-regulated by 1.4-fold (Student’s t test, P < 0.05) (Fig. 4, A and B, and table S3), showing that nitrifier denitrification might be enhanced. However, isotope analysis showed that the N₂O production via NO₂⁻ reduction and the contribution of NO₂⁻ reduction to total N₂O production were reduced under 30-nm AgNP (500 µg liter⁻¹) exposure (Fig. 4C). It is probable that, under AgNP exposure, NO formed from NO₂⁻ reduction was released into the surrounding environment, thus being oxidized rather than reduced to N₂O (58, 59). In addition, 94 and 92% of nitrate reductase transcripts were affiliated with nitrifiers in the AgNP treatment and the no-silver control samples, respectively, further suggesting that NO₂⁻ reduction via heterotrophic denitrifiers was negligible. Transcription of nitrifying bacterial nitric oxide reductase (norQ) increased 1.4-fold in the AgNP treatment, consistent with N₂O production, although the nitric oxide reductase norB and norC genes were not observed. qPCR confirmed that the norQ expression was significantly up-regulated under AgNP exposure (Student’s t test, P < 0.01) (table S3). Alternative enzymes might have also contributed to nitrifier N₂O production, such as cytochrome c₃₋β (encoded by cytS) (60) and cytochrome P₄₅⁰ (61), but expressions of these genes were not observed in this study.

Transcripts encoding Cu(I)/Ag(I) efflux membrane protein (cusA), Cu(I)/Ag(I) efflux periplasmic protein (cusB), and Cu²⁺-exporting adenosine triphosphatase (ATPase) (copB) were up-regulated by 8.6-, 5.5-, and 1.9-fold, respectively, under AgNP exposure (Fig. 4, A and B). Ag and Cu have similar coordination chemistries and can be treated interchangeably (62). Thus, the up-regulation of these genes (Student’s t test, P < 0.01; table S3) indicated an urgent demand for transporting Ag⁺, as no extra Cu was added, from the cytoplasm to the exterior of the nitrifying cells. This result also suggests that nitrifiers might have been invaded by AgNPs or released Ag⁺, although no apparent physical cell damage was observed via transmission electron microscopy (TEM) (Fig. 4, D and E). Transcripts encoding mercuric reductase [merA, converting toxic heavy metal ions into their relatively inert elemental forms (63)] were up-regulated by 1.2-fold in the AgNP treatment (Student’s t test, P < 0.05) (Fig. 4B and table S3). These results imply that the nitrifying cells were under AgNP stress, and the enzymes discussed above might help them cope with this stress.

Transcriptional response to oxidative stress was detected (Fig. 4B). Transcripts encoding superoxide dismutase (SOD2, Fe-Mn family), peroxiredoxin (BCP), thiol peroxidase (TPX), and cytochrome c peroxidase (CCP), all of which convert superoxides and hydroperoxides into innocuous products, were up-regulated by 1.4-, 1.4-, 1.2-, and 1.2-fold, respectively. These genes were up-regulated (Student’s t test, P < 0.05; table S3) to detect and defend the cell from oxidative damage. Thus, we speculate that ROS generation and subsequent oxidative damage to nitrifying cells occurred during the AgNP treatment. Transcripts encoding heat shock protein and cold shock protein were up-regulated by 1.2- and 1.5-fold, respectively, when exposed to AgNPs (Fig. 4B). These proteins were first described in relation to temperature shock (64), but the observed up-regulation of these genes (Student’s t test, P < 0.05; table S3) under AgNP exposure suggests that they might be commonly expressed to protect the organism from stress. In contrast, transcripts of genes encoding glutathione peroxidase (GPX) and alkyl hydroperoxide reductase (ahpC) were both down-regulated by approximately 1.3-fold (Student’s t test, P < 0.05) (Fig. 4B and table S3), indicating that some catalase gene expressions might also be inhibited by AgNP exposure.

