Addition of silver nanoparticles has no long-term effects on natural phytoplankton community dynamics in a boreal lake

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Abstract

Lake phytoplankton communities are dynamic with well-documented seasonal variability in taxonomic composition and biomass. However, this variability has been largely overlooked when assessing the risk posed to aquatic ecosystems by the antimicrobial agent, silver nanoparticles (AgNPs). Here, we report results from a whole lake AgNP addition study at the IISD-Experimental Lakes Area that assessed the effects of AgNPs on phytoplankton. Phytoplankton communities were largely unaffected in terms of taxonomy, pigment concentration, and biomass by AgNP additions. These negative toxicological results are due to community changes being more strongly affected by natural processes, such as temperature and dissolved nutrients. We conclude that AgNP exposure at environmentally relevant concentrations for 2 yr did not affect phytoplankton communities in boreal lakes, which emphasizes the importance of incorporating natural variability into analyses that attempt to determine the effect of contaminants on aquatic ecosystems.

Silver nanoparticles (AgNPs) are an antimicrobial agent used in many industrial and consumer products. Given recent increases in their use and their high likelihood of entering wastewater streams after washing out of products (Massarsky et al. 2014; Sharma et al. 2014), AgNPs are considered an emerging threat to aquatic ecosystems. Thus,
there is a need to study the fate and toxic effects of AgNPs to assess their effects on aquatic ecosystems. Phytoplankton in particular seem to have extremely varied responses when exposed to AgNPs. Both AgNPs and the silver ions they release can negatively affect phytoplankton at short time scales by decreasing photosynthetic abilities, decreasing Adenosine triphosphate (ATP) production, and increasing lipid peroxidation, primarily through the generation of reactive oxygen species (Oukarroum et al. 2012; Pillai et al. 2014). At predicted environmentally relevant concentrations (below 1–2 μg L$^{-1}$; Massarsky et al. 2014), algae may experience some toxicity as their EC$_{50}$ values for growth inhibition and reduced chlorophyll $a$ (Chl $a$) content range between 0.02–11 mg L$^{-1}$ and 3–500 μg Ag L$^{-1}$, respectively (Griffitt et al. 2008; Das et al. 2014; Baptista et al. 2015; Leonardo et al. 2016). However, phytoplankton also have response mechanisms to heavy metals such as increasing carotenoid pigments and to silver such as increasing silver efflux mechanisms to cope with the stress of metallic contaminants (Okamoto et al. 2001; Pillai et al. 2014; Leonardo et al. 2016).

Seasonal variability in the taxonomic composition of phytoplankton is a prominent feature of lakes. However, these dynamics are underrepresented in literature surrounding the effects of AgNPs on phytoplankton communities. Based on changing light, nutrient availability, and temperature, phytoplankton populations experience periods of growth and decline throughout the year (Fee 1976; Carpenter et al. 2001), which may connect to their ability to cope with the stress of AgNPs or act as more important drivers of change in terms of algal dynamics throughout the season. Few previous studies have been long enough in duration to understand how phytoplankton will react to AgNPs when seasonal variability and cellular repair mechanisms have time to play a role. In one of the longest studies to date, Vincent et al. (2017) detected no response to AgNPs in the phytoplankton pigments of boreal lake mesocosms, but did not examine the taxonomy of the algae. Small experimental scales (e.g., microcosms and small mesocosms) also may constrain shifts in taxonomic composition caused by contaminant exposure due to small and isolated species pools and insufficient exposure times to allow for changes in community composition (Schindler 1998; Benndorf et al. 2002). Consequently, the environmental risk of AgNPs to natural phytoplankton communities remains uncertain. In a multiyear study in a whole lake ecosystem, with environmentally relevant concentrations of AgNPs, the phytoplankton may change the community composition as a result of stress caused by the AgNPs.

To test this idea, we conducted a whole-lake experiment by adding AgNPs to a boreal lake at the IISD-Experimental Lakes Area (IISD-ELA) over two ice-free seasons. We sampled phytoplankton communities regularly for 2 yr before and 2 yr during the additions in the experimental lake and a reference lake to determine if taxonomic diversity, algal biomass, pigments concentrations, or pigment ratios changed following exposure to AgNPs. We assessed how changes during AgNP additions were related to natural seasonal drivers of algal communities to determine if the effects of AgNPs were relatively greater than those of known natural drivers.

