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RESEARCH ARTICLE

Ecotoxicity of nanosilver on cladocerans and the role of algae provision

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Abstract

Silver nanoparticles (AgNPs) are applied in diverse industries due to their biocide and physicochemical properties; therefore, they can be released into aquatic systems, interact with environmental factors, and ultimately exert adverse effects on the biota. We analyzed AgNPs effects on *Ceriodaphnia reticulata* (Cladocera) through mortality and life-history traits, considering the influence of food (*Tetradesmus obliquus*, Chlorophyceae) presence and concentration. *C. reticulata* was exposed to AgNPs in acute (absence and two algae concentrations plus five AgNPs treatments) and chronic assays (two algae concentrations plus three AgNPs treatments). AgNPs did not affect algae flocculation but increased Ag^+ release, being these ions less toxic than AgNPs (as proved by the exposure to $AgNO_3$). A reduction in AgNPs acute toxicity was observed when algae concentration increased. Acute AgNP exposure decreased *C. reticulata* body size and heart rate. The chronic AgNP exposure reduced *C. reticulata* molt number, growth, heart rate, and neonate size:number ratio, being these effects mitigated at the highest algae concentration. Increases in relative size and number of neonates were observed in AgNP treatments suggesting energy trade off. The increased Ag^+ release with food presence suggests that the AgNP-algae interaction might be responsible of the decreased toxicity. Although algae reduced AgNP toxicity, they still exerted adverse effects on *C. reticulata* below predicted environmental concentrations. Since algae presence reduces AgNP effects but increases Ag^+ release, studies should be continued to provide evidence on their toxicity to other organisms.

Keywords Silver nanoparticles · Environmental behavior · Ceriodaphnia · Tetradesmus · Mortality · Life-history traits

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Introduction

The nanotechnology industry is constantly increasing and diversifying due to its wide potential applications (Jeevanandam et al. 2018; Turan et al. 2019). Silver nanomaterials are one of the most produced due to their biocide capacity and physicochemical properties such as electrical and thermal conductivity, catalytic activity, and surface plasmon resonance (Jones et al. 2018; Ahmad et al. 2020; Corsi et al. 2022). Currently, more than 50% of the commercial products inventoried by the Woodrow Wilson Project on Emerging Nanotechnologies contain silver nanoparticles (AgNPs)

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(http://www.nanotechproject.org/). AgNPs are applied in several fields such as medicine, cosmetics, food industry, electrical and optical devices, catalysis, textiles, paints, agriculture, and water treatment (Antezana et al. 2021; Islam et al. 2021; Municoy et al. 2021; Zahoor et al. 2021). The extensive application of AgNPs inevitably leads to their release into aquatic environments making it a rising issue of concern for eco-toxicologists (Bour et al. 2015; Skjolding et al. 2016; Zhang et al. 2019; Devasena et al. 2022). Although quantification methods for nanoparticles in the environment are still limited, AgNPs are expected to reach about 8.8×10^{-5} – 10 µg/L on surface water (Nowack and Mueller 2008; Gottschalk et al. 2013; Maurer-Jones et al. 2013).

AgNPs released in aquatic systems could affect aquatic microorganisms such as zooplankton in different ways (Baun et al. 2008; Gutierrez et al. 2021). This community plays a key role as is a direct link between primary producers and consumers and contributes to organic matter and nutrient cycling (Mano and Tanaka 2016). Within zooplankters, micro-crustaceans are particularly sensitive to environmental disturbances as they have small size and short generation times (Resh 2008; Ferdous and Muktadir 2009). AgNPs have been shown to impair freshwater cladoceran survival, growth, and reproduction in a wide range of concentrations from 1 to > 100 µg/L (Gaiser et al. 2011; Völker et al. 2013; Ribeiro et al. 2014). These effects are usually attributed to the "Trojan horse" mechanism as Ag^+ released by AgNPs into organism cells cause oxidative stress as reviewed by de Souza et al. 2019, who pointed out the need for developing new AgNPs considering their toxicity and environmental behavior. Nevertheless, these studies mainly focus on *Daphnia magna*, which do not represent neither the different functional groups of micro-crustaceans (copepods and cladocerans) nor holotropic region scenarios (Gutierrez et al. 2021). In this sense, there is a gap of knowledge as model test species are usually holarctic and little is known about species representatives from other regions. Particularly, *Ceriodaphnia reticulata* (Jurine) is a planktonic Daphniidae that was recorded in Palearctic, Nearctic, Neotropical, Oriental-Indomalaya, and Afrotropical regions (Kotov and Forró 2019). It inhabits large streams, reservoirs, and lakes and is easily grown under laboratory conditions. It has a short life cycle that allows completing a three-brood life cycle test in seven days (Mount and Norberg 1984). Toxicity tests of different compounds have been reported for *C. reticulata* (e.g., Jaser et al. 2003; Mangas-Ramirez et al. 2007; Mano et al. 2010); nevertheless, the effects of nanomaterials have not been previously evaluated for this species. In this scenario, there is a need for studies focusing on chronic endpoints over species that inhabit different regions under environmentally relevant AgNP concentrations to understand the population consequences of this kind of pollution (Baun et al. 2008; Gutierrez et al. 2021).

Several physical, chemical, and biological factors in aquatic systems may alter AgNP behavior, in terms of aggregation, chemical speciation, and adsorption (Corsi et al. 2022; Kansara et al. 2022), which in turn, can modify their toxicity to aquatic organisms. Nevertheless, few studies have analyzed the incidence of environmental factors in AgNPs toxicity on zooplankton leading to a gap in the knowledge of potential attenuators (Zhang et al. 2019; Gutierrez et al. 2021). For instance, dissolved organic matter (DOM) such as humic and fulvic acids are known to reduce AgNP toxicity, as DOM can be adsorbed and reduce their dissolution (Wang et al. 2015; Jung et al. 2018; Ale et al. 2021). The presence of algae as food supply has also been proposed to be an attenuator of AgNP toxicity for some cladocerans, but the underlying mechanisms remain under discussion. Ribeiro et al. (2014) attributed this toxicity reduction to the alga-AgNP interaction as the particles can be agglomerated by DOM or adsorbed to the algae, and also algae can uptake Ag^+ released by AgNPs. Besides, the attenuation of AgNPs toxicity could be explained due to both interaction alga-AgNPs and better nutritional condition of exposed cladocerans, as in the presence of food organisms may be more tolerant due to their greater energy status compared to standardized bioassays without food (Harmon et al. 2017). Algal exudates and metabolic products (such as oxygen peroxide) could also contribute to the dissolution of AgNPs (Navarro et al. 2015; Sigg and Lindauer 2015; Chen et al. 2019; Ponton et al. 2019), and thus, modify their toxicity.

