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Impacts of Silver Nanoparticles on a Natural Estuarine Plankton Community

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ABSTRACT: Potential effects of metal nanoparticles on aquatic organisms and food webs are hard to predict from the results of single-species tests under controlled laboratory conditions, and more realistic exposure experiments are rarely conducted. We tested whether silver nanoparticles (Ag NPs) had an impact on zooplankton grazing on their prey, specifically phytoplankton and bacterioplankton populations. If Ag NPs directly reduced the abundance of prey, thereby causing the overall rate of grazing by their predators to decrease, a cascading effect on a planktonic estuarine food web would be seen. Our results show that the growth rates of both phytoplankton and bacterioplankton populations were significantly reduced by Ag NPs at concentrations of ≥500 $\mu g\ L^{-1}$. At the same time, grazing rates on these populations tended to decline with exposure to Ag NPs. Therefore, Ag NPs did not cause a cascade of effects through the food web but impacted a specific trophic level. Photosynthetic efficiency of the phytoplankton was significantly reduced at Ag NPs concentrations of ≥500 $\mu g\ L^{-1}$. These effects did not occur at relatively low concentrations of Ag that are often toxic to single species of bacteria and other organisms, suggesting that the impacts of Ag NP exposure may not be apparent at environmentally relevant concentrations due to compensatory processes at the community level.

INTRODUCTION

The impact of nanoparticles (NPs) on natural ecosystems is a growing concern in environmental science and management.[1] Silver nanoparticles (Ag NPs) are some of the most widely used NPs in consumer and industrial products, mainly because of their antibacterial properties, with applications in cosmetics, fabrics,[2] medicine, and hygiene.[3] Nanomaterials discharged into the environment find their way through waste disposal and other routes and ultimately into estuaries and near-shore marine environments.[4] Predicted environmental concentrations (PEC) in surface waters are in a range in ng L^{-1} (parts per trillion) but are expected to continue increasing.[5] The PEC of Ag NPs in surface waters is higher than those of other metal oxide nanomaterials including ZnO, TiO_2, fullerenes, or carbon nanotubes and may pose risks to aquatic organisms.[5] Our knowledge of nanomaterial toxicity largely comes from in vitro or single-species assays that may not reflect responses of natural ecosystems and associated food webs. Impacts of anthropogenic contaminants on aquatic food webs can cause complex ecological effects: grazers or predators can be reduced through toxicity, thereby releasing their prey from predation and increasing the prey's abundance. If the prey also acts as predators on smaller species, increasing the numbers of these mid-level predators can cause decreases in the smaller prey species. This combination of direct and indirect interactions, in a food web with three levels or more, is called a trophic cascade. At the same time, toxicants can directly affect prey populations. Pollutants can cause trophic cascades,[6] but such cascades have not been observed for nanomaterials. Research to examine the direct and indirect ecological effects of nanomaterials in aquatic ecosystems is important to develop a more realistic understanding of the environmental implications of nanotechnology.[7]

In vitro toxic effects of Ag NPs have been documented for organisms in practically every major taxonomic group, including mammals,[8] insects,[9] snails,[10] plants,[11] algae,[12] and bacteria.[13] In the aquatic environment, Ag NPs negatively affect prokaryotes, invertebrates, and fish.[14,15] Toxicity mechanisms have not yet been well-defined for most NPs including Ag but can include destabilization of outer-membrane integrity,[16] disruption of membrane potential,[13] cytotoxicity,[17] genotoxicity,[18] interruption of energy transduction,[19] and formation of

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reactive oxygen species.[20] Due to their small size, NPs can penetrate biological systems in novel ways.[21] Aggregated nanoparticles of varying sizes are expected to result from discharges of NPs into natural waters and may mitigate some such effects.[22] However, even aggregated particles are likely to eventually dissolve, releasing toxic Ag^+ ions.[23,24]