**Methods**

**Whole lake AgNP additions**

We conducted a whole lake AgNP addition in Lake 222 at the IISD-ELA in northwestern Ontario, Canada. During the 2014 and 2015 ice free seasons, we added a total of 15 kg of polyvinylpyrrolidone (PVP)-capped AgNPs with additional gum arabic stabilization from a point source near the lake in-flow. 10 kg were added in 2014 and 5 kg were added in 2015. Preparation and characterization of these AgNPs is described in depth elsewhere (Martin et al. 2017) but yielded AgNP suspensions that consisted of 30–50 nm diameter particles. We pumped concentrated AgNP suspensions into the lake every 6 h using a peristaltic pump on a timer. AgNPs were detected at all sampled locations throughout the lake within 24 h of the first dose. Total silver concentrations (TAg) in the lake epilimnion were highly variable and ranged between 0 μg L$^{-1}$ and 17.4 μg L$^{-1}$ and the silver in sestonic particles (including algae) was high at first (3.8 μg Ag mg C$^{-1}$), then stabilized to between 0.6 μg Ag mg C$^{-1}$ and 1.6 μg Ag mg C$^{-1}$ (Fig. 1). Mesocosm studies at the IISD-ELA using PVP-capped AgNPs have found that silver has high sedimentation rates, but also is found in variable amounts in the water column, sediments, and all food-web levels, but not as silver ions (Furtado et al. 2015).

**Field sampling/preservation**

We collected single samples of phytoplankton communities from the epilimnion (integrated 0.5–1.5 m depth) at the center of the experimental lake and the reference lake (Lake 221) at monthly intervals in 2012, 2013, and 2015 and every 2 weeks during the first year of additions (2014). Lake 221 and 222 are shallow (5.5 m and 6.5 m maximum depth, respectively) dimictic lakes of similar size that are located next to each other and exposed to nearly the same weather. These lakes were chosen from the available lakes at the IISD-ELA due to their close geographical proximity, general physical and chemical similarity, and to comply with Canadian government regulations restricting intentional addition of contaminants to freshwater ecosystems. They both have relatively high dissolved organic carbon (DOC), low nutrients, and diverse food-webs with multiple species of zooplankton grazers and fish (Supporting Information Table 1). We collected water from both lakes using a Van Dorn sampler and brought it back to the lab for processing. We screened the water through a 35 μm filter to remove zooplankton and large detritus before preserving subsamples for algal pigments, algal counts, and total and particulate silver. We collected pigments on pre-ashed glass fiber filters (GFF) that were immediately frozen and stored in the dark. We...
preserved algal taxonomy samples in ~ 2% Lugol’s acid solution. We sampled total silver by preserving screened water and acidifying the sample to 4% nitric acid. Particulate silver and particulate mass (carbon) were filtered onto 0.8 μm filters and preserved in nitric acid or dried, respectively. All silver samples were digested using a nitric acid/hydrogen peroxide digestion and analyzed by inductively coupled plasma mass spectrometry (ICP-MS) (Furtado et al. 2015).

Particulate carbon was measured using an elemental analyzer. Limnological variables including temperature, dissolved oxygen (DO), DOC, stratification strength (Brunt Vaisala buoyancy frequency), pH, total dissolved phosphorus, and total dissolved nitrogen were collected and measured concomitantly with the algal community and analyzed using multiprobes or standard colorimetric methods (APHA 1992; Crumpton et al. 1992).