In this article, we assessed the effects of AgNPs on *Ceriodaphnia reticulata* (Cladocera) through mortality and life-history traits, considering the influence of food (*Tetradesmus obliquus* -before *Scenedesmus obliquus*-, Chlorophyceae) presence and concentration. We expected that AgNPs would be toxic for *C. reticulata* by affecting their mortality and life-history traits such as growth, reproduction, and heart rate. According to previous findings reported in the bibliography, we hypothesized that these toxic effects would be reduced by algae presence and it would depend on its concentrations.

Materials and methods

Materials and reagents

Silver nanoparticles were obtained from Nanotek S.A. (20—40 nm, colloidal suspension of 1% w/v, nanArgen®, CAS no. 7440–22-4), and the main ingredient of the product was stated as silver (purity ≥ 99.0%). The capping agent was made of glucose oligomers (mainly nanocrystalline cellulose) and the stabilizing agent was made of polyvinyl

pyrrolidone (PVP). Silver nitrate was purchased from Tetrahedron® (CAS no. 7761–88-8), purity ≥ 99.0%.

Test organisms

Ceriodaphnia reticulata was collected from a shallow lake of the middle Paraná River flood plain and gradually adapted to laboratory conditions by maintaining it for several months as the initial stock. One parthenogenetic female was isolated from this initial stock and cultured for more than ten consecutive generations in laboratory to prepare the unique strain for the toxicological experiments. *C. reticulata* was identified under an optical microscope (Nikon E100), and the species was confirmed through observation of post-abdominal claw ornamentations (Rogers et al. 2020). This culture was maintained at 21 ± 1 °C, 12/12 day/night photoperiod, in dechlorinated and aerated tap water (pH: 7.1, conductivity: 1020 µs/cm, total hardness: 180 mg/L $CaCO_3$, alkalinity 120 mg/L $CaCO_3$, 39 mg/L Ca^{++}, 20 mg/L Mg^{++}, and 146 mg/L HCO_3^-). Culture water was changed three times a week and the organisms were fed with *Tetradesmus obliquus* every other day.

The pure strain of *Tetradesmus obliquus* (Turpin) MJ Wynne (before *Scenedesmus obliquus*) was isolated from a natural shallow lake of the middle Paraná River flood plain. The species was confirmed by Sanger sequencing and defined based on the NCBI database (www.ncbi.nlm.nih.gov) and taxonomic keys (Wynne and Hallan 2015). The axenic culture was growth in Detmer modified medium for green algae (Watanabe 1960) (KCl: 50, KH_2PO_4: 50, $Ca(NO_3)_2$-$4H_2O$: 360, $MgSO_4$-$7H_2O$: 360, Cl_3Fe^+: 5, $C_4H_6O_6$: 5, H_3BO_3, 2.86, $MnCl_2$-$4H_2O$: 1.81, $ZnSO_4$-$7H_2O$: 0.23, Cl_2Cu: 0.05 mg/L), at 25 °C with warm-white LED light (50 µmol/m^2.s, 2600 lx) and constant aeration. The culture was cropped in the exponential growth phase and algae were resuspended in sterile distilled water and stocked at -4 °C in the dark. Cell concentration was estimated under an optical microscope (Nikon E100) with a Neubauer chamber.

Exposure conditions

Nanoparticle characterization

In order to assess the optical properties of AgNPs, a suspension of the particles was monitored by UV–vis spectroscopy using a Jasco V-730 Spectrophotometer (Jasco Analytica Spain, Madrid, Spain). Fourier transform infrared (FTIR) spectra of AgNPs were obtained over the range of 4000–500 cm^{-1} using an FTIR-Raman Nicolet iS 50 (Thermo Scientific). A dynamic light scattering (DLS) analysis was performed to calculate the AgNP average hydrodynamic diameter (Z-average, nm) and polydispersity index (PDI, dimensionless) in both ultrapure water and culture water with a detection angle of 173° at 25 °C using a Zetasizer Nano-Zs Laser Light Scattering Instrument (Malvern Instruments, UK). AgNPs were characterized through transmission electron microscopy (TEM) and scanning electron microscopy (SEM) in both ultrapure and culture water. The Ag^+ release from AgNPs was followed by analyzing the dissolved fraction during 96 h in culture water with the three algae concentrations employed in the bioassays (A0 = 0, A1 = 10×10^4, and A2 = 50×10^4 cel/mL) and a concentration of 44 µg/L AgNPs. Briefly, 1 mL of the solution was placed in the upper chamber of Vivaspin® 20 centrifugal concentrator (30 kDa molecular weight cutoff, Sartorius Stedim Biotech GmbH, Göttingen, Germany) and was then centrifuged at 5000 rpm for 15 s at 25 °C. The nanoparticles remained in the upper chamber, while the aqueous filtrate contained the dissolved fraction. The concentration of Ag in the filtrate was measured consecutively in time for 96 h by atomic absorption. Cumulative doses were calculated using a standard curve and expressed as a function of time. In all cases, results were expressed as mean ± SD from triplicate experiments.

Algae flocculation test

In order to assess the effect of AgNPs on the algae flocculation, which may have influenced their availability for cladocerans, *T. obliquus* flocculation was assessed at the same AgNP concentrations tested in the chronic toxicity test (C1, C2, and C3) after 48 h of exposure (chronic assay renewal time). Algae flocculation was determined by measuring the absorbance of the supernatant at 750 nm (Griffiths et al. 2011) both at the beginning and final time following Beuckels et al. (2013):

$$\text{Flocculation}(\%) = \frac{OD_{t0} - OD_{tf}}{OD_{t0}} \times 100$$

where OD = optical density of the supernatant (750 nm), $t0$ = initial time (0 h), and tf = final time (48 h).