CONCLUSIONS

In conclusion, our findings suggest that AgNPs potentially have the capability to reduce nitrification rates while enhancing N₂O production. Size-dependent impacts of AgNPs on nitrification and the associated N₂O production were observed, and our analyses suggest that the toxicity was due to the Ag⁺ released from AgNPs. The toxicity data indicate that considerable hormesis occurred after low-dose AgNP exposure,
which stimulated N₂O production up to twofold. The isotopomer analysis suggests that N₂O production was mainly from NH₂OH oxidation, which was enhanced by exposure to low doses of AgNPs. The metatranscriptional data illustrate that AgNPs affected the gene expression of nitrifying communities, particularly those involved in the stress response and nitrogen metabolism. Consistent with the stimulation of N₂O production, the expression of the nitric oxide reductase norQ gene was up-regulated, as were many stress-response genes. Although the estimated

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**Fig. 4. Response of nitrifying communities to AgNP exposure.** (A) Schematic model depicting the effects of AgNPs on the expression of gene families involved in nitrification. N₂O can be produced through NO₂⁻ reduction (the bold pink arrows) or incomplete NH₂OH oxidation (the bold blue arrows). Upward green arrows indicate that the gene expressions were up-regulated when exposed to AgNPs, the downward red arrows indicate that the gene expressions were down-regulated, and “N” denotes that gene expression was not affected by AgNP exposure. CusA, Cu(I)/Ag(I) efflux system membrane protein cusA; CusB, Cu(I)/Ag(I) efflux system periplasmic protein cusB; amo, ammonia monooxygenase; hao, hydroxylamine oxidase; Cyt554, cytochrome c554; mCyt552, cytochrome cm552; nir, nitrite reductase (NO-forming) nirK; nor, nitric oxide reductase norQ; nxr, nitrite oxidoreductase; Cyt551, cytochrome c551; Cyt552, cytochrome c552; Cyt553, cytochrome c553; Q, ubiquinone; QH₂, ubiquinol. The roman numbers refer to the enzyme complex I (NADH-ubiquinone reductase), complex III (ubiquinol-cytochrome c reductase), complex IV (cytochrome c oxidase), and complex V (F-type ATPase) in the respiratory chain (related gene expression regulations by AgNPs are shown in fig. S12). Colored proteins were detected in the cDNA libraries, whereas those in dark gray were not identified but are included in the model of electron transport for reference (74). Dotted blue arrows show the movement of electrons, and white arrows show movement of protons. The membrane was broken by dotted line, as nitrite oxidation did not often occur in the same organism with ammonia oxidation, with the exception of recently discovered comammox Nitrospira (55, 56). (B) Fold change (FC) of transcripts encoding proteins involved in heavy metal stress, oxidative stress release, and the nitrogen transformation process of nitrifying organisms when exposed to 30-nm AgNP (500 μg liter⁻¹) for 12 hours. FC in relative gene expression was calculated by comparing AgNP-treated samples to the no-silver control. Gene expression levels were calculated on the basis of FPKM. (C) Contribution of different pathways to N₂O emission in the no-silver control and the 30-nm AgNP (500 μg liter⁻¹) treatment. (D) TEM image of the no-silver control at 12 hours. (E) TEM image of the 30-nm AgNP (500 μg liter⁻¹) treatment at 12 hours. No apparent physical damage to the cell surface was observed.

concentrations of AgNPs released into the environment are lower than those used here, our work contributes to a mechanistic understanding of AgNP toxicity to nitrification. Our study reveals that further increases in AgNP concentrations in the environment could disrupt a critical link in the nitrogen cycle and could increase the production of a potent greenhouse gas (N₂O). Future work is needed to explore the chronic effect of low doses of AgNPs on nitrification. In addition, future work could expand this study to different salinity conditions, as the changing salinity in estuarine environments has an important influence on the aggregation and dissolution kinetics of AgNPs as well as the ammonia-oxidizer community structure. Last but not least, we should pay attention to the environmental risk of the sulfidation process of AgNPs and focus on the activation of Ag₂S driven by estuarine environmental conditions and associated toxic effects on nitrification.