Plankton is a polyphyletic group of aquatic organisms, including nearly 25 000 morphologically defined species distributed among eight major divisions or phyla.[25] In oceans and estuaries, phytoplankton and bacterioplankton provide many ecological services, including in particular the biogeochemical cycling of carbon, nutrients, and trace metals.[26] Coastal marine phytoplankton are major primary producers,[27] and changes to the structure of these communities has the potential to impact higher trophic levels including fisheries species.[28] Plankton communities are highly dynamic, and their productivity is influenced by nutrient availability, water quality (including toxicants), and grazing, which is defined as the consumption of phytoplankton and bacterioplankton cells by other heterotrophic (or mixotrophic) plankton species. Protozoan or zooplankton grazers can regulate the trophic transfer of energy from planktonic production to higher trophic levels because smaller grazers are consumed by larger organisms.

NPs have been shown to depress phytoplankton population growth rates.[29−31] However, NPs may also impact grazers such as relatively large copepods (>500 μm)[32] and protozoans (5−15 μm),[33] potentially changing the outcome of toxicity across the community. In this study, we tested whether and how Ag NPs affect a community composed of zooplankton predators and bacterioplankton and phytoplankton prey in a shallow estuarine lagoon. We predicted that Ag NPs would be toxic phytoplankton and bacterioplankton, reducing their abundance and the overall rate of grazing of their predators, specifically zooplankton, triggering a trophic cascade. Our alternative hypothesis was that Ag NPs would not cause a cascading effect but instead impact specific trophic levels. To evaluate these effects, we conducted dilution experiments[34] to simultaneously estimate phytoplankton and bacterioplankton growth rates and zooplankton grazing rates as well as the effect of exposure to Ag NPs on these critical ecological processes.

MATERIALS AND METHODS

Study Site and Sampling. The study was conducted at an estuarine lagoon on the University of California Santa Barbara (UCSB) campus. The shallow lagoon (~2 m in depth) covers 0.125 km^2. Saltwater is pumped into the lagoon from the ocean, and freshwater is input from runoff.[35] Water samples were collected in June of 2011, when the average air temperature was 16 °C and the salinity of the lagoon was 33‰.

Dilution Experiments. Grazing rates were assessed in serial dilution experiments.[34] Samples were collected from a depth of 0.5 m using 20 L carboys. A portion of the water was filtered through 0.45 μm nitrocellulose filters (Millipore). Dilutions were established using 100%, 75%, 50%, and 25% lagoon water diluted with 0.45 μm filtered lagoon water and were executed in triplicate in 0.5 L polycarbonate bottles. The carboys and bottles were soaked overnight in a 5% (v/v) HCl bath and rinsed with deionized water filtered with a 30 Da filter (nanopure water) prior to use.

Nutrient (phosphate and nitrogen) concentrations were measured spectrophotometrically, in duplicate, in 50 mL of water filtered by 0.45 μm nitrocellulose filters (Millipore) and were in the millimolar range (PO_4^{3-} = 969 ± 2, NO_x = 671 ± 13, NH_4^+ = 292 ± 70 mmol L^{-1}); therefore, nutrients were not supplemented. All of the bottles were incubated for 24 h partially submerged in an outside holding tank plumbed with circulating lagoon water to maintain natural temperature and light levels.

Exposure to Ag NPs. To assess the effect of Ag NPs to the lagoon plankton community, we exposed triplicate bottles to 0.1, 0.5, and 1 mg L^{-1} Ag NPs. The same serial dilution experiments described above were employed for the bottles with Ag NPs. Concentrations were chosen on the basis of previous experiments with aquatic organisms.[15]

Ag NPs > 99.9% trace metal basis were obtained from Sigma-Aldrich and were characterized by the University of California Center for Environmental Implications of Nanotechnology (UC CEIN). The Ag NPs were semispherical and averaged 57 ± 20 nm in primary size as measured by TEM. Hydrodynamic diameter was 143 ± 9 nm (DLS). Purity was measured as 100% (XRD). The ζ potential was −44.7 ± 1.6 nV, and the electrophoretic mobility (EPM) was $-3.36 \pm 0.12 \times 10^{-8}$ (m^2 V^{-1} s^{-1}) (ZetaPALS in DI water). To produce 100 mg L^{-1} stock dispersions, we added 10 mg of Ag NPs to 1 mL of deionized water, sonicated it for 30 min, vortexed it for 30 s, diluted it into filtered (0.2 μm Millipore) natural seawater containing 10 mg L^{-1} of alginate, and again vortexed it for 30 s.