Acute toxicity tests

In order to obtain the LC50 of AgNPs on *C. reticulata*, three acute toxicity tests were developed with different food conditions: without algae (A0) and with two concentrations of *T. obliquus*: 10×10^4 and 50×10^4 cel/mL (A1 and A2, respectively). These concentrations were based on a preliminary growing and reproduction experiment (Supplementary Material 1) and bibliographic information (Savaş and Erdoğan 2006; Rodgher and Espíndola 2008). The acute toxicity of AgNPs was tested following APHA (2017) guidelines with some

modifications by exposing 20 neonates (> 24 h) (four replicates, with five neonates each) during 48 h to five concentrations of AgNPs (shown in Table 1) (dilution factor: 1.3) plus a control for each algae concentration (A0, A1, and A2). AgNP concentrations were chosen based on preliminary exploratory toxicity tests and bibliographic information. Fresh stock solutions (400 µg/L) were prepared in ultrapure water before each bioassay and stored in the dark to prevent any prior transformation (e.g., aggregation, agglomeration, or dissolution). The nominal detected Ag concentration correlated with the product description. We used 50 mL beakers for each replicate with dechlorinated and aerated tap water at 21 ± 1 °C. The acute toxicity tests were performed in darkness to avoid algae growth and AgNP degradation (Li et al. 2013). Mortality, determined as immobility after a gentile stimulus, was assessed at 24 and 48 h of exposure. Conductivity (µs/cm), dissolved oxygen (DO, mg/L), and pH were measured (Hanna multi-parameter portable meter) at the beginning and the end of each bioassay.

In addition to mortality, two other biological variables were measured at 48 h of exposure: body size and heart rate. Body size was measured on three surviving individuals per replicate (when possible) by a microscale attached to the microscope eyepiece (Nikon E100) to identify potential effects of the AgNPs on growth. The methodology to assess heart rate (beats per minute -bpm-) was adapted from Baylor (1942). Briefly, three surviving individuals per replicate (when possible) were individually placed on a microscope slide, and most of the water was withdrawn; thereby, the animals were kept in the microscope's field by the surface tension of the remaining fluid. Organisms were recorded with an iPhone 7 (1080p, 30 fps) mounted on an optical microscope (Nikon E100) for 10 s. For greater precision, each video was cut to a length of 6 s (MKVToolNix 66.0.0), and heartbeats were recorded with a manual counter at a low video speed (0.12x) (Villegas-Navarro et al. 2003; Borase et al. 2019).

Additional experiment with $AgNO_3$

The acute toxicity of $AgNO_3$ was also tested under the three algae concentrations (A0, A1, and A2) (Table 1) to identify the toxicity of dissolved silver ions and elucidate whether the toxicity of AgNPs is due to the Ag^+ release or the AgNPs themselves. This experiment was developed as described for AgNPs, but only mortality was assessed.

Table 1 Concentrations of AgNPs, $AgNO_3$ (µg/L), and algae (cel/mL) employed in the acute and chronic toxicity tests

		Acute assay					
		AgNPs			$AgNO_3$		
Algae (cel/mL)		0 (A0)	10×10^4 (A1)	50×10^4 (A2)	0 (A0)	10×10^4 (A1)	50×10^4 (A2)
Test concentration (µg/L)	0.00	x	x	x	x	x	x
	0.20	x					
	0.27	x					
	0.35	x					
	0.46	x	x				
	0.60	x	x				
	0.78		x	x	x		
	1.00		x	x	x	x	
	1.30		x	x	x	x	x
	1.70			x	x	x	x
	2.20			x	x	x	x
	2.86					x	x
	3.72						x
	Chronic assay						
	0 (C0)		x	x			
	0.07 (C1)		x	x			
	0.37 (C2)		x	x			
	0.56 (C3)		x	x			

x indicates the concentrations applied in each assay and exposure condition

Chronic toxicity test

Chronic toxicity test followed APHA (2017) guidelines with some modifications. Briefly, *C. reticulata* < 24 h neonates were exposed to three AgNP concentrations plus a control (C0) for ten days in order to cover three broods of neonates according to the life cycle of *C. reticulata* (Mount and Norberg 1984). Exposure concentrations were based on the A1 48 h-LC50, thus representing the 10, 50, and 75%: C1, C2, and C3, respectively (Table 1). In each treatment, five replicates were performed. Each replicate corresponded to one neonate, which was placed individually in 50 mL beakers with dechlorinated and aerated tap water. Laboratory conditions were the same as described for the acute toxicity test. Culture water (control and treatments) was completely renewed every 48 h. This methodology was replicated simultaneously with two algae concentrations (A1 and A2) as described for the acute toxicity test.

Every 24 h, molts and neonates were quantified, measured as described for the acute test, and removed. In addition, the adult body size and heart rate were also measured at the end of the experiment, as described before.

Data analysis

The dissolved fraction of AgNPs was compared among each algae concentration (A0, A1, and A2) through analysis of variance (ANOVA, Tukey post-test). The mean flocculation percentages of algae in AgNPs treatments were compared to control through ANOVA (Dunnett post-test) or Kruskal–Wallis test (KW), as appropriate, in both algae concentrations (A1 and A2). Mean physicochemical variables (conductivity, DO, and pH) were compared between treatments by ANOVA (Tukey post-test) and through time by paired t-test.

With the mortality of the acute exposure assay, probit analyses (Finney 1971) were performed to obtain the 24 and 48 h LC50. The mean size and heart rate of treatments in acute assays were compared to control through ANOVA (Dunnett post-test).

The molt sizes of each replicate of the chronic assay were plotted in relation to time, and their linear regression slopes were considered as growth rates. The neonate relative size and number were calculated per each replicate by dividing the mean neonate size and mean brood number by mother size, respectively. The neonate size:number ratio was calculated for each replicate by dividing the mean neonate size by the mean brood number. The mean of these variables from treatments, in addition to molt number and heart rate means, was compared to control through ANOVA (Dunnett post-test).