MATERIALS AND METHODS
Sample collection and AgNP preparation
Sediment samples and overlying tidal water were collected from intertidal flats of the Yangtze Estuary. Briefly, surface sediments (0 to 5 cm) were collected using PVC tubes from six to eight plots and then stored in sterile plastic bags. Overlying tidal water was collected at the respective sampling sites with a sterile plastic carboy. After collection, the sediment and overlying water samples were transported to the laboratory on ice within 2 hours. Upon arriving at the laboratory, the sediment samples were homogenized, and the overlying tidal water was filtered through cross-flow ultrafiltration to remove microorganisms and nanoparticles (65) and stored at 4°C. Nitrate (NO₃⁻) and ammonium (NH₄⁺) concentrations and the salinity of the overlying water at the sampling site were approximately 80 μM, 10 μM, and 2‰, respectively, whereas the sediment characteristics were as follows: NO₃⁻ (NO₂⁻ plus NO₃⁻), ca. 0.6 μmol g⁻¹; NH₄⁺, ca. 1.1 μmol g⁻¹; organic carbon, ca. 0.8%; and total nitrogen, ca. 0.07% (66).

Three different sizes (10, 30, and 100 nm) of AgNPs coated with PVP were purchased from Coldstone Tech. The average particle diameters were confirmed by TEM inspection (fig. S14). The amount of PVP coating in the AgNPs was less than 0.5% by weight. PVP is a hydrophilic, neutral, and high–molecular weight polymer that protects nanoparticles by steric stabilization. Here, PVP-coated AgNPs were chosen, because they are commonly used. Stock solutions of AgNPs were prepared immediately before the experiment by dispersing AgNPs in Milli-Q water at a known concentration. In addition, Ag⁺ was prepared as silver nitrate (AgNO₃) to compare its toxicity with that of AgNPs.

Nitrification inhibition
The concentrations of AgNPs and Ag⁺ used in the present study were selected on the basis of a range-finding test. Three sizes of AgNPs (10, 30, and 100 nm) and dissolved Ag⁺ were tested. AgNP stock solutions were added to the overlying water (filtered overlying tidal water with a salinity gradient of 0, 0.05, 0.1, 0.5, 1, 5, 10, and 15 μg liter⁻¹ PVP was chosen on the basis of the amount of PVP on the AgNPs (less than 0.5% by weight). After 12 hours of incubation, the NO₂⁻ in the headspace, DO, pH, NO₃⁻, and NO₂⁻ of the suspension were determined.

Stable isotope analysis
To identify the pathway of N₂O production, N₂O isotopomer ratios (δ¹⁵N²O, δ¹⁸O²O, and δ¹⁵N³O) were measured during the nitrification inhibition experiment. Briefly, sediment slurries with AgNPs (100 μg liter⁻¹, 10 nm; 500 μg liter⁻¹, 30 nm; 1000 μg liter⁻¹, 100 nm; 2000 μg liter⁻¹, 10 nm; and 3000 μg liter⁻¹, 30 and 100 nm), Ag⁺ (5 μg liter⁻¹), and Ag⁺ (50 μg liter⁻¹) or no-silver control were prepared and immediately transferred into three replicate 120-ml gas-tight glass vials, as described above. These vials were placed on an orbital shaker at 180 rpm and incubated in the dark at room temperature (20°C) for 12 hours. N₂O isotopomer ratios of the headspace gas were then determined using an Isotopic N₂O Analyzer (Los Gatos Research), with a precision of <1‰. ¹⁵N-site preference (SP) is defined using the following equation

\[ SP = \delta^{15}N^a - \delta^{15}N^b \]  

where ¹⁵N⁰ and ¹⁵N⁰ represent the ¹⁵N/¹⁴N ratios at the central and end sites of the nitrogen atoms, respectively. Characteristic SP values of 33‰ for NH₂OH oxidation and 0‰ for NO₂⁻ reduction, which were estimated in pure cultures (46, 47), were used to estimate the contribution of each process, assuming that each process is linearly proportional to the SP value using the following equations (47)

\[ \text{Contribution of NH₂OH oxidation (%) } = \frac{SP \times (SP \text{ for NH₂OH oxidation } - SP \text{ for NO₂⁻ reduction})}{100} \]  

\[ \text{Contribution of NO₂⁻ reduction (%) } = 100 - \text{Contribution of NH₂OH oxidation} \]
Metatranscriptomes were obtained from no-silver controls and AgNP-qPCR analyses. For metatranscriptome sequencing, whereas the other part was used for resulting in qualified mRNA samples. One part of the mRNA was used (rRNA) was removed using a Ribo-Zero Magnetic Kit (Epicentre), thus Nanodrop2000 (Thermo) and Agilent2100 (Agilent). Ribosomal RNA purity, and RNA integrity number of the RNA were determined using a 909R (5