Community Composition. Water samples were preserved with 2.5% paraformaldehyde,[36] frozen at −80 °C for 24 h, and analyzed using a BD LSR II flow cytometer (Biosciences) equipped with a 488 nm excitation laser and standard filter set. For counts of autofluorescent cells, the samples were diluted 1:5 with nanopure water and analyzed with a flow rate of 2 μL s^{-1} (total volume of 200 μL). For counts of total cells, the samples were diluted 1:100 with nanopure water, incubated for 20 min with 1% SYBR Green I stain (Sigma-Aldrich), and analyzed with a flow rate of 0.5 μL s^{-1} (total volume of 45 μL). Dilutions were chosen[36] to keep the counting rate <500 cells s^{-1}. Cell enumeration was performed at the beginning of the incubation period and after 24 h of exposure. Initial samples were taken from the bulk dilutions, whereas final samples were taken from each replicate bottle. Controls of sheath fluid (nanopure water), sheath fluid with SYBR Green, sheath fluid with Ag NPs (in a final concentration of 1 mg L^{-1}), and sheath fluid with the stain and the Ag NPs were run in parallel. 3.0 mm Rainbow beads (Spherotech Inc.) were run at the beginning of every measurement. Nanopure water was run daily to check the consistency and precision of the system.

Acquired data were analyzed using FACS Diva software (BD Biosciences) as described in Ewart et al.[37] Cell abundance in cells per mL was calculated from the sample flow rates and the number of events recorded. Flow-cytometry cell counts stained with the SYBR green were considered to account for the total number of bacteria, whereas the autofluorescent cell counts were considered to account for phototrophic bacteria. The difference between both values was considered the number of heterotrophic bacteria.

Chlorophyll *a* concentration was used as an estimate of eukaryotic phytoplankton biomass. Approximately 200 mL of water was filtered with 45 μm nitrocellulose filters (Millipore). The filter was then extracted with acetone and chlorophyll *a*, and phaeophytin content was measured following Parsons et al.[38] Initial samples were taken from the bulk dilutions, whereas final samples were taken from each replicate bottle.

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Chlorophyll Fluorescence. Chlorophyll fluorescence kinetics was measured with a pulse-amplitude-modulated fluorometer (WATER PAM, Heinz Walz, Germany). Saturating light pulses (800 ms, 3000 μmol m^{-2} s^{-1}) given every 30 s provided an estimate of the effective quantum yield (Y), the electron transport rate (ETR), and nonphotochemical quenching (NPQ) in light-adapted samples. NPQ was measured across a range of light intensities using standard settings for light curves. The NPQ response to increasing light intensities was tested on the assumption that NPQ exhibits a simple exponential saturation curve with a value of zero at the origin and an asymptotic maxima NPQ = NPQ_{max} $(1 - e^{-kE})$, where k is a constant and E is light intensity.[39] Calculations were made using Wincontrol software (v3.18).

Data Analysis. Landry and Hassett[34] developed the dilution technique to estimate the mortality rate of the plankton community due to predator grazing. The technique assumes that plankton growth rate (μ) is independent of the dilution, that the rate of plankton mortality due to grazing (g) is proportional to the dilution effect on grazer abundance, and that the plankton grows exponentially over time t. For each bottle, the growth and grazing rates were calculated as