**Fig. 1.** Biomass of algal groups over the 2 yr pre-study (2012 and 2013) and the 2 yr during AgNP additions (2014 and 2015) in both the experimental lake (panels a, b, c, and d) and the reference lake (panels e, f, g, and h). Dashed vertical lines refer to the start and stop of AgNP additions during that year. Numbers above the bars indicate the total silver concentrations (μg L⁻¹, top number) and the particulate silver per particulate mass (μg Ag mg C⁻¹, bottom number) at the time algal samples were taken.
Table 1. BACI results for pigments per unit biomass and pigment ratios between the reference lake (L221) and experimental lake (L222) before and during AgNP additions in the spring, summer, and fall. Bonferroni corrected \( p \) value for significance is 0.005 for biomass and 0.006 for pigment ratios. Bold values indicate marginally significant results with \( p \) values between 0.05 and the corrected value.

| Pigment                  | Spring (ng pigment mg biomass\(^{-1}\)) | Summer (ng pigment mg biomass\(^{-1}\)) | Fall (ng pigment mg biomass\(^{-1}\)) |
|--------------------------|----------------------------------------|----------------------------------------|------------------------------------|
|                          | Ref Pre AgNPs | Exp Pre AgNPs | Ref Pre AgNPs | Exp Pre AgNPs | p value | Ref Pre AgNPs | Exp Pre AgNPs | Ref Pre AgNPs | Exp Pre AgNPs | p value |
| Alloxanthin               | 143 235 155 28 0.46 | 139 200 140 283 0.11 | 265 265 277 273 0.99 |
| Aphaxanthophyll           | 200 102 132 199 0.37 | 98 84 132 302 0.08 | 305 469 294 94 0.39 |
| \( \beta \)-carotene      | 168 165 216 245 0.40 | 121 138 152 227 0.01 | 93 132 118 170 0.89 |
| Canthaxanthin             | 160 326 89 57 0.47 | 149 255 97 250 0.72 | 266 238 135 102 0.94 |
| Chl a                     | 650 3046 891 193 0.29 | 1361 1793 1572 2533 0.76 | 983 2864 989 736 0.43 |
| Chlorophyll b             | 2345 3402 1099 1074 0.98 | 2271 3919 1273 3058 0.99 | 4862 4740 2978 1711 0.53 |
| Diadinoxanthin            | 46 129 49 42 0.40 | 57 155 56 91 0.32 | 87 67 27 65 0.52 |
| Echinonone                | 90 158 28 79 0.82 | 64 114 44 58 0.37 | 72 106 43 28 0.44 |
| Fucoxanthin               | 245 729 370 336 0.47 | 386 639 430 765 0.58 | 721 730 301 488 0.76 |
| Myxoxanthophyl            | 154 376 128 120 0.35 | 146 340 169 390 0.89 | 305 236 98 81 0.82 |

**Algal biomass**

We assessed algal biomass and species composition using the Utermöhl method (Utermöhl 1958). Ten milliliters of preserved water was settled for 24 h and an inverted microscope at 400X was used to identify and measure at least 400 algal units per sample. We estimated biovolume using measurements taken for at least 15 individuals of each taxa, which was then converted to wet biomass using a specific gravity of 1 (Hillebrand et al. 1999). We identified algae to genus and separated cells in this group into size categories (large, medium, and small; \(<10\ \mu m, 10–15\ \mu m, >15\ \mu m\), respectively). Similarly, we found some cyanobacteria too small to identify. The colonial cyanobacteria genera *Aphanocapsa, Microcystis,* and *Gomphosphaeria* were grouped as “cyanobacteria colonies.”

**Pigments**

We analyzed frozen samples on filters to determine the concentration and relative ratios of pigments indicative of different algal groups (Leavitt and Hodgson 2001). We extracted pigment from filters for 24 h in an 80% acetone and 20% methanol solution, then removed filters and dried the samples in the dark with N\(_2\) gas, before resuspension in injection fluid consisting of 70% acetone, 25% ion pairing reagent, and 5% methanol. We analyzed samples using high performance liquid chromatography (HPLC) with a reverse phase column (Alliance Waters 2695 Separations Module, Waters 2475 Multi \( \lambda \) Fluorescence Detector). We determined pigments concentrations using known pigments standards for each pigment (Table 1) and divided them by biomass estimates to determine the concentration of pigments within cells. Pigment ratios of carotenoid pigments to Chl \( a \) were calculated to determine if the phytoplankton were experiencing oxidative stress from reactive oxygen species.