A continuous-flow membrane nitrifying bioreactor with a working volume of 4.0 liter was set up with approximately 50 g of fresh sediment as inoculum from the Yangtze Estuary. Filter-sterilized and nanoparticle-free overlying tidal water, with an addition of NH4+ to a final concentration of 3 mM, was supplied at a flow rate of approximately 2 liters day−1. The temperature and pH were maintained at 20°C (room temperature) and 7.8, respectively, with a water bath and a 1 M KHCO3 solution. The DO concentration was maintained at approximately 8.0 mg liter−1 by flushing continuously with air. The reactor was stirred at 250 rpm with two stirrers installed at the bottom and above the inlet airflow. The reactor tank and other related materials were heat-sterilized by autoclaving at 121°C and 15 psi for 20 min, whereas the reagent solutions were filter-sterilized through filters with pore size of 0.2 μm (Waterman) before use. Liquid samples (10 ml) were collected from the effluent of the reactor every day and filtered immediately using filters with pore size of 0.2 μm (Waterman). Filtrates were stored at −20°C for the measurement of NH4+, NO3−, and NO2−. Triplicate sediment slurries (5 ml) were harvested from the reactor every day and pelleted by centrifugation for 5 min at 20,000g. Pellets were immediately used for total DNA extraction, and subsequent qPCR and pyrosequencing to detect the enrichment level of nitrifying prokaryotes in the bioreactor (Supplementary Materials and Methods).

When the nitrifying bioreactor achieved a steady state (figs. S9 and S10), samples were taken from the bioreactor for the nitrification inhibition test. Briefly, nitrifying-enriched sediments were harvested from the reactor by centrifugation (20,000g, 5 min). The precipitate was washed three times using 40 mM KH2PO4 buffer (pH 7.8) and resuspended in culture medium. Then, 100 μl of the suspension was transferred immediately into 120-ml gas-tight glass vials in which 10 ml of culture medium was prepared with AgNPs or no-silver control. Inhibition of nitrification rate and effects of AgNPs on N2O emission based on the nitrifying-enriched sediments were determined as described above.

**Nucleic acid extraction**

The total RNA was extracted from the triplicate no-silver controls and AgNP-treated enrichments using the EZNABio Soil RNA kit (Omega Bio-tek, Norcross) and purified by removing residual genomic DNA with the Turbo DNA-free kit (Ambion). Contamination of DNA was ruled out using PCR based on primers 515F (5′-GTCCAGCMGGCCGCTA-3′) and 909R (5′-CCCGGCAATTCMTTTRAGT-3′) (69). The concentration, purity, and RNA integrity number of the RNA were determined using a Nanodrop2000 (Thermo) and Agilent2100 (Agilent). Ribosomal RNA (rRNA) was removed using a Ribo-Zero Magnetic Kit (Epigenics), thus resulting in qualified mRNA samples. One part of the mRNA was used for metatranscriptome sequencing, whereas the other part was used for qPCR analyses.

**Metatranscriptomic analysis**

Metatranscriptomes were obtained from no-silver controls and AgNP-treated (500 μg liter−1 of 30-nm AgNPs) nitrifying enrichments after the 12-hour incubation (table S2), in which a 25% nitrification inhibition (Student’s t test, P < 0.01) and a hormetic stimulation (80%) on N2O production (Student’s t test, P < 0.01) were observed. Triplicate mRNA samples were pooled and used for library construction with the TruSeq RNA Sample Prep Kit (Illumina) and sequenced on an Illumina HiSeq4000 platform (70).