Data analysis was performed with R Studio (version 1.2.5042), packages "drc" (Ritz et al. 2015), and "rstatix" (Kassambara 2020).

Results

Exposure conditions

Nanoparticle characterization

The characteristics of AgNPs were analyzed in ultrapure water through FTIR and UV–visible spectroscopy. FTIR spectra of the AgNPs stabilized with PVP showed a wide band at 3246 cm^{-1} (H-bonded OH), a peak at 1288 cm^{-1} (N–OH complex), and a strong peak at 1060 cm^{-1} (C-N of pyrrolidone) (Wang et al. 2005) (Fig. 1a). The UV–visible absorption spectrum presented the typical surface plasmon of AgNPs, with a maximum peak close to 410 nm (Fig. 1b). The observed asymmetry in the plasmon has been reported by several authors and is due to the presence of nanoparticles that are not spherical, but have triangular or elongated shapes (Tak et al. 2015), as can be seen in TEM and SEM analyses (Fig. 2a and c). TEM analysis showed that the average size of the spherical AgNPs was 24 ± 7 nm, while for the non-spherical shapes, it was 80 ± 13 nm. This correlates with what was observed in the DLS (Table 2 and Fig. 2e).

The AgNPs tended to agglomerate in culture water, but the z-potential remained constant (-13.7), as can be observed in TEM, SEM (Fig. 2b and d), and DLS analyses (Table 2 and Fig. 2f).

The dissolved fraction of AgNPs increased significantly at higher algae concentration (ANOVA $F = 59.46$ $p = 0.004$) (Fig. 1c). At 48 h, the mean dissolved fraction was $11.17 \pm 0.61\%$ in absence of algae (A0) and increased to $15.95 \pm 1.18\%$ ($p < 0.05$) and $23.35 \pm 0.15\%$ ($p < 0.01$) at A1 and A2 algae concentrations, respectively.

Algae flocculation test

The AgNP treatments did not induce algae flocculation (A1: KW $H = 6.83$, $p = 0.078$, A2: ANOVA $F = 0.06$, $p = 0.981$). The percentage of algal flocculation was significantly lower in A2 control compared to A1 control (ANOVA $F = 14.65$, $p = 0.009$) (Supplementary Material 2).

Physicochemical variables

Physicochemical variables did not vary significantly neither between treatments (ANOVA $F = 2.07$, 2.78, and 1.09, $p = 0.21$, 0.14, and 0.39, for conductivity, DO, and pH, respectively) nor through time (paired t-test $t = 2.06$, 1.27, and 1.51, $p = 0.1$, 0.33, and 0.19, for conductivity, DO, and pH, respectively); conductivity: 1018 – 1089 µs/cm, DO: 7.7 – 8.6 mg/L, and pH: 7.1 – 7.7.

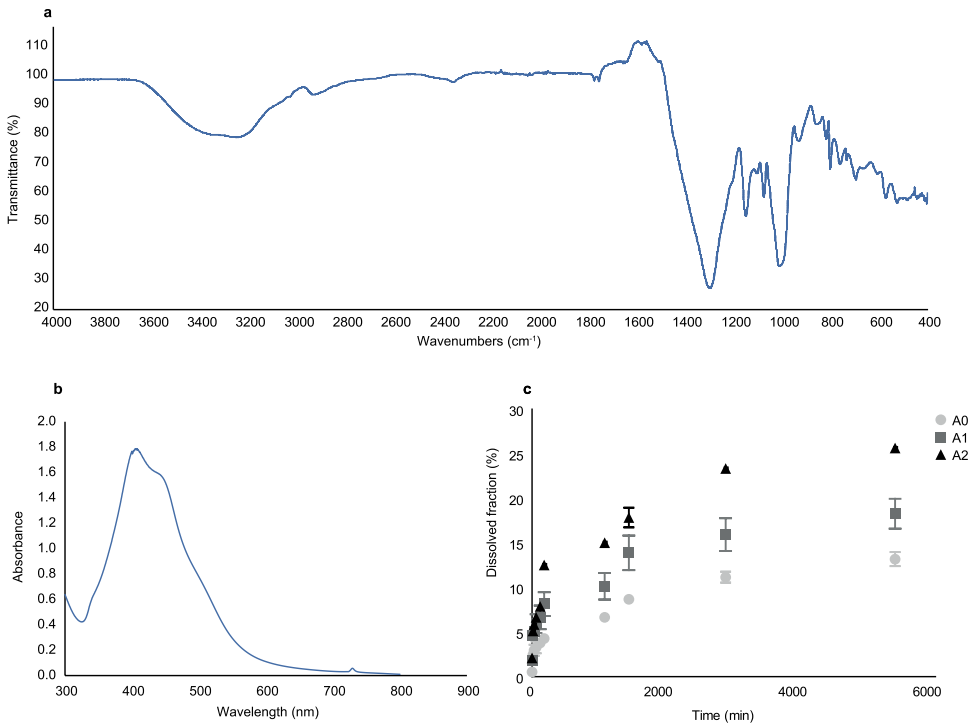

Fig. 1 (**a**) Fourier transform infrared (FTIR) spectra of AgNPs. (**b**) Surface plasmon resonance of AgNPs. (**c**) dissolved fraction of AgNPs in culture water. A0: absence of algae, A1: low algae concentration, and A2: high algae concentration