$$\frac{1}{t}\ln\left(\frac{B_t}{B_0}\right) = \mu - gD \quad (1)$$

where B_0 and B_t are, respectively, the initial and final bacteria concentration (cell count) or the chlorophyll a concentration, t is 1 day, and D is the dilution. The dilution series consisted of unfiltered to filtered lagoon water in the ratios 1:0 (100% unfiltered water); 3:1 (75%); 1:1 (50%); and 1:3 (25%). Plotting the linear regression of the dilutions versus the apparent growth rates allowed the estimation of grazing rates. Originally, Landry and Hassett[34] proposed that a linear relationship would be obtained, but later work has shown that this is not always the case. Gallegos[40] fit nonlinear models to the results of dilution experiments, and since then, a number of authors have attempted to improve the fitting of nonlinear models to experimental data.[41−43] We used the approach of Redden et al.,[44] which assumed a grazing rate linearly proportional to the plankton concentration up until a value for which the plankton concentration becomes independent from the grazing rate. When this concentration that saturates grazing (B_s) is reached, eq 1 can be split as

$$\frac{dB}{dt} = \begin{cases} \mu B - gDB & \text{if } B < B_s \\ \mu B - gDB_s & \text{if } B \geq B_s \end{cases} \quad (2)$$

The slope of the linear relationships was assessed with a t-test. A one-way ANOVA was used to test for the effect of Ag NPs toxicity after testing for homoscedasticity using Levene's test. When ANOVA revealed significant differences among treatments, a Dunnett's test was conducted to test for pairwise differences between each treatment and the control. GraphPad Prism 6 was used for the calculations.

RESULTS

The flow cytometry counts allowed the distinction of two groups of cells: autofluorescent phototrophs and nonautofluorescent heterotrophs. In oceanic samples, the cyanobacteria *Synechocystis* sp. and *Prochlorococcus* sp. can commonly be separated from other phototrophs using flow cytometry, primarily due to differences in size and fluorescence.[36] However, in our work no such distinction could be seen, and these cyanobacteria were considered to be absent from the samples. Data from the flow cytometry counts showed that heterotrophic bacteria represented 95% of the bacterioplankton community at the UCSB lagoon. Values for the unfiltered lagoon water were 10^8 and 10^6 cells mL^{-1} for heterotrophic and phototrophic bacteria, respectively.

Heterotrophic bacteria populations not exposed to Ag NPs grew at a mean rate of 1.41 ± 0.16 d^{-1}, while phototrophic bacteria populations grew slower, averaging 0.96 ± 0.21 d^{-1} (Figure 1). Exposure to Ag NPs repressed population growth rates of both heterotrophic and phototrophic bacteria at the 0.5 and 1 mg L^{-1} concentrations (Figure 1). This decrease was significant for both concentrations in the case of heterotrophic bacteria and was significant for 1 mg L^{-1} for phototrophic bacteria (Dunnett's test, $p \leq 0.05$). Eukaryotic phytoplankton, estimated as chlorophyll a concentration, exhibited slightly negative growth rates in the absence of Ag NPs, indicating that the number of individuals decreased throughout a 24 h period (Figure 2). Growth was even more depressed upon exposure to Ag NPs. Although exposure to Ag NPs affected chlorophyll a concentration in phytoplankton, it apparently did not affect phaeophytin concentrations because the ratio of phaeophytin to chlorophyll a was constant in the Ag NPs exposures (Figure 2).