The quality of the raw reads was visualized using FastQC and cleaned and trimmed using SeqPrep (https://github.com/jstjohn/SeqPrep). Reads that were shorter than 50 base pairs (bp), contained ambiguous (N) bases, and were of low quality (below 20) were discarded using Sickle (https://github.com/najoshi/sickle). RNA reads were further screened using SortMeRNA (http://bioinfo.lifl.fr/RNA/sortmerna/). The trinity de novo assembly pipeline was used to assemble the high-quality paired-end mRNA reads (71, 72). Resulting sequences were filtered to remove contigs of less than 300 bp in length. TransGeneScan (http://sourceforge.net/projects/transgenescan/) was used to predict open reading frames. Transcripts were assigned to taxonomic affiliations by binning to the best hit (BLASTP, e value ≤ 10−5) in the nr database. The potential function was assigned on the basis of the best homology (BLASTP, e value ≤ 10−5) to proteins within the Kyoto Encyclopedia of Genes and Genomes database. Sequences with hits to proteins in eukaryotic organisms and viruses were identified and removed before statistical analysis. Expression levels of transcripts were calculated with RNA-Seq by Expectation-Maximization (http://deweylab.github.io/RSEM/) based on the fragments per kilobase of transcript per million mapped reads (FPKM) (73). The FC in the relative gene expression was calculated by comparing the AgNP-treated samples to no-silver control samples.

**Quantitative polymerase chain reaction**

Corroboration of gene expression in metatranscriptomic method was performed by qPCR with cDNA as template on an ABI 7500 Sequence Detection System (Applied Biosystems). The cDNA was obtained from the triplicate mRNA using Superscript Double-Stranded cDNA Synthesis kit (Invitrogen) and a random hexamer primer. Before qPCR, all cDNA samples were normalized to a concentration of 10 ng μl−1. The quantitative standard for each gene was constructed as described previously (67). The qPCRs were performed in triplicate with corresponding primers as given in Supplementary Materials and Methods and table S4.

**Membrane integrity testing**

Nitrifying enriched samples were harvested and pelleted for thin-section TEM imaging after the nitrification inhibition experiment (22). Briefly, biomass from no-silver controls and AgNP treatments was harvested by centrifugation at 700g for 5 min. The obtained flocs were fixed at 4°C for 12 hours in a solution of 2.5% glutaraldehyde, 0.1 M phosphate buffer (pH 7.0), and ultrapure water. The flocs were washed three times with phosphate buffer (0.1 M, pH 7.0) and fixed with 1% osmic acid for 2 hours and then washed three times with phosphate buffer cycle. Samples were dehydrated in an increasing ethanol series (15 min each in 30, 50, 70, 80, 95, and 100% ethanol) at room temperature and in 100% acetone for 20 min at 4°C. Subsequently, they received penetrating treatment with epoxy resin and acetone at a volume ratio of 1:1 and 3:1 for 1 and 3 hours, respectively, at room temperature. Samples were then embedded in pure epoxy resin for 12 hours at 70°C. Ultramicrotome-cut thin sections of the resin sample and slices were dyed for 5 to 10 min with lead citrate and a solution of uranyl acetate saturated in 50% ethanol, and inspected at 60 kV with a FEI Tecnai G2 Twin TEM. Over 10 images were taken for each sample after surveying a large amount of each TEM grid.

**Analytical methods**

Total Ag and dissolved Ag+ were quantified using an Agilent 7700 inductively coupled plasma mass spectrometer with a detection limit of 0.03 μg liter−1. In brief, total Ag was measured by adding concentrated trace metal-grade nitric acid (HNO3) at 70°C overnight to dissolve AgNPs and then diluted with 2% HNO3 followed by filtration with a 0.2-μm membrane (Waterman) to remove impurities (4). To measure...
Dissolved Ag⁺ concentration was quantified in the filtrate after a dilution (fig. S6). Effects of AgNPs and Ag⁺ on N₂O emission during nitrification in the 30-hour incubation period.

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**Statistical analysis**

The unpaired, two-tailed Student’s t test was applied using SPSS (version 16.0) to identify the statistical differences among differently treated groups. Pearson correlation analysis was also conducted to explore any underlying correlations. Results were considered significant when P < 0.05.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/

**REFERENCES AND NOTES**


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Effects of silver nanoparticles on nitrification and associated nitrous oxide production in aquatic environments

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