**Statistical analysis**

We used the reference condition approach (RCA) and nonmetric multidimensional scaling (NMDS) to compare communities at sampling times after addition of AgNPs. We defined the reference condition as all sampling times in the reference lake and all sampling times in the 2 yr prior to AgNP additions in the study lake to satisfy recommendations for the number of reference sites using the RCA approach (Bowman and Somers 2005). We performed NMDS by comparing Bray-Curtis dissimilarity indices among each sampling
point. Bray-Curtis dissimilarity determines differences between community composition based on presence and abundance of each taxa. We calculated the NMDS of the reference condition points using 50 iterations of the data starting at randomized points to minimize stress. We placed a 95% confidence interval around the reference condition points and then plotted the experimental points on the same NMDS axes (White et al. 2011). We determined points that fell outside of the confidence interval to be statistically different from those of the reference condition. These analyses were performed at the genus level to avoid incidences of rare species biasing the results. We performed the NMDS analyses using the vegan package in RStudio version 3.

Individual species (or genera when species were unavailable) biomass, pigment concentrations, and pigment ratios in the spring (May and June), summer (July and August), and fall (October) were compared before and after AgNP exposure using Before-After-Control-Impacted (BACI) analysis based on differences between the experimental and reference lakes. At all paired time intervals, the variable of interest in the experimental lake was subtracted from the variable in the reference lake and then a t-test with Bonferroni table wide correction was performed comparing the differences between pre-exposure and exposure years. Adjusted p values for significance were 0.0001, 0.005, and 0.006 for taxa biomass, pigments, and pigment ratios, respectively. As Bonferroni corrections can sometimes be deemed overly conservative for large numbers of comparisons such as ours (Perneger 1998), we considered any p value between 0.05 and the adjusted p value to be marginally significant. BACI analysis was performed using SigmaPlot 12.5 software.

Pearson’s correlation coefficients were used to determine if there was any relationship between lake total silver concentrations and particulate silver concentrations and indicators of community function. Shannon’s diversity based on genera biomass was calculated using the vegan package in RStudio version 3. Total algal pigments and total algal biomass calculations and the Pearson’s correlations were performed using SigmaPlot version 12.5. Additionally, we performed a canonical correspondence analysis (CCA) to determine if natural limnological parameters influenced the community composition of the experimental lake more or less than TAg. All data were log(x + 1) transformed prior to analysis and the CCA was performed using the vegan package in RStudio version 3.

**Results**

**Community and individual dynamics**

There were no differences in the biomass of phytoplankton between the two lakes but instead considerable seasonal and yearly variability in both lakes (Fig. 1). We used the RCA and NMDS to determine a 95% confidence interval around the reference conditions based on data from 2 yr in the study lake (L222) prior to AgNPs and 4 yr of data from the reference lake (L221; Fig. 2A). We found no differences in community composition (based on the biomass of algal genera) between the experimental period in L222 and the reference conditions. One sampling event from the late summer in the second year of AgNP addition was slightly outside of the reference conditions due to a slightly greater biomass in L222 of the very large sized, but generally rare dinoflagellate, *Ceratium*.

We also compared concentrations of different algal pigments using the same statistical approach of NMDS and RCA. Communities in L222 exposed to AgNPs were not different to reference conditions except for one point that occurred 5 d after the first dose of AgNPs (Fig. 2B). This algal community experienced a large decrease in all measured pigments and an increase in pigment : Chl a ratios (Table 1) from the previous sampling event, but levels returned to reference levels by the next sampling 2 weeks later.

BACI analysis was used to determine if there were changes in biomass of individual phytoplankton species, pigment...
concentrations, and pigment ratios before and after additions in comparison to the reference lake. No species, pigments per biomass, or pigment ratios exhibited a significant difference before and after additions in any season (Table 1 and Supporting Information Table 2). The green algae *Chlamydomonas* sp., *Pediastrum tetras*, and *Quadrigula closterioides* the diatoms *Cyclotella stelligera*, *Rhizosolenia eriensis*, and *Tabellaria fenestrata*, as well as the pigment β-carotene all had marginally significant differences when exposed to AgNPs in at least one season, but not in a consistent direction and typically not a decrease in biomass (Table 1; Fig. 3).