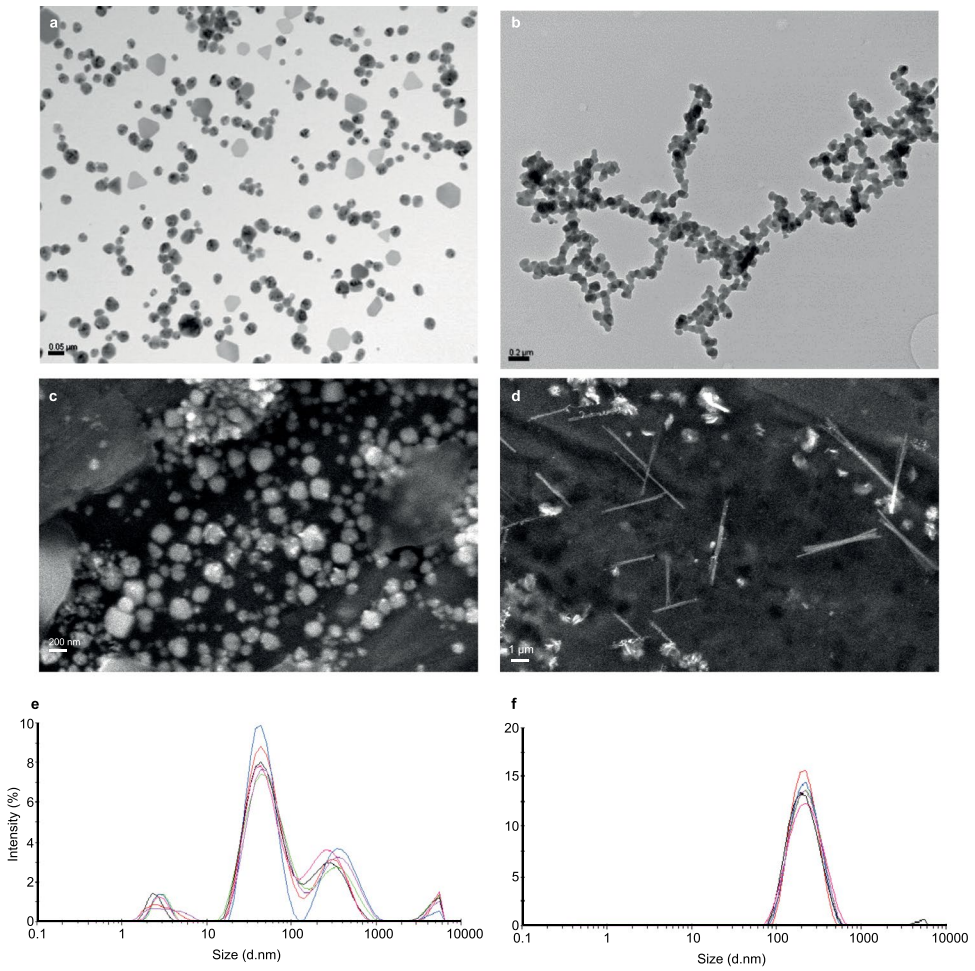

Fig. 2 Transmission electron microscopy (TEM) of AgNPs in (**a**) ultrapure water and (**b**) culture water. Scanning electron microscopy (SEM) of AgNPs in (**c**) ultrapure water and (**d**) culture water. Size distribution by intensity of AgNPs in (**e**) ultrapure water and (**f**) culture water

Table 2 Dynamic light scattering (DLS) analysis showing the AgNP average hydrodynamic diameter (Z-average, nm), polydispersity index (PDI, dimensionless), and z-potential (mV) in both ultrapure water and culture water

Sample	Average hydrodynamic diameter (nm) (±S.D.)						PDI (±S.D.)	z-potential (mV) (±S.D.)
	Peak 1	%	Peak 2	%	Peak 3	%		
Ultrapure water	53.0 (3.9)	65.6 (3.1)	343.9 (49.6)	26.2 (3.4)	3.0 (0.3)	5.3 (0.3)	0.594 (0.148)	-13.7 (0.8)
Culture water	230.7 (7.3)	100.0 (0.0)	-	-	-	-	0.146 (0.027)	-13.7 (0.8)

S.D.: standard deviation, PDI: polydispersity index

Acute toxicity tests

A reduction in both AgNPs and $AgNO_3$ acute toxicity on *C. reticulata* was observed; meanwhile, algae concentration increased. $AgNO_3$ was less toxic than AgNPs in all treatments (Table 3 and Fig. 3a and b).

C. reticulata body size increased significantly in controls with algae (A1 and A2) in comparison with the control without algae (A0) (ANOVA $F=115.04$, $p<0.001$). The organism size was significantly reduced at higher AgNP concentration (i.e., ≥ 1 µg/L) compared to control in the presence of algae (ANOVA A1: $F=8.8$, $p=0.001$; A2 $F=7.46$, $p=0.002$) (Fig. 3c). The heart rate was significantly higher in the controls with algae (A1 and A2) than the control without algae (A0) (ANOVA $F=115.04$, $p<0.001$). In both treatments with algae (A1 and A2), a significative decrease in heart rate was observed at higher AgNP concentrations compared to control (i.e., ≥ 0.78 and 1 µg/L for A1 and A2, respectively) (ANOVA A1: $F=6.13$, $p=0.006$; A2 $F=12.67$, $p<0.001$) (Fig. 3d).

Chronic toxicity test

The growth rate decreased significantly in the lower algae concentration (A1) in the three AgNP treatments compared to control (ANOVA $F=7.34$, $p=0.004$) (Fig. 4a). Also, a smaller number of molts were observed at 0.07 and 0.56 µg/L AgNPs compared to control in the lower algae concentration (A1) (ANOVA $F=3.83$, $p=0.032$) (Fig. 4b). Under the higher algae concentration (A2), a significant increase in growth rate was observed at 0.07 and 0.56 µg/L AgNPs compared to control (ANOVA $F=21.46$, $p<0.001$) (Fig. 4a).

At the end of the experiment, the heart rate of adults was significantly lower in the three AgNP treatments compared to control in the case of the lower algae concentration (A1) (ANOVA $F=10.29$, $p=0.001$). Under the higher algae concentration (A2), no significative effects of the AgNP treatments were observed (ANOVA $F=2.44$, $p=0.111$) (Fig. 4c).

The neonate relative size significantly increased in the three AgNP treatments compared to control at the lower algae concentration (A1) (ANOVA $F=7.98$, $p=0.002$). In the case of the higher algae concentration (A2), this relation was significantly higher at 0.37 and 0.56 µg/L AgNPs than control (ANOVA $F=5.06$, $p=0.013$) (Fig. 4 d). Also, under both algae concentrations (A1 and A2), the neonate relative number was significantly higher at 0.37 and 0.56 µg/L AgNPs compared to control (ANOVA A1: $F=10.58$, $p=0.001$, A2: $F=13.78$, $p<0.001$) (Fig. 4e).