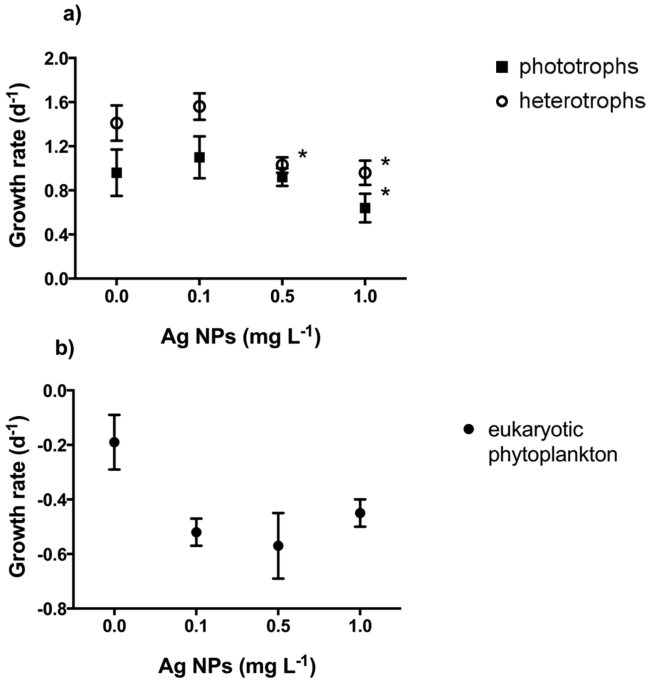

Figure 1. Growth rates of (a) phototrophic and heterotrophic bacteria and (b) eukaryotic phytoplankton exposed to Ag NPs. Symbols are mean values, and bars are the SEM of three replicates. Asterisks in panel a identify means that are significantly less than controls (Dunnett's test, $p \leq 0.05$).

Heterotrophic bacteria populations not exposed to Ag NPs showed a grazing rate of 1.21 ± 0.23 per day, whereas for the phototrophic bacteria, the grazing rate was 1.91 ± 0.24 per day (Figure 3). These data show that the grazing rate was higher for the phototrophic bacteria, suggesting a zooplankton preference for this prey. Moreover, the heterotrophic bacteria could have compensated for the grazing pressure because the growth rate was higher than the grazing rate, whereas for the phototrophic

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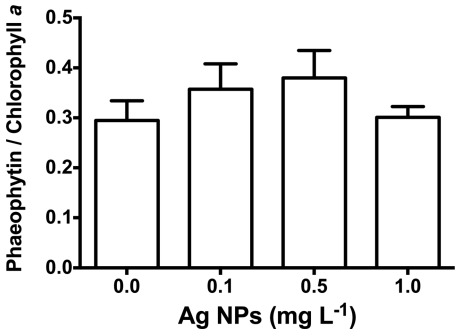

Figure 2. Ratio of photosynthetic pigments phaeophytin and chlorophyll *a* of the unfiltered lagoon water after 24 h exposure to different concentrations of Ag NPs. Columns are mean values, and bars are the SEM of three replicates.

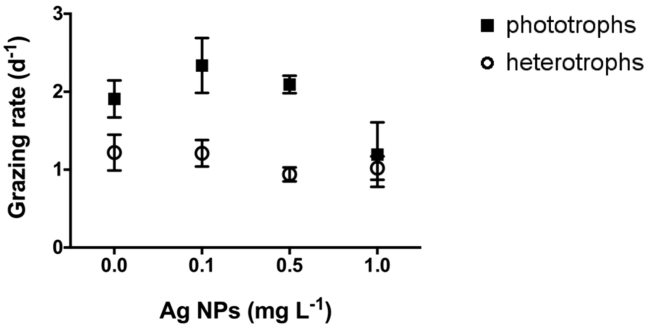

Figure 3. Grazing rates on phototrophic and heterotrophic bacteria exposed to Ag NPs. Symbols are mean values, and bars are the SEM of three replicates.

bacteria, the grazing rate was almost twice as high as the growth rate.

In treatments exposed to Ag NPs, both heterotrophic and phototrophic bacteria grazing rates declined with Ag concentration up to 1 mg L^{-1} but did not differ significantly from the control (Dunnett's test, $p \leq 0.05$) (Figure 3). Given that eukaryotic phytoplankton growth was slightly negative in every dilution tested, the grazing rates were not calculated because phytoplankton growth over time was required for grazing rate calculations through the dilution technique.

The kinetics of the chlorophyll fluorescence showed that both the effective quantum yield and the electron transport rate were significantly reduced (Dunnett's test, $p \leq 0.05$) in the 0.5 and 1 mg L^{-1} Ag NPs concentrations (Figure 4). Moreover, for the NPQ values increased with increasing light intensities, the exposure to 1 mg L^{-1} Ag NPs concentration resulted in the highest increase (Figure 4).

DISCUSSION

The toxicity of NPs has been studied in a number of simple cellular systems under controlled exposure conditions. In natural communities, organisms interact through processes including competition, predation, mutualism, and facilitation,[45] increasing the complexity of possible outcomes and making predictions of the overall effects of toxicants difficult. For this reason, and because tracking multiple types of organisms can be difficult, the effects of NPs on natural communities are poorly known. For example, we do not know if NPs can cause trophic cascades, or other complex multitrophic-level impacts, in aquatic food webs. Here, for the first time, we evaluated the effect of Ag NPs on a natural estuarine plankton community.

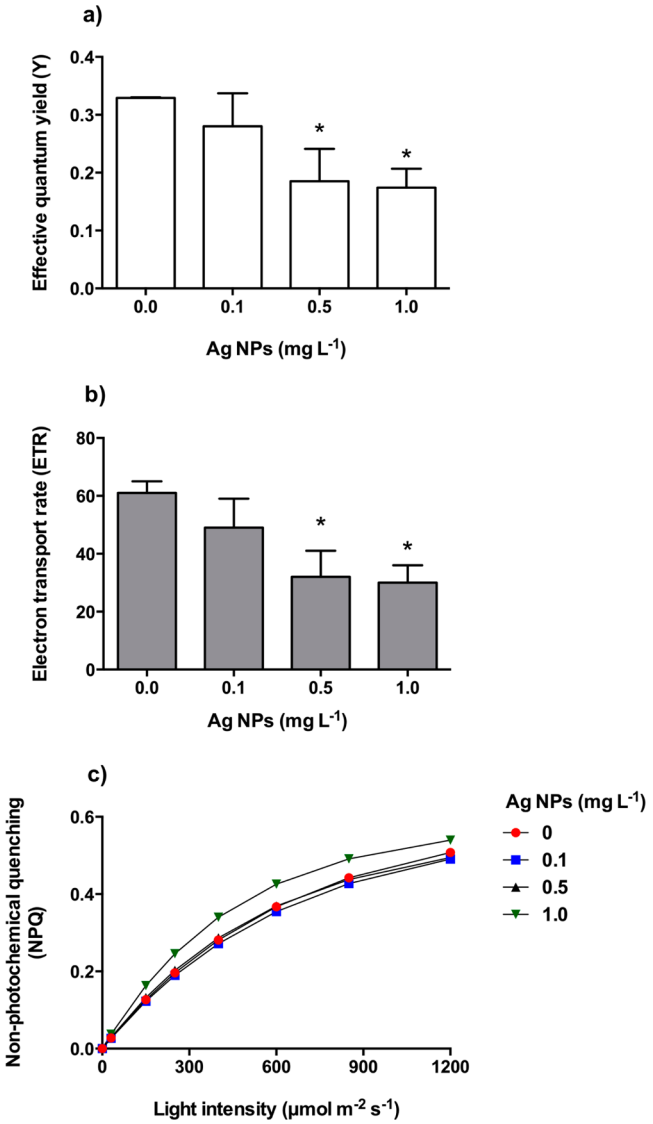

Figure 4. Chlorophyll fluorescence parameters of the unfiltered lagoon water after 24 h of exposure to Ag NPs. (a) Effective quantum yield, Y; (b) electron transport rate, ETR; and (c) nonphotochemical quenching, NPQ. Columns and symbols are mean values, and bars are the SEM of three replicates. In panel c, error bars are omitted for reading simplicity. Asterisks identify means that are significantly less than controls (Dunnett's test, $p \leq 0.05$).

We found that the numerically dominant organisms, heterotrophic bacterioplankton, declined in growth rate with increasing Ag NP exposure, but that grazing rates on these organisms by zooplankton also tended to decline, thus possibly reducing the direct negative effect of the NP toxicity. Grazing rates on another group of prey, phototrophic bacteria, also declined slightly with exposure to Ag NPs, offsetting the declining growth rate but not enough to counteract the overall negative effect. Therefore, the prediction that a trophic cascade would result from toxic effects of Ag NPs was not supported.

Populations of eukaryotic phytoplankton, as measured by chlorophyll *a* concentration, were in slow decline in our controls, and this decline was accelerated under exposure to Ag NPs. The decline in control populations apparently was not due to low nutrient availability because nutrient concentrations were relatively high throughout the experiment. Instead, the probable cause was bacterial-induced lysis of senescing cells.[46]

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This decline made grazing rates unmeasurable, and grazing was likely low. This situation has occurred in previous studies employing the dilution technique to estimate grazing rates.[47,48] Murrel and Hollibaugh[47] proposed that larger grazers not represented in dilution experiments could sometimes control the grazing rate of the phytoplankton community. In this study, a visual inspection of water samples (via light microscopy) showed that copepods were the dominant large grazer in the study lagoon. The copepod *Acartia tonsa* exhibited reduced reproduction and increased mortality following the consumption of ionic Ag-contaminated diatoms.[49] This implies that Ag NPs could also impact the consumption of phytoplankton by these large grazers.

The negative impact of Ag NPs on the growth of phytoplankton and phototrophic bacteria prompted the analysis of the effect of Ag NPs on the photosynthetic capacity of these primary producers. Photosynthesis is a fundamental process, making carbon available to phytoplankton cells and ultimately food webs, and its inhibition by toxicants can be an indicator of sublethal effects.[50] Quantum yield is a measure of the photons absorbed and, therefore, the photosynthetic capacity of the cell.[51] Electron transport rate is a parameter proportional to Y and is expected to vary in accordance with Y.[39] Our results show that both indicators of photosynthetic performance were affected by Ag NPs at concentrations of 0.5 mg L^{-1} and greater. Furthermore, during photosynthesis the energy absorbed is divided between the fraction used in photochemistry and that lost nonphotochemically.[52] The quenching by so-called nonphotochemical processes (NPQ) is the degree to which photons are lost in the photosynthetic process.[52] NPQ is, therefore, a competing nonproductive pathway.[39] We found that at 1 mg L^{-1} Ag NPs, losses of photons to NPQ increased compared to the controls. The measurement of NPQ as a function of the light intensity showed that as the light level increased, the system lost increasingly more photons to NPQ processes, and this loss was higher in Ag-exposed populations. This suggests that photoinhibition increases with exposure to Ag NPs.

Metallic nanomaterials are transformed as they travel through the environment, undergoing changes in aggregation and oxidation state, precipitation of secondary phases, and sorption of organic and inorganic species.[53] Nanosilver may undergo partial or complete sulfidization that may affect toxicity.[53,54] Our study was executed in natural eutrophic lagoon water. Therefore, the Ag NPs in our experiment were almost assuredly exposed to natural transformation processes, likely including partial sulfidization. Ag NPs are employed mainly because of their antimicrobial properties, and microbial communities are perhaps most at risk of toxicity. Our results show the clearest impacts at Ag NP concentrations ≥0.5 mg L^{-1}, an order of magnitude higher than the levels predicted to occur in surface waters.[55] In contrast, several studies have shown impacts on aquatic and marine bacterial and other organisms at concentrations as low as only a few μg per liter.[15,56] For example, Doiron et al.[57] showed evidence of reduced population growth in marine bacterial communities exposed to Ag NPs and ionic silver at only 5 μg per liter. This was evident as a lengthened lag phase in the growth curves that recovered after ~48 h. However, species richness was reduced, suggesting that certain species were more tolerant of Ag exposure. Whatever the mechanism, our results suggests that at the community level, species interactions and feedbacks may dampen any small effects that are seen at the individual organisms level at lower concentrations.

Dilution and ecotoxicity experiments are necessarily short-term to avoid artifacts, sometimes called "bottle effects".[58] In long-term exposures to Ag NPs in the environment, we would expect that the continued grazing of bacteria, combined with decreases in growth rate and photosynthesis, could eventually lead to the depletion of the food supply for the grazers and the collapse of the system to a simpler and less productive food web. Combined with other stressors, including the effects of overfishing and nutrient loading, chronic inputs of toxicants including Ag NPs could therefore cause significant changes in food-web structure and the loss of ecosystem services.[59−61] Nevertheless, the effects seen in this study were only evident at higher concentrations of Ag NPs than those that are often toxic to individual species, suggesting that species sorting or other processes may ameliorate toxic effects at the community level.

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Notes

The authors declare no competing financial interest.

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