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EFFECTS OF SILVER NANOPARTICLES TO THE FRESHWATER SNAIL *PHYSA ACUTA*: THE
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Running title: Effects of silver nanoparticles to the snail *Physa acuta*

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Abstract: Silver nanoparticles (AgNP) are highly used worldwide, which will most likely lead to their release to the environment and subsequently increase environmental concentrations. Therefore studying AgNPs' deleterious effects to organisms is crucial to understand their environmental impacts. The freshwater snail *Physa acuta* was chosen to evaluate the potential deleterious effects of AgNP and counterpart AgNO₃, through water only exposures. AgNPs' toxicity is greatly influenced by medium composition. Thus, two test media were tested: Artificial Pond Water (APW) and modified APW (modified by removing calcium chloride from medium). Acute tests (96h) were performed with juvenile and adult snails, in both media, to assess lethality, and egg mass chronic tests, with APW medium only, to assess embryo viability and mortality, carried out until reaching 90% hatching success in the control. Acute toxicity increased with decreasing shell length for both silver forms (ion and nanoparticle), i.e. juveniles were more sensitive than adults. Different test media led to dissimilar LC₅₀ values, with chloride playing an important role in toxicity, most probably by complexation with silver ions and thus reducing silver's bioavailability, uptake and toxicity. Chronic tests showed that hatching success was more sensitive to silver in the ionic form than in the particulate form. Different forms of silver, exposure media and life cycle stages led to different patterns of toxicity, highlighting an impairment in the snails' life cycle. This article is protected by copyright. All rights reserved

Keywords: Silver nanoparticles, Silver ions, *Physa acuta*, chloride, Test media, Life cycle

INTRODUCTION

The production and commercialization of silver nanoparticles (AgNP) has grown rapidly in the last decade. AgNP are found in several different products such as domestic disinfectants, clothes, cleaning products, hygienic products and pharmaceuticals (due to their bactericidal properties) [1, 2]. This will inevitably lead to the release of nanoparticles (NPs) into the environment and consequently increase environmental AgNP concentrations over time [3, 4]. The predicted environmental concentration (PEC) of AgNP in aquatic environments was reported to range between 0.03 µg/L for AgNP and 100 µg/L for latex NPs [5, 6]. Lower PEC levels were modelled in other studies: 0.18 ng/L [7] and around 0.70 ng/L [8] for AgNP and 40 ng/L [9] for total Ag. Even though PEC levels are low, rates of AgNPs' discharge may increase as a result of the growing application of AgNP in daily used products. There are scarce modelled scenarios estimating environmental concentrations and limited amount of data regarding engineered nanoparticle (NP) releases [10]. In the environment AgNP are naturally formed from silver ions (Ag^+) and the surrounding conditions [11]. This natural formation will also influence AgNP levels and thus may lead to inaccurate PEC values, since these values only account for engineered NPs.

There is an urgent need to increase AgNP research studies in order to improve our knowledge about potential deleterious effects of AgNP to the environment [12, 13, 14], as it is still unclear whether the toxicity of AgNP is due to the release of Ag^+ or to their unique properties, or even due to a combination of both silver forms (ion and particulate) [15] and to what extent [3]. In a study with zebrafish models, Asharani et al. [16] demonstrated that the toxicity of AgNP to fish embryos was not due to the release of Ag^+ , since the phenotypic defects observed in AgNP treatments were not observed in the Ag^+ treatments. Griffitt et al. [17] also concluded that the effects of copper and silver NPs were not exclusively consequence of the release of soluble metal ions in zebrafish gill acute exposures. Thus, it is imperative to focus on the specific characteristic properties of the NPs, such as effects of different

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sizes and coatings [1, 16, 18] and transformation products [19]. On the other hand, Kennedy et al. [20] studied the acute toxicity of different AgNP suspensions using three aquatic test organisms (*Daphnia magna*, *Pimephales promelas*, *Pseudokirchneriella subcapitata*) and concluded that dissolved silver played a critical role in acute toxicity. AgNP release Ag^+ over time, with rates of release depending on AgNP characteristics. Kittler et al [21] demonstrated that the % weight loss of citrate-stabilized and poly(vinylpyrrolidone)-stabilized AgNP due to the release of Ag^+ varied from approximately 2 to 50% after 8 days of dispersion in ultrapure water and between 10 to 70% after 125 days. Silver ions complex with ligands such as sulphides, chlorides and also organic matter [4]. The concentration of chloride varies between aquatic environments, affecting silver speciation. As result, several chloride complexes are formed, such as AgCl (aqueous - aq), AgCl_2^- , AgCl_3^{2-} , and AgCl_4^{3-} , which play an important role on silver's bioavailability and uptake rates [22]. To better understand the behaviour, mode of action, and potential hazardous issues associated to AgNP, it is important that ecotoxicological studies focus on different forms of silver, such as dissolved, nanoparticle and bulk [12] and also different species, with different life traits, e.g. different feeding behaviours will consequently influence the uptake of silver.

Ecotoxicological studies evaluating the potential toxic/deleterious effects of AgNP in freshwater environments are mostly based on assays performed with fish, algae and crustaceans (namely daphnids, the most common species used in ecotoxicological studies), representing a narrow range of species. Also, due to AgNP antifungicidal/antibacterial properties, microbes have been targets of study. Thus, to expand the range of test species, the hermaphroditic freshwater pulmonate snail *Physa acuta* was the aquatic model organism chosen for the present study [23]. It is an invasive snail species originated from North America that has widely spread its distribution in the last two centuries [24]. *Physa* potentially serves as an important link between primary producers and higher level organisms in freshwater food webs [25], in both lentic and lotic systems, since different life cycle stages of *Physa* can serve as quarry to fish and other organisms. Previous studies have demonstrated the suitability of *P. acuta* for toxicity testing, due to their high sensitivity to different metals and

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pharmaceuticals [25, 26]. More recently, not only *P. acuta* but also *Lymnaea* snail species were used as model organisms in nanoparticle toxicity studies [27-30].

The present study examines the impact of different chloride concentrations in exposure media (Artificial Pond Water (APW) medium [31] and modified APW medium (section 2.2)), on the toxicity of silver in the particulate (AgNP) and aqueous form (AgNO₃) to the freshwater snail *Physa acuta*. The use of both silver forms helps to determine if toxicity is mainly due to the particulate form or the ionic/complexes [32]. The whole life cycle of *P. acuta* was approached and each stage was examined upon water only exposures of the egg masses, juveniles and adults (Figure 1). Silver toxicity was therefore assessed by measuring several endpoints at each life cycle stage after acute and chronic exposures to AgNP and silver nitrate (AgNO₃).

MATERIAL AND METHODS

Chemicals

Silver nitrate was purchased as a crystalline powder from Sigma Aldrich Co. (CAS number 7761-88-8), 99% purity. The silver nanoparticles were supplied dispersed in ultrapure water in a concentration of 1000 mg/L by AMEPOX Enterprise (90-268 Łódź, Jaracza 6, Poland), as described by Ribeiro et al. [4]. The range of particle diameter in the supplied dispersion was between 3 and 8 nm, negatively charged and coated with an alkane protection layer. This polymer was also provided as a powder and tested for toxicity. The initial AgNP dispersion was always shaken before use to homogenise the batch suspension. For each AgNP bioassay, test dispersions were prepared immediately before use by dispersion in Artificial Pond Water (APW) or modified APW medium. The AgNO₃ exposures were prepared by diluting a 50 mg Ag/L stock solution, from the 99% purity AgNO₃ powder, in ultrapure water and posteriorly diluted in APW or modified APW medium to the desired concentration. APW was prepared by adding 100 mL of each stock solution (prepared with ultrapure water) to a 20L carboy filled with distilled water: 1) calcium chloride (58.8 g/L CaCl₂·2H₂O); 2) magnesium sulphate (24.65 g/L MgSO₄·7H₂O); 3) sodium hydrogen carbonate (12.95 g/L NaHCO₃);

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and 4) potassium chloride (1.15 g/L KCl) [31]. In the acute toxicity tests, modified APW was also used as a test media, differing from APW only on the absence of stock solution 1) calcium chloride.

Physa acuta

Cultures of *Physa acuta* have been maintained in aquariums (6L glass), in laboratory conditions, for several years, with 30 to 50 snails per aquarium in 3L of APW [31]. Culture snails were fed every other day with grinded fish food (Tetramin®) *ad libitum*, maintained in a controlled-temperature room ($22^{\circ}\text{C} \pm 1$), with a photoperiod of 16h: 8h light: dark cycle, pH kept above 7.9 (± 0.3) as acidic environments cause shell fractioning, and continuous aeration (dissolved oxygen above 8 mg/L). Culture media was partially renewed every other day and fully renewed once a week. New snail cultures were initiated from newly hatched offspring using egg masses deposited by adult snails and collected to 1L glass vials. After 8 to 10 days the egg hatching started and the newly hatched snails were transferred to aquariums, as described above.

Acute toxicity tests

Both adult ($> 5\text{mm}$) and juvenile ($< 1\text{mm}$) snails were exposed to AgