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Dissolution of Silver Nanoparticles in Stratified Estuarine Mesocosms and Silver Accumulation in a Simple Planktonic Freshwater Trophic Chain

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Abstract: The increasing presence of nanomaterials in consumer products has led the scientific community to study the environmental fate of these contaminants of emerging concern. Silver nanoparticles, used mainly for their antibacterial properties, are among the most common nanomaterials. Understanding their transformations and interactions with living organisms, especially under environmentally relevant conditions that can modify metal bioavailability, is a crucial step in the study of their impacts on aquatic ecosystems. In the present study, citrate-coated silver nanoparticles (20 nm; 10 µg/L) were added to the surface freshwater layer of mesocosms simulating a stratified estuary. The investigation by dialysis of the nanoparticle dissolution showed that a large amount of total silver was found in the freshwater layer (and a very low amount in the seawater layer) and that 5–15% was in the form of dissolved silver. These results indicate that the halocline, separating fresh water from seawater, acted as a strong density barrier limiting the sedimentation of the nanoparticles. A simple trophic chain, composed of the freshwater alga *Chlamydomonas reinhardtii* and the invertebrate *Daphnia magna*, was used to determine silver bioavailability. This study suggests that citrate-coated silver nanoparticles do not significantly contribute to Ag accumulation by algae but may do so for invertebrates.

Keywords: silver nanoparticles; dissolution; accumulation; fresh water; *Chlamydomonas reinhardtii*; *Daphnia magna*

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1. Introduction

More than 50% of biocidal silver products such as nanotextiles, registered by the United States Environmental Protection Agency (USEPA), contain silver nanoparticles (AgNPs) [1]. Due to the rapid growth in their commercialization, AgNPs are likely to be released into the aquatic environment. However, there are still no fully developed analytical techniques that can specifically detect anthropogenic nanoparticles in complex matrices such as a natural environment [2,3]. Their predicted environmental concentrations (PEC) have been estimated by environmental fate models or material flow analysis in several studies (see [4] for a recent review). Gottschalk et al. predicted that silver nanoparticle concentrations would be between 0.09 and 0.43 ng/L and between 0.59 and 2.16 ng/L in U.S. and European surface waters, respectively [5]. The transformations of silver nanoparticles in the environment, mainly aggregation and dissolution [6], were reviewed recently [2,7,8]. These transformations depend on several physicochemical factors including particle size, concentration and coating, temperature, dissolved oxygen levels, pH, presence of ligands and ionic strength. They also strongly affect the bioavailability of AgNPs towards aquatic organisms [9,10]. Therefore, it is essential to carry out studies on nanoparticles under environmentally relevant conditions so as to better assess their behavior in complex natural

environments and the potential combined effects of these factors on the bioavailability and toxicity of nanoparticles [11,12]. It was hypothesized that under conditions close to those encountered in a natural system, AgNPs would be highly scavenged by suspended particles and have thus a very low bioavailability to aquatic organisms.

Among the possible approaches to mimic natural systems, mesocosms have been used on several occasions to study the environmental fate and biological effects of AgNPs for both freshwater [13,14] and marine ecosystems [15,16]. Although these studies focused on benthic organisms or macrophytes, in this study the bioavailability to two planktonic species was examined. Furthermore, this study examined the behavior and fate of AgNPs at relatively low concentrations in a model stratified and dynamic estuarine system, generated in mesocosms.

Two model organisms were selected, *Chlamydomonas reinhardtii* and *Daphnia magna*, to examine the bioavailability of Ag after addition of AgNPs to these mesocosms. *Chlamydomonas reinhardtii* is relatively easy to culture and is tolerant to changes in pH [17] and salinity [18]. It has also been used repeatedly in the study of interactions with silver nanoparticles [19,20] and ionic silver [18,21,22]. *Daphnia magna* is often used in standard toxicity protocols (e.g., [23]) as it is highly sensitive to metals. Daphnids are used as benchmark organisms to provide an estimate of the impacts of metals on freshwater zooplankton. Moreover, they can be exposed to both waterborne and diet-borne metals, allowing a better understanding of the relative contributions of food and water to the bioaccumulation and toxicity of metals.

In this context, the objectives of this study were (1) to determine the release of silver ions from citrate-coated silver nanoparticles (c-AgNPs) that were introduced at low concentrations into a natural fresh water overlying a saline layer, and (2) to assess the bioavailability of Ag to phytoplankton and zooplankton in the freshwater component of a model stratified estuary.

2. Materials and Methods

2.1. Mesocosm Design

This study was part of a collaborative project conducted over a period of 35 days. A schematic diagram of the installation is presented in Figure 1. Flow-through mesocosms were located at the Rimouski Institute of Marine Sciences (ISMER) aquaculture station (Pointe-au-Père, QC, Canada). Fresh water injected into the mesocosms came from the Rimouski (QC, Canada) aqueduct. The city tap water was dechlorinated prior to its use in the mesocosms. To approximate the natural composition of the Saint Lawrence River, clay (10 mg/L, SWy-2, The Clay Minerals Society, Boulder, CO, USA) and dissolved organic matter (DOM) were continuously added to the mesocosms. Every 10 days, 2200 L of water was taken from the Sainte-Anne River at Pointe-au-Père (QC, Canada). This water, rich in dissolved organic matter (60 $\pm$ 10 mg C/L), was added to the mesocosms at a flow rate of 50 mL/min in order to obtain a final organic carbon concentration of approximately 5 mg C/L in the freshwater layer. The temperature was measured daily at the surface (between 13.8 and 16.5 °C), at the interface between the two layers and at the bottom of the mesocosms (between 9.7 and 13.1 °C). Fluorescent tubes were placed above the mesocosms, creating a light/dark photoperiod (16/8) followed throughout the duration of the experiment. Fresh water, stored in a separate tank, was oxygen saturated by continuous air bubbling. The filtered seawater came from the Saint Lawrence River estuary (Pointe-au-Père, QC, Canada).

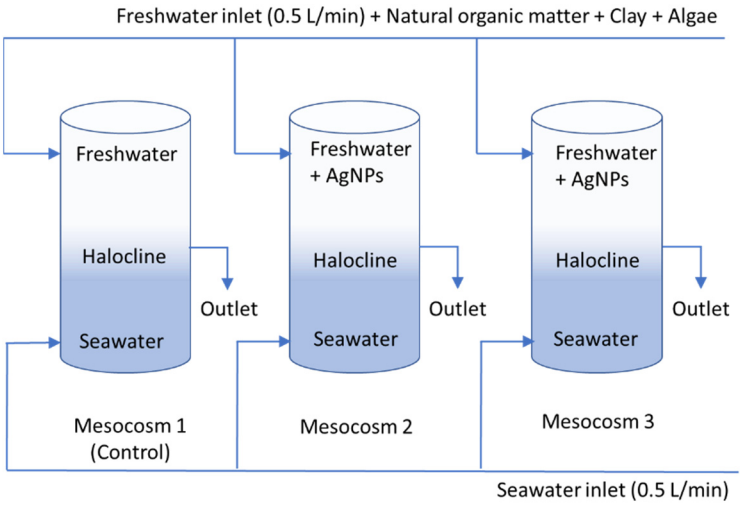

Figure 1. Mesocosms (diameter: 1.45 m; height: 2.1 m; volume: 3.4 m^3) set up using a continuous flow system mimicking interaction between fresh water and seawater. Mesocosm 1 was the control unit without silver nanoparticles added. Silver nanoparticles (20 nm; 10 µg Ag/L) were maintained constant by continuous additions to the surface of mesocosms 2 and 3. Algae (*Chlamydomonas reinhardtii*, 1500 ± 500 cells/mL), clay (10 mg/L) and dissolved organic matter (5 mg C/L) were also added continuously. The halocline was located at the level of the outlet.

Citrate-coated silver nanoparticles (c-AgNPs) of about 20 nm in diameter, were prepared by reducing silver nitrate with citrate (synthesis described in Millour, et al. [24]). After c-AgNPs synthesis, the suspension was cleaned by ultracentrifugation to remove more than 99% of the dissolved silver. The suspension was kept in the refrigerator and in the absence of light (for a maximum of 3 weeks before use). The c-AgNPs suspension was injected continuously at the top of mesocosms 2 and 3, mesocosm 1 serving as a control without silver addition. The final targeted c-AgNP concentration in the mesocosms was set at 10 µg Ag/L.

2.2. Determination of Dissolved Ag in the Surface Freshwater Layer

A dialysis device with a nominal molecular mass cutoff (MWCO) of 100–500 Da (Spectra/Pro® Float-A-Lyzer® G2, 10 mL, VWR, Mississauga, ON, Canada) was used to determine the dissolved (or dialyzable) silver concentration in each mesocosm (1, 2 and 3, see Figure 1) as described in Chen et al. [25]. Briefly, the device was soaked in 10% isopropanol for 10 min, and then flushed and soaked with deionized water for 15–20 min. Each dialysis device was initially filled with Milli-Q water, and inserted in the mesocosm water. Aliquots inside and outside the device were collected every 3 to 10 days to be analyzed by inductively coupled plasma—mass spectrometry (ICP-MS, Model XSeries 2, Thermo Scientific, Bremen, Germany; detection limit of 1.5 ng/L). Previous tests indicated that the equilibrium between Ag concentrations inside and outside the dialysis device could be established within 2 days [25]. The dialysis devices were sealed again after sampling and returned to each mesocosm until the dialysis volume had decreased by 20%, at which time the device was replaced. Replacement was conducted to avoid an increase in the equilibrium time. The dissolved silver concentration was defined as the Ag concentration in the dialysis devices. The total Ag concentration in the outer water was determined by sampling at 10 cm under the surface of the three mesocosms. It should be noted that all the samples from the three mesocosms in this study were pumped from a point 10 cm below the surface of the freshwater layer in order to avoid surface agitation and disruption of the biofilm formed at the air/water interface. Unless otherwise mentioned, the samples were kept in polypropylene containers at 4 °C in the dark.

Samples to be analyzed by ICP-MS were acidified (2% HNO_3; trace metal grade). Instrument response was calibrated with standard solutions obtained from PlasmaCAL SCP Science, Baie D'Urfé, QC, Canada. An internal standard solution of ^{103}Rh (58 nM) was used to correct analytical signal suppression (or enhancement) due to matrix effects or signal fluctuations. Corrections were made for both ZrO^+ and NbO^+ spectral interferences. Custom ICP Standards (SCP Science, Baie D'Urfé, QC, Canada) were used as controls to verify the precision and accuracy of the method. The inorganic carbon concentration was measured using a total organic carbon analyzer (TOC-VCPH, Shimadzu, Kyoto, Japan). Chloride and sulfate ion concentrations were determined by ion chromatography in the three mesocosms (ion exchange resin: Ion PAC AS18 4 mm, Dionex ICS-2000, Bannockburn, IL, USA).

2.3. Algal Culture and Cell Density in Mesocosms

In order to avoid bacterial contamination, all the material used for the algae cultures or their transfer was sterilized by autoclaving at 121 °C for 15 min (Sanyo Labo Autoclave, Osaka, Japan). The green alga *Chlamydomonas reinhardtii* (CPCC 11, wild type, Canadian Phycological Culture Center, University of Waterloo, Waterloo, QC, Canada) was grown in a chemostat initially placed in an environmental chamber (Conviron CMP4030, Winnipeg, MB, Canada) at 22 °C under 100 ± 10 $\mu E/m^2/s$ illumination and rotary agitation (50 rpm), with constant bubbling with filtered ambient air (Millex Syringe Filter Units, Sterile, 50 mm, Hydrophobic PTFE, Bedford, MA, USA). Fresh culture medium (modified high salt medium; MHSM-1), the composition of which is shown in Table S1, was continuously added to the chemostat. The algae were removed at the same time and at the same rate so as to keep a constant volume in the chemostat. The culture medium was prepared from six stock solutions stored in the dark at 4 °C and pH was buffered to pH 7 using MOPS (1 mM). These solutions were previously filtered through a 0.2 µm filter in order to eliminate any bacteria, in addition to any particles that could create a bias when counting the algae or adsorb the metals in solution. The culture solution, before addition of the trace elements (AAP: algal assay procedure), was sterilized in the autoclave. At the end of the algal growth cycle (stationary phase, ~1.5×10^6 cells/mL), the pumping rate was set at ~1 mL/min so that the cell density remained constant. Cell density in the chemostats was measured with a particle counter (Multisizer 3, Beckman Coulter, Brea, CA, USA; 70 µm aperture). An aliquot of the culture was taken and diluted in an isotonic solution (Isoton® II Diluent, Beckman Coulter, Brea, CA, USA) with a dilution factor depending on the cell density to be analyzed. The cell density in the chemostat, expressed as the number of cells/mL, was then deduced from the number of cells given by the particle counter, taking into account the dilution factor and the volume analyzed.

Three of these chemostats were transferred to the ISMER aquaculture station for use. At the beginning of the experiment, an appropriate quantity of algae was directly introduced into the mesocosms in order to reach a cell concentration of 1500 ± 500 cells/mL. Subsequently, the algae, produced by the chemostats, were continuously added to the mesocosms using a peristaltic pump in order to maintain a relatively stable cell concentration in the mesocosms. In order to determine the cell concentration in the mesocosms, a fluorometric determination of chlorophyll-*a* was carried out. Fresh water recovered from the mesocosms and stored in amber borosilicate glass containers (250 or 500 mL) in the dark and at 4 °C was filtered through a 3 µm nitrocellulose filter. Chlorophyll-*a* was then extracted from the filters with 10 mL of a 90% acetone solution. The samples were kept for a minimum of 24 h in the dark at −20 °C followed by 2 min vortex agitation. They were finally centrifuged at 1500 to 2000 rpm for 10 min before analysis (excitation and emission wavelength: 436 and 680 nm, respectively) on a fluorometer (model 10, Turner Designs, San Jose, CA, USA). A calibration curve was obtained by diluting the initial algae culture solution of known cell concentration several times in mesocosm 1 (control) water and by measuring the chlorophyll-*a* in each of the solutions as indicated above (Figure S1).

2.4. Silver Bioaccumulation by the Green Algae

Mesocosm water was first centrifuged to remove most of the suspended clay. This initial step was required to facilitate the harvesting of algal cells subsequently added for the short-term exposure experiment. The effective centrifugation speed was determined in preliminary trials by comparing the turbidity of the supernatant with that of the filtrate (0.2 µm polycarbonate filters), using a Turbidimeter (model 2100A, Hach, London ON, Canada). Total Ag concentration and total organic concentration (TOC) were also measured before and after centrifugation, to evaluate losses due to centrifugation, using an ICP-MS and a TOC analyzer, respectively.

Cells (~40,000 cells/mL) were then exposed for 1 h to centrifuged mesocosm water. Three replicates per mesocosm were performed. To consider the potential effect of particles that may not be removed by the centrifugation process, supernatants from the three mesocosms without algae (three replicates) were also used as controls. At the end of each exposure, cells were separated from the medium by filtration through two 2 µm polycarbonate filters. Following this filtration step, the algae were rinsed by 4 × 10 mL 200 µM $Na_2S_2O_3$ to remove Ag bound to the algal surface [18]. The upper filter (collected algae) and the lower filter (adsorption control) were then separated, dried for 24 h at 70 °C, and digested with concentrated HNO_3 at 90 °C for 1 h. The samples were analyzed by ICP-MS after appropriate dilution.

The thiosulfate rinse solution was also collected after filtration to determine the amount of silver adsorbed on the surface of the cells. The dissolved Ag concentration before and after exposure was determined by centrifugal ultrafiltration [26]. The centrifugal filter devices (Amicon ultra, Millipore, Bedford, MA, USA; 3 kDa MWCO) were centrifuged at 3700× *g* for 20 min. In order to control for potential silver adsorption on the ultrafiltration membrane, a known concentration of radioactive silver (85.8 and 145 ng/L, ^{110m}Ag) was added to the supernatant of mesocosm 1 (control). The silver losses after one to four cycles of ultrafiltration by centrifugation were then determined (Figure S2). The filter membrane had previously been rinsed with 4.5 mL of the same supernatant without addition of silver.

2.5. Silver Bioaccumulation in Daphnia Magna

The water flea *Daphnia magna* was obtained from the Centre d'expertise en analyse environnementale du Québec (CEAEQ, Quebec City, QC, Canada). Daphnids were cultured in 16 L aquaria containing 10 L of dechlorinated municipal water, and placed in an environmental chamber or a suitable breeding enclosure. The water used was at a temperature of 20 ± 2 °C and a pH ranging from 6.5 to 8.5. It was previously saturated with filtered oxygen and the bubbling was maintained at all times. The water hardness was between 160 and 180 mg/L and was adjusted if necessary. A 16 h of light/8 h of dark photoperiod was maintained. Light was provided by Cool White fluorescent tubes placed above the aquaria. The luminous intensity was maintained at all times between 6.2 and 13.7 µE/m^2/s as measured at the water surface. Daphnids were fed 7 days a week using a concentrated green algae culture (*Pseudokirchneriella subcapitata*) and a solution of beef and dextrose extract. In order to ensure a stable production, physicochemical analysis of water (temperature, dissolved oxygen, pH, and hardness) and controls of daphnids' health (absence of ephippium, lack of mortality, and average number of neonates) were regularly performed. Adult daphnids (between day 12 and 37) were used for bioaccumulation experiments.

To study Ag transfer to *Daphnia magna*, five daphnids were added to 500 mL of mesocosm water from each mesocosm. Five replicates were conducted per mesocosm. Samples were then taken from each flask after 22 h exposure at 20 ± 2 °C and 9.9 µE/m^2/s (14 h light—8 h darkness). After exposure, collected daphnids were transferred to 500 mL dechlorinated tap water for 6 h depuration to empty their intestinal tract. No mortalities were observed during the exposure and depuration periods. Daphnids were then dried overnight at 80 °C and digested with 1 mL of concentrated HNO_3 in the autoclave (125 °C, 45 min). Digested samples were diluted with Milli-Q water for ICP-MS analysis. DOLT4 (National Research Council of Canada, Ottawa, ON, Canada), a certified reference material,

was used as a digestion control. Blanks, with and without daphnids, were also tested to take potential contamination into consideration.

3. Results and Discussion

3.1. Dissolution of c-AgNPs in the Surface Freshwater Layer

Time series analyses of Ag showed that Ag could be found mostly in the freshwater layer (4–7 µg/L; Figure 2a). This value exceeds the concentrations estimated to be found in the environment, but it was essential to be able to detect the silver nanoparticles once introduced into the mesocosms. The background Ag concentration was as low as 0.004 ± 0.002 µg/L in mesocosm 1 (control), where no c-AgNPs were added. Silver nanoparticles could actually settle to the halocline located at the interface between fresh and salt water but did not cross it. Indeed, very low concentrations of silver were found in the saltwater layer (with an average of 0.1 µg/L, 25× higher than the concentration found in control mesocosm 1), indicating that the halocline acts as a very dense barrier (average conductivity 20.2 mS/cm versus 1.2 mS/cm for fresh water), limiting passage of the c-AgNPs through the halocline [27].

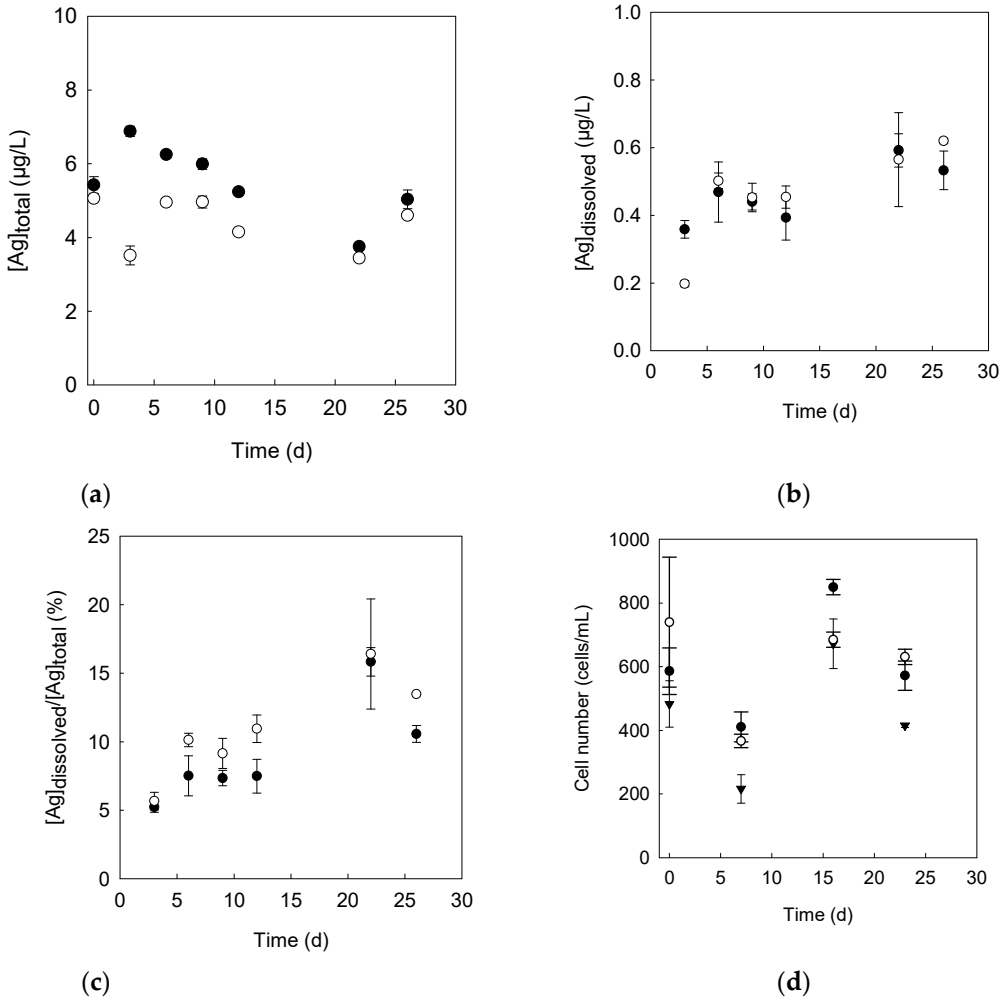

Figure 2. (**a**) Total Ag concentrations in the freshwater layer of mesocosms 2 and 3 as a function of time (samples were not filtered or centrifuged); (**b**) dissolved Ag concentrations in the freshwater layer of mesocosms 2 and 3, as measured by in situ dialysis, as a function of time; (**c**) the percentage of dissolved concentration versus time in mesocosms 2 and 3; (**d**) cell densities of *Chlamydomonas reinhardtii* over time in all mesocosms. Error bars represent the standard deviation of three replicates. Total and dissolved Ag concentrations in mesocosm 1 (control) were negligible (<0.01 µg/L). Symbols show mesocosms 1 (▼; control, for cell numbers only), 2 (●) and 3 (o).

Dissolved Ag concentrations, which were determined by dialysis to separate dissolved Ag from c-AgNPs, were observed to be between 0.2 and 0.6 µg/L over time (Figure 2b), which was 5–15% of total Ag concentration (Figure 2c). Total Ag and dissolved Ag concentrations in mesocosm 1 (control) could be considered negligible (<0.01 µg/L) compared to those observed in mesocosms 2 and 3. The dissolution of c-AgNPs might be expected to vary considerably depending on ambient conditions. For example, significant proportions of dissolved silver (82–99%) were found to be released from AgNPs (20 and 80 nm, max. 8.2 µg/L) in a long-term mesocosm treatment in seawater [15]. In the present study, dissolved Ag concentrations were defined as the Ag concentrations measured in the dialysis devices. Silver nanoparticles (20 nm) and silver complexes with natural organic matter (>100 nm; [28]) would not be expected to pass the dialysis membrane, because the pore size of the 100–500 Da MWCO dialysis devices was estimated to be lower than 1.5 nm [29]. Dialysis membranes have been previously used to differentiate between dissolved forms of Ag and PVP-AgNPs (3 kDa MWCO; [30]).

Mean concentrations of inorganic carbon, chloride and sulfate were 35.1 ± 0.1, 263 ± 85 and 29.9 ± 9.9 mg/L, respectively, in the three mesocosms at all times during the experiment (Figure S3). Silver speciation was calculated using Visual Minteq 3.0 (for absence of precipitation of Ag mineral phases) and WHAM 7 (for expected complexation of Ag) at 18 °C and pH 8.0. The maximum concentrations of inorganic carbon, chloride and sulfate (36.0, 392 and 45.4 mg/L) were used as input parameters. Organic carbon, with a concentration of 4.5 ± 0.6 mg C/L in mesocosms, was considered for WHAM speciation calculations to be composed of 50% carbon and entered as fulvic acid and considering that 65% of the fulvic acid is active in metal binding, as proposed by Bryan, et al. [31]. Calculated dissolved silver speciation was dominated by chloro-complexes (53% $AgCl^0$, 36% $AgCl_2^-$) due to the relatively high concentration of chloride, whereas free silver (4% Ag^+) and silver bound to organic matter (8%) were predicted to remain low. Thermodynamic calculations also indicated an undersaturation with respect to cerargyrite (AgCl(s)) or any other mineral phases under the tested conditions. In addition, dynamic light scattering (DLS) analysis of the fresh water layer in the mesocosms showed that particles had a multimodal distribution with two main populations, one between 0 and 190 nm and the other between 220 and 5000 nm [27,32]. In the presence of silver nanoparticles, the population between 0 and 190 nm increased: it corresponded to 10% of the total particles before and 36% after addition of the nanoparticles. Within the 0–190 nm size range, two size populations were found. The first (10–44 nm) corresponds to the original size of AgNPs, showing that a proportion of the added nanoparticles persisted several hours after the AgNPs were introduced into the mesocosms. The second (50–142 nm) likely corresponds to the aggregates formed between the silver nanoparticles and the natural organic matter [27].

3.2. Determination of Ag Concentrations in Green Algae

A linear relationship between the chlorophyll-*a* concentration and algal density was established in the algal growth medium solution (Figure S1). This plot was used as a calibration curve to determine the cell density of *Chlamydomonas reinhardtii* in mesocosms from the chlorophyll-*a* concentration as determined by fluorescence. The cell densities in the freshwater layer were found to be lower than the nominal density (measured: 200–900 cells/mL, expected: 1000–2000 cells/mL; Figure 2d). The difference is likely due to the sedimentation of algae to the interface between fresh and salt water.

Marine clay (SWy-2) and algae, both present in the mesocosms, were recovered simultaneously by filtration. However, silver nanoparticles are known to adsorb strongly on the surface of clays [33], and consequently the presence of clay would prevent determining the internalized or adsorbed silver on the surface of the algae. A centrifugation approach was thus applied to eliminate clay particles from the solutions prior to the algae exposure tests. The most effective centrifugation conditions were determined to be for 15 min at 5000× *g* or 10,000× *g* to diminish the turbidity of the supernatant after centrifugation of the mesocosm solution (Student's *t*-test, $p < 0.05$, Figure S4). In order to remove as many

clay particles as possible, a speed of 10,000× *g* was used. The Ag concentration in the supernatant decreased sharply with filtration or centrifugation (from 5.30 to 0.63 µg Ag/L, Figure S5). The supernatant from the centrifugation was then used to conduct all algal exposures and their controls. The total organic carbon concentration was also analyzed before (4.60 ± 0.26 mg/L) and after (4.53 ± 0.59 mg/L) centrifugation and no significant difference was observed (Student's test, $p > 0.05$, $n = 3$).