**Effect of silver concentration on community indices**

We used Pearson correlations to determine if silver concentrations in L222 were related with community metrics including total biomass, total pigments, and Shannon’s diversity (Fig. 4). There was no evidence that variability in these responses was associated with total or particulate Ag concentrations, as there were no significant correlations detected. CCA analysis yielded a model where axis 1 explained 56% of the variation and was most related to temperature, stratification, DO, and DOC. Axis 2 explained 23% of the variation and was most related to DO, TDN, and TAg (Supporting Information Fig. 1).

**Discussion**

Our results suggest that AgNPs do not have an effect on algal communities over a 2 yr period, a longer time scale than those previously tested. We did not find any removal or large blooms of any taxa during the 2 yr of experiments, and the seasonal progression of total algal biomass and the yearly variability in community composition is comparable to those of other unimpacted boreal lakes at the IISD-ELA (Fee 1976; Findlay et al. 2001). We see evidence of the naturally dynamic processes of ice-free seasons and years in our CCA analysis as temperature, stratification, DO, and DOC. Diversity, total biomass, and total pigments also showed no correlation with total silver concentrations or particulate silver concentrations indicating a higher influence of natural variables. Similar to the results of a several week long, lake mesocosm study with AgNPs, at the longer temporal scale natural variability in our ecosystem outweighs any effects the AgNPs may be causing on algal communities (Vincent et al. 2017). It is also possible that in both of these studies natural variables such as dissolved organic matter (DOM) and dissolved ions are helping to increase AgNP stability, which can decrease toxic effects to organisms such as algae (Sharma et al. 2014).

At shorter timescales such as individual seasons and sampling points, we saw marginally significant small effects that are more similar to those found in the AgNP literature. BACI analysis showed that three species of green algae and three species of diatoms had marginally different dynamics than that of the reference lake in either the fall, spring, or summer months, but never across the whole year. Interestingly, none of these differences were associated with a decrease in the species in the experimental lake during additions compared to before additions. All of the differences were characterized by either a change in the reference lake and no change in the experimental lake or a slight increase in the biomass of the species during AgNP additions. Previous studies of stream periphyton using mesocosms have seen similar increases in green algae species or no response to green and diatom species in response to AgNPs (González et al. 2015; Kroll et al. 2016). Other small scale studies with stream and temperate lake water have seen decreases in biomass of all measured algal species, particularly diatoms and cyanobacteria, however, these studies used AgNPs with...
different, typically more toxic capping agents (carboxy-functionalized polyacrylate and citrate), and higher concentrations than those in our experiment (Das et al. 2014; González et al. 2015).

We also found small changes in algal pigments during our study with a decrease in all pigment concentrations and an increase in particulate silver and pigment ratios after the first dose of silver. These responses may be linked to the cellular adaptive responses that some algae exhibit when exposed to silver, where they experience decreases in photosynthesis and ATP concentrations before being able to upregulate silver detoxification mechanisms that can vary depending on silver concentrations (Pillai et al. 2014; Leonardo et al. 2016). The highest particulate silver concentration, twice any other measured concentration, occurred on the day when we saw low pigments and high pigment ratios. These adaptive responses to silver and their associated reactive oxygen species could be an explanation for the small scale transient responses in pigments seen in this study, though our temporal resolution is not fine enough to confirm it.

While our data point to no observable response in the algal community in the longer term when exposed to AgNPs, there are limitations to these conclusions. Our algal identification methods and use of only single samples may have...
caused us to miss effects on some species due to insufficient number in the sample, low magnification and/or loss of very large species due to filtration to remove zooplankton. We also did not account for the effect of AgNPs on other food-web components, specifically zooplankton grazers which may be more susceptible to AgNPs than phytoplankton (Griffitt et al. 2008). However, a lake mesocosm study at the IISD-ELA found that PVP-capped AgNPs only had subtle effects on the zooplankton community and that these effects had no impact the phytoplankton, so we feel this is likely the case for our study as well (Vincent et al. 2017). Despite these limitations, we nonetheless provide strong evidence that phytoplankton communities do not respond to environmentally relevant concentrations of AgNPs at seasonal scales across 2 yr and that natural seasonal and climatic drivers exert stronger effects on community dynamics at longer time scales. Our results indicate that results from short term experiments are unlikely to capture responses of algal communities to AgNPs in boreal lakes over longer time periods, especially at environmentally relevant concentrations of AgNPs.

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