The neonate size:number ratio was lower at the higher algae concentration (A2) control (i.e., many smaller neonates) compared to the lower algae concentration (A1) control (ANOVA $F=12.93$, $p=0.016$). In A1, a significant

Table 3 Acute toxicity values (24 and 48 h LC50, µg/L) of both AgNPs and $AgNO_3$ in *C. reticulata*, and confidence interval (95%). A0: absence of algae, A1: low algae concentration, and A2: high algae concentration (0, 10×10^4, and 50×10^4 cel/mL, respectively)

			LC50 (µg/L)	CI 95%		
AgNP	24 h	A0	0.46	0.42	-	0.49
		A1	0.73	0.68	-	0.78
		A2	1.18	1.10	-	1.25
	48 h	A0	0.44	0.39	-	0.48
		A1	0.72	0.67	-	0.77
		A2	1.10	1.01	-	1.20
$AgNO_3$	24 h	A0	1.19	1.08	-	1.29
		A1	2.35	2.03	-	2.66
		A2	2.94	2.79	-	3.08
	48 h	A0	1.12	1.01	-	1.23
		A1	2.32	2.18	-	2.46
		A2	2.89	2.73	-	3.05

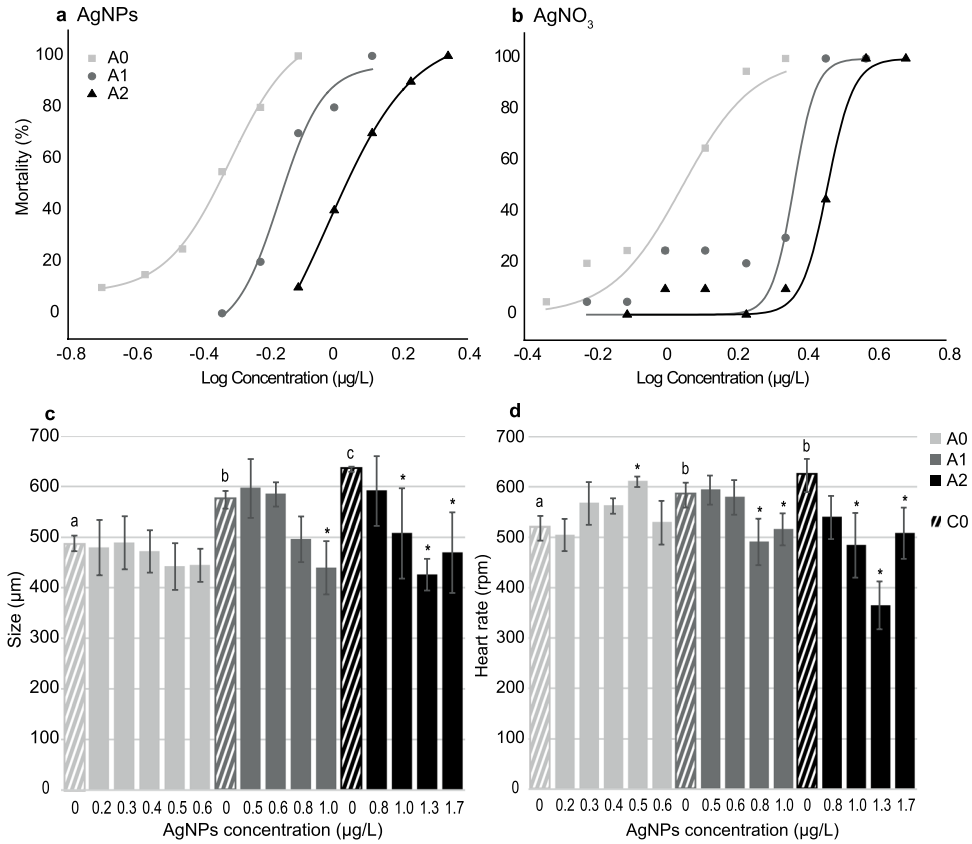

Fig. 3 Dose response curves of acute exposure of *C. reticulata* to (**a**) AgNPs and (**b**) $AgNO_3$. A0: absence of algae, A1: low algae concentration, and A2: high algae concentration. Mean values ± SD of (**c**) size (µm) and (**d**) heart rate (bpm) of surviving neonates of the acute toxicity test with AgNPs (48 h). A0: absence of algae, A1: low algae concentration, and A2: high algae concentration. Letters: significative differences between controls, *Significative differences between treatments and control

increase in this relation was observed at 0.07 µg/L (i.e., fewer bigger neonates) compared to control, while at 0.37 and 0.56 µg/L AgNPs, this relation decreased significantly (i.e., many smaller neonates) (ANOVA $F = 27.76$, $p < 0.001$). In A2, no significative effects of the AgNPs treatments were observed (ANOVA $F = 1$, $p = 0.421$) (Fig. 4f).

Discussion

Exposure conditions

Nanoparticle characterization

Although no agglomeration was observed in ultrapure water, AgNPs tended to form medium-sized agglomerates in the culture water. This may be a consequence of the interaction with the salts present in the media, as observed in numerous studies (Griffitt et al. 2008; Li et al. 2013; Borase et al. 2019).

Several authors reported that algae might induce AgNP dissolution by increasing Ag^+ release, given that algae exudates and reactive oxygen species (ROS) metabolites—such as hydrogen peroxide—can destabilize AgNPs (Navarro et al. 2015; Sigg and Lindauer 2015; Chen et al. 2019; Ponton et al. 2019). This process might occur in the algae cell boundary layer since, in general, algal wall pores have an average diameter of 5—20 nm; therefore, only smaller nanoparticles could enter cells (Chen et al. 2019). Moreover, *Tetradesmus obliquus* has the typical cell wall of the Chlorococcales, which is more resistant due to the trilaminar structure (Burczyk 1973; Allard and Templier 2001), which would make it even more difficult for the AgNPs to enter the cells. Thus, in our case, it is also likely that the mechanism of Ag^+ release occurred in the algae wall.

Algae flocculation test

AgNPs did not affect algae flocculation in the chronic exposure concentrations used in the present study (up to 0.56 µg/L). Therefore, algae were equally available for *Ceriodaphnia reticulata* in treatments and control. This agrees with the toxicological data on the effect of AgNPs on *T. obliquus*, as the reported EC50 growth inhibition concentrations are several orders of magnitude higher (between 38.5 and 1000 µg/L (Zouzelka et al. 2016; Pham 2018).

Acute toxicity tests

The AgNPs were highly toxic for *C. reticulata* even under the presence of food (LC50 0.44 – 1.1 µg/L). Although the AgNP toxicity has not been previously evaluated for this

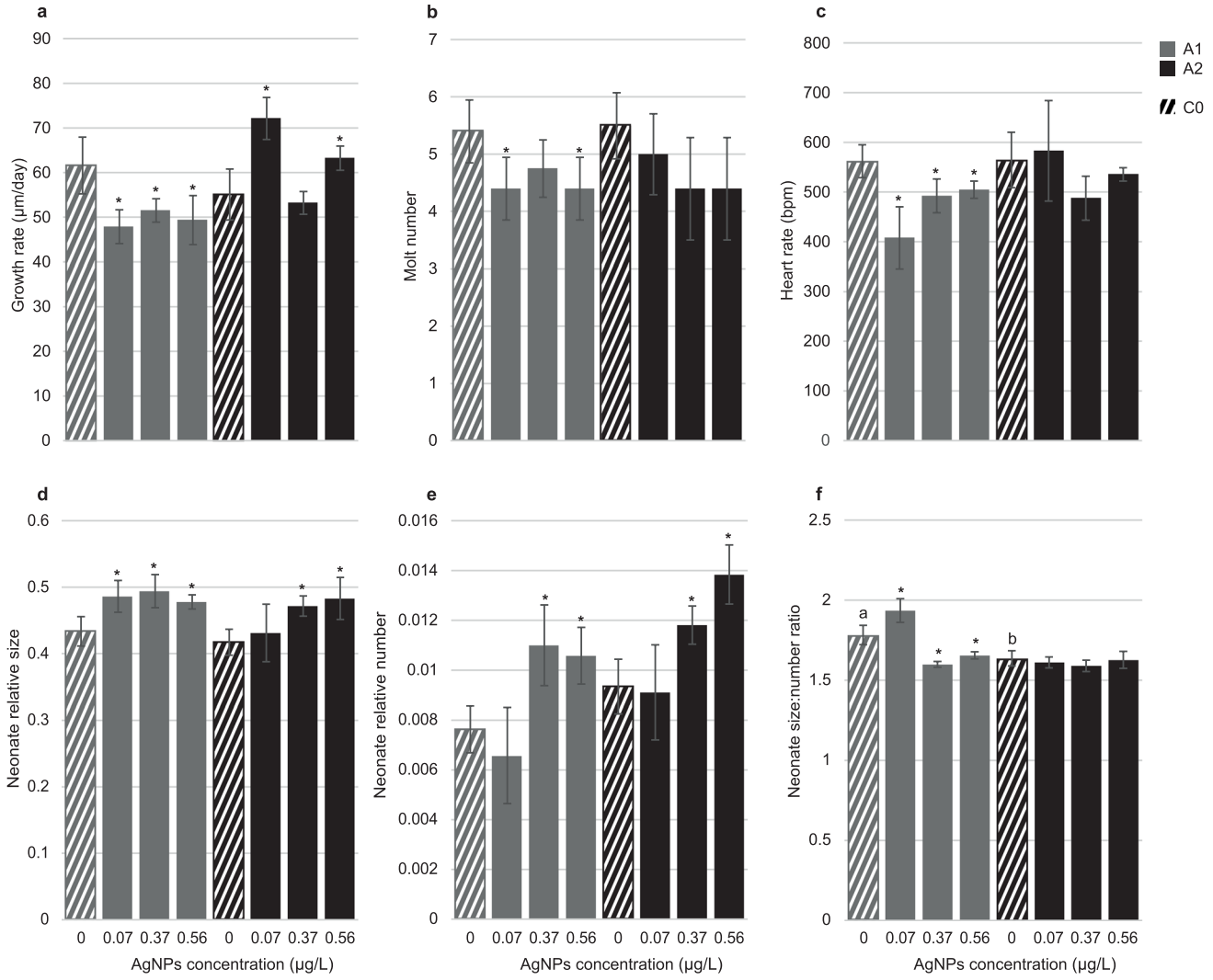

Fig. 4 Chronic toxicity test with AgNPs results: *C. reticulata*: (**a**) growth rate (µm/day), (**b**) molt number, (**c**) heart rate (bpm), (**d**) neonate relative size (dimensionless: mean neonate size µm/mother size µm), (**e**) neonate relative number (dimensionless: mean brood number/mother size µm), and (f) neonate size:number ratio (dimensionless: mean neonate size µm /mean brood number)

species, the LC50 values found in the present study are among the lowest toxicity values reported for *C. dubia*: LC50 between 0.15 and 67 µg/L (Griffitt et al. 2008; McLaughlin and Bonzongo 2012; Angel et al. 2013; Kennedy et al. 2015; Harmon et al. 2017) and *Daphnia* spp.: LC50 between 0.26 to 30 µg/L (Hoheisel et al. 2012; Zhao and Wang 2012; Silva et al. 2014; Becaro et al. 2015; Assis da Silva et al. 2022). Moreover, the LC50 informed in the present study is below the estimated exposure scenarios for freshwater bodies (8.8×10^{-5} – 10 µg/L) (Nowack and Mueller 2008; Gottschalk et al. 2013; Maurer-Jones et al. 2013), which may imply a high environmental risk for cladocerans. The AgNPs imposed higher toxicity than $AgNO_3$ for *C. reticulata*; therefore, the nanoparticles themselves showed to be more toxic than the Ag^+ ions they release. This constitutes a relevant result as is opposite to previous studies (e.g., Angel et al. 2013; Hu et al. 2017; Ribeiro et al. 2014; Zhao and Wang 2011), which tend to subordinate the toxicity of AgNPs to the current regulation for silver and may underestimate the toxic effects and the different mechanisms of action of nanoparticles. The higher toxicity of AgNPs could have been due to different aspects related to their nanometric properties. Several studies reported that their greater reactivity could promote their permeability through biological membranes (McShan et al. 2014), and then, AgNPs can release Ag^+ into organism cells, generating oxidative stress, a process called "Trojan horse" mechanism (Ulm et al. 2015; de Souza et al. 2019; Galhano et al. 2022). Also, several reports showed that AgNPs can impose mechanical effects such obstruction of filter setae and digestive system and adhesion to antennas and carapace (Zhao and Wang 2010; Asghari et al. 2012).