The Ag concentration in the exposure solutions from mesocosms 2 and 3 decreased by 25 to 30% during the 1 h exposure, both in the absence and presence of algae (Figure 3a). The Ag concentration after exposure is defined as the Ag concentration in the filtrate (two superimposed polycarbonate filters, 2 µm porosity). Because Ag concentrations after exposure were determined after the harvest of algal cells, the decrease in Ag may be due to the adsorption of silver on particles (such as residual clay), large enough to be retained on the 2.0 µm filter used to recover algae. A mass balance analysis showed that between 85 and 90% of Ag initially present in the exposure solution was recovered (Table 1).

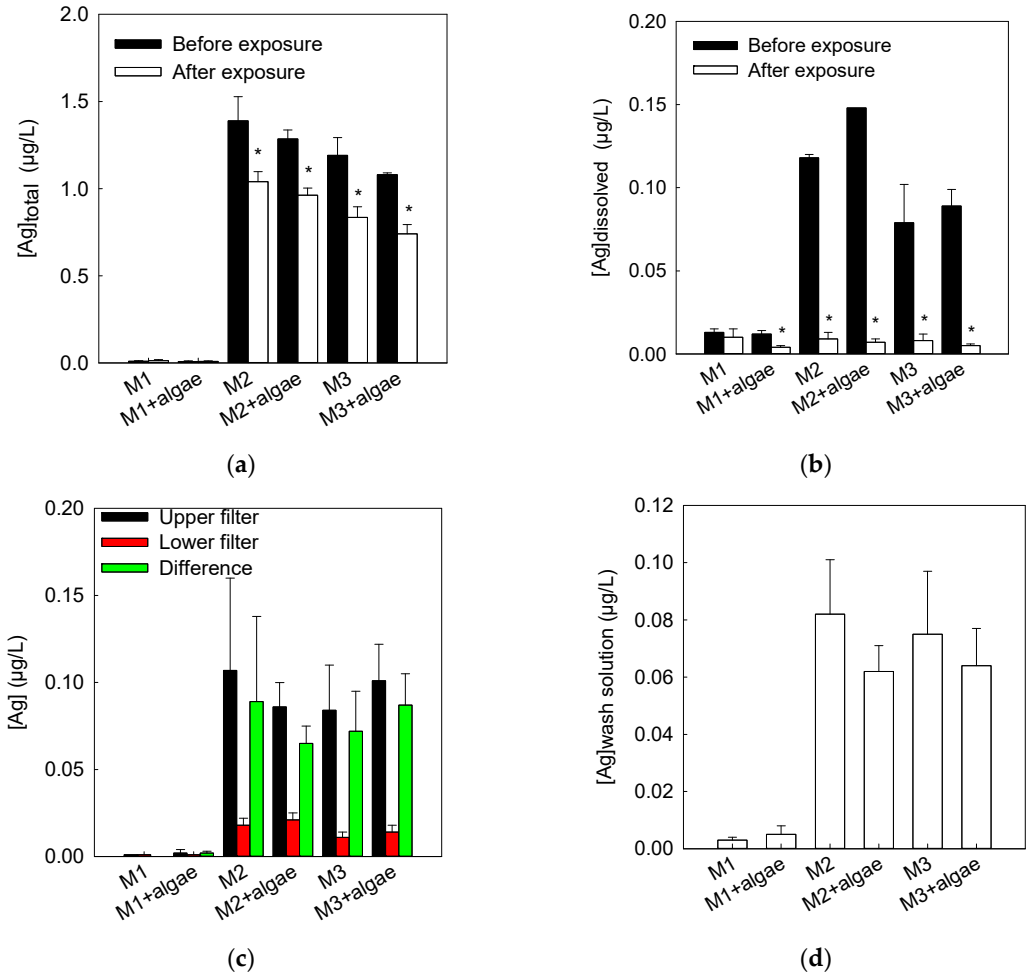

Figure 3. (**a**) Total silver concentrations before (black) and after 1 h exposure (white) with or without algae. The exposure solutions were samples of the supernatants of each mesocosm, after centrifugation for 15 min at 10,000× *g*; (**b**) dissolved Ag concentrations in water from three mesocosms before (black) and after (white) exposure with or without algae; (**c**) silver concentrations in solution after digestion of the upper filter (black), lower filter (red) and the difference between the two filters (green) after passage of exposure solutions from the three mesocosms with or without algae; (**d**) silver concentrations in the wash solution of the filters used to filter the three mesocosm media with or without algae. Error bars represent the standard deviation of three replicates. M1, M2 and M3: Mesocosms 1 (control), 2 and 3. Asterisks indicate significant differences (Student's *t*-test, * means $p < 0.05$) among treatments.

Table 1. Mass balance of silver (µg) calculated for a 1-L solution for the silver exposures of algae (mean ± 1 standard deviation).