The lethal toxicity of AgNPs in *C. reticulata* decreased when algae concentration increased. Some authors have attributed this toxicity reduction to two main factors: better nutritional condition and the interaction algae-AgNPs (Allen et al. 2010; Ribeiro et al. 2014; Harmon et al. 2017). In this sense, Conine and Frost (2017) tested the AgNP toxicity under the presence of *T. obliquus* with different phosphorus content and concluded that the decrease of AgNP toxicity was primarily due to the algae binding and uptake abilities and, to a lesser extent, to their effects on *Daphnia magna* nutrition. Although both factors (nutritional condition and AgNPs-algae interaction) can act simultaneously, the observed increase in Ag^+ release in relation to the algae concentration in the present study indicated that algae actually interacted with the AgNPs. Therefore, as AgNPs were more toxic than Ag^+ for *C. reticulata*, the AgNP-algae interaction could have been one of the main factors conditioning the particle toxicity.

The observed increase in *C. reticulata* size in relation to food concentration in controls was expectable due to the increase in energy supply (Savaş and Erdoğan 2006). However, AgNP concentrations ≥ 1 µg/L caused development inhibition (i.e., decreased final size) together with a decreased heart rate. As AgNPs did not affect algal flocculation, food availability for *C. reticulata* was not affected in treatments. Nevertheless, several studies reported that algae can uptake Ag^+ or adsorb AgNPs; therefore, they may have less nutritional quality and constitute another Ag uptake route for cladocerans, which could have affected their metabolism and growth (Zhao and Wang 2010; Yoo-iam et al. 2014; Wang et al. 2019a; Dang et al. 2021). In addition, the potential mechanical effects of AgNPs on micro-crustaceans previously mentioned (obstruction of filter setae and digestive system, and adhesion to antennas and carapace) can affect their feeding and locomotion (Zhao and Wang 2010; Asghari et al. 2012).

Chronic toxicity test

In the case of low algae concentration, *C. reticulata* heart rate, number of molts, and growth rate were lower in all AgNP treatments. However, these effects were not observed at the highest algae concentration. Furthermore, an opposite trend was observed as the growth rate increased in the highest algae concentration treatments compared to control; thus, the underlying mechanisms of this trend remain unclear and need to be further explored. Our findings clearly show a mitigation on AgNP toxicity when the algae concentration increased, which, as discussed above, might be due to both nutritional condition and algae-AgNP interaction (Harmon et al. 2017). Concomitantly, Ribeiro et al. (2014) reported no effects of AgNPs on *D. magna* growth exposed to up to 5 µg/L AgNPs (21 d). Conversely, Zhao and Wang (2011) registered an inhibition of *D. magna* growth when exposed to higher AgNP concentrations (over 5 µg/L, 21 d) and attributed these results to a poor food quality because algae can adsorb the particles or uptake Ag^+ ions (Wang et al. 2019b; Dang et al. 2021).

Cladocera heart rate is considered a sensitive physiological indicator (Villegas-Navarro et al. 2003; Corotto et al. 2010), and recent studies showed negative effects of AgNPs on this variable. For instance, Borase et al. (2019) reported that *Moina macrocopa* heart rate decreased when exposed for 15 min to a high concentration (500 µg/L) of AgNPs. Park et al. (2022) found a significant decrease in *D. magna* heart rate after a 3 h exposure to a lower concentration (10 µg/L) of AgNPs.

Regarding reproductive parameters, an increase in neonate relative size and number in relation to the mother size was observed in AgNP treatments under both low and high algae concentrations. These results may indicate that *C. reticulata* has suffered energy allocation compromises, investing its energy in reproduction instead of growth. In accordance, Li et al. (2011) reported energy allocation compromises in *C. dubia* exposed to titanium oxide and aluminum oxide nanoparticles due to a reduction in energy assimilation. Also, Sun et al. (2022) found that zinc oxide nanoparticles affect energy allocation on reproduction and growth of *D. magna* under different food (*Chlorella pyrenoidosa*) concentrations.

In the present study, energy allocation compromises were also observed in the offspring characteristics. At low algae concentration, the neonate size:number ratio was variable depending on AgNP concentration: at 0.075 µg/L AgNPs, fewer bigger neonates were observed compared to control, whereas the opposite was registered at concentrations ≥ 0.373 µg/L AgNPs. At high algae concentration, this ratio was lower (i.e., many smaller neonates) but AgNPs did not change this pattern, indicating that this effect was mitigated by the algae. Several studies reported a decrease in *D. magna* offspring when exposed to AgNPs concentrations ≥ 1 µg/L (Ribeiro et al. 2014) and 50 µg/L (Zhao and Wang 2011). Those effects were attributed to reduced food consumption and nutrient absorption (Park et al. 2021).

Conclusions

AgNPs negatively affected *C. reticulata* life-history traits, including mortality, growth, reproduction, and heart rate. The presence of algae mitigated most of these negative effects in a concentration-dependent manner. Nevertheless, even in presence of algae, negative effects of AgNPs on *C. reticulata* were imposed below the predicted environmental concentrations. Our study demonstrated that algae promote the release of Ag^+ from AgNPs, which could negatively

affect other organisms and ecological processes. This study highlights the importance of assessing realistic exposure scenarios considering potential environmental effects on AgNP behavior and toxicity.

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Declarations

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