Exposure		Total (µg)	Filtrate (µg)	Wash Solution (µg)	Filter 1 (µg)	Filter 2 (µg)	Recovery (%)
Mesocosm 2	Without algae	1.39 ± 0.10	1.04 ± 0.06	0.082 ± 0.019	0.107 ± 0.053	0.018 ± 0.004	89.8 ± 2.2
	With algae	1.29 ± 0.05	0.96 ± 0.04	0.062 ± 0.009	0.086 ± 0.014	0.021 ± 0.004	88.0 ± 1.6
Mesocosm 3	Without algae	1.19 ± 0.10	0.84 ± 0.06	0.075 ± 0.022	0.084 ± 0.026	0.011 ± 0.003	84.5 ± 1.9
	With algae	1.08 ± 0.01	0.74 ± 0.05	0.064 ± 0.013	0.101 ± 0.021	0.014 ± 0.004	85.3 ± 3.0

The dissolved Ag concentration obtained before exposure by ultrafiltration (i.e., Ag concentration in the ultrafiltrate after centrifugal ultrafiltration at 3700× *g* for 20 min) was shown to be between 7 and 11% of total Ag concentration. This is comparable to the dissolved Ag concentration obtained by dialysis (5–15%). The dissolved Ag concentration decreased dramatically at the end of the exposure (Figure 3b). The decrease was unexpected in the absence of algae, and may be due to the effect of remaining clay particles. There was no significant difference between the amount of Ag on the filters with or without algae (Student's *t*-test, $p > 0.05$, $n = 3$, Figure 3c). Similarly, no significant difference was found between the adsorbed silver concentrations (the Ag concentration in the wash solution (200 µM $Na_2S_2O_3$, 4 × 10 mL) includes Ag adsorbed on the filters) with or without algae (Student's test, $p > 0.05$, $n = 3$, Figure 3d). This indicates that no significant Ag accumulation by *Chlamydomonas reinhardtii* had occurred. Under these conditions, Ag was thus not bioavailable, i.e., it was not taken up by the alga.

3.3. Determination of Ag Concentration in Daphnia Magna

No significant difference was found in Ag concentrations in the water taken from mesocosms 2 or 3 and used for the daphnid exposures, before and after the 24 h exposure (Student's *t*-test, $p > 0.05$; Figure 4a). No mortality occurred during the exposure. The Ag content in *Daphnia magna* after exposure was determined to be ~0.5 ng/daphnid in both mesocosms containing c-AgNPs (Figure 4b), a value markedly higher than for daphnids in the control mesocosm, ~0.01 ng/daphnid, for which the background level of Ag (0.003 µg/L) was very low. It was also previously demonstrated that silver nanoparticles in suspension and bound to algae (adsorbed or absorbed) could be found in *Daphnia magna* at relatively high concentrations ([34–36]). The levels of accumulation in AgNP-exposed daphnids observed in this study are higher than those observed by McTeer et al. in 5-day exposures to 100 µg/L AgNP (polymer coated) [35]. However, Ribeiro et al. observed higher accumulation of Ag in conditions similar to the present study with an uptake of ~2.7 ng Ag/daphnid (assuming a dry weight of 180 µg/daphnid; [37]) after 24 h of exposure to 5 µg/L AgNP (3–8 nm, unknown coating; [34]). From the data obtained in this study, it is difficult to conclude whether Ag was directly transferred from the solution and internalized in the organisms or simply adsorbed on the animal's surface or in its digestive tract. Because Ag in the mesocosms was not significantly accumulated by *Chlamydomonas reinhardtii*, but quantified in daphnids, these results suggest that the daphnids likely took up Ag from abiotic particles. Yan and Wang recently demonstrated that c-AgNPs could indeed accumulate in *Chlamydomonas reinhardtii* and then be trophically transferred to *Daphnia magna* [36]. The conditions of their experiment were, however, quite different from those used in this study. In addition to working with larger nanoparticles (57.2 nm on average versus 20 nm here), their concentration (1000 µg/L AgNPs) and the exposure time of the algae to the nanoparticles (12 h) were much greater than in the experiment presented here (3.0–3.5 µg/L and 1 h of exposure, respectively, in this study). The combined effect of these last two parameters could explain why they were able to quantify Ag uptake in algae and its subsequent trophic transfer whereas no significant uptake and trophic transfer was observed in the present study. A longer exposure time of the algae to the nanoparticles might have provided a quantifiable accumulation in *Chlamydomonas reinhardtii* and, in

turn, in *Daphnia magna*. Nevertheless, the objective of this study was to simulate real environmental conditions as closely as possible. To do this, it was important to keep the exposure concentrations as close to real-world exposure scenarios as possible. If the exposure time proved to have no significant effect on the bioaccumulation of silver nanoparticles by *Chlamydomonas reinhardtii*, this might mean that no significant accumulation in algae, and subsequently no trophic transfer to daphnids, would be expected at the ng/L levels anticipated to occur in receiving water bodies.

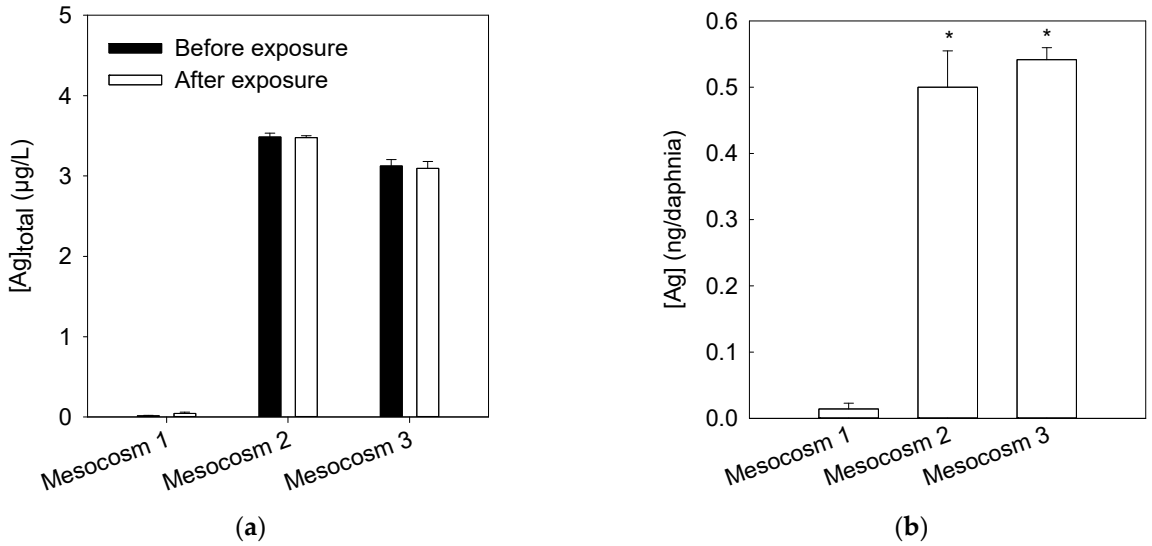

Figure 4. (**a**) Total Ag concentrations in the three mesocosm exposure solutions (grab samples, no filtration or centrifugation; $n = 3$) before (black) and after 24 h exposure of *Daphnia magna* (white). There was no significant difference between total Ag concentration in the solution before and after daphnia exposure (Student's *t*-test, $p > 0.05$). (**b**) Silver uptake from all mesocosms by *Daphnia magna* after a 24 h exposure ($n = 5$). Error bars represent the standard deviation. Asterisks (*) indicate significant differences with the control (mesocosm 1) (Student's *t*-test, $p < 0.05$).

4. Conclusions

The dissolution of citrate-coated silver nanoparticles of about 20 nm in diameter was followed after their addition to mesocosms mimicking a stratified estuary. The total silver concentration in the freshwater layer over 12 days varied between 4 and 7 µg/L, against 0.1 µg/L in the saltwater layer. This latter concentration is close to the concentrations found in the reference mesocosm and indicates that in the absence of appreciable turbulence, the halocline acts as a barrier that AgNPs could not cross. The dialyzable silver concentrations in the freshwater layer ranged from 0.1 to 0.6 µg/L, corresponding to a small fraction of the total Ag concentration. It was shown that silver adsorbed strongly on clay particles present in the water column, suggesting that, at low concentrations, AgNPs would likely be scavenged by natural suspended matter.

Accumulation results showed that Ag was not bioavailable to algae under the tested conditions. Because no significant accumulation was observed in algae, this indicates that the daphnids likely did not assimilate silver via ingestion of algae. Silver could have been associated with daphnids by their ingestion of non-algal particles such as clay and/or by adsorption on their outer surface.

This model estuarine mesocosm has some limitations, mostly the use of a silver concentration that exceeded that expected to be found in a large estuary, and absence of tides and currents, which are typical of natural estuarine environments and can influence silver distribution and fate in both water layers. However, the water composition was chosen to be as close as possible to that of the Saint Lawrence River, especially with regards to the concentrations of dissolved organic matter and clay, which would be expected to attenuate the bioavailability of silver nanoparticles and dissolved silver. This could be the

case in this study as it was shown that silver adsorbed strongly on clay particles, strongly decreasing silver bioavailability to algae and possibly also to daphnids. Considering that the nanoparticulate silver concentrations used in this work are well over three orders of magnitude higher than the highest nanosilver concentration expected in surface water, and that silver uptake was either not detectable (in algae) or low (in daphnids), AgNPs likely represent a low-risk hazard for these species in estuarine waters.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/environments9020020/s1, Figure S1: Relationship between chlorophyll-*a* concentration and *Chlamydomonas reinhardtii* cell number in mesocosm 1 (control) surface water, Figure S2: Mean proportion of silver in the ultrafiltrate ($[Ag]_f$) measured for successive centrifugation cycles at total silver ($[Ag]_t$) concentration of 85.8 (•) and 145 ng/L (o) with 4.5 mL at 3700 fre× g for 20 min. Error bars represent the standard deviation of three replicates, Figure S3: Mean concentrations of inorganic carbon, chloride and sulfate in the three mesocosms as a function of time. Error bars represent the standard deviation of concentrations (IC, Cl^-, SO_4^{2-}) from mesocosms 1, 2 and 3 (n = 3; one measurement per mesocosm), Figure S4: Turbidity of supernatant from the freshwater layer of mesocosms after centrifugation or filtration (0.2 µm polycarbonate filter). Turbidity is expressed as Nephelometric turbidity units (NTU). Error bars represent the standard deviation of turbidity measurements on samples from the three mesocosms; one sample was tested from each mesocosm. Asterisks (*) indicate there was no significant difference with respect to the 0.2 µm filtered solution (Student's t-test, $p > 0.05$), Figure S5: Ag concentrations in mesocosms 2 and 3 at the beginning of the experiments (no filtration or centrifugation; labeled "control"), compared with Ag concentrations in the filtrate after passing through 0.2 µm polycarbonate filters, as well as Ag concentrations in the supernatants after centrifugation for 15 min at different speeds (g-values). Error bars represent the standard deviation of Ag concentrations from mesocosms 2 and 3 (n = 2; one sample per mesocosm), Table S1: Composition of culture medium (MHSM-1). All reagents were of analytical grade or better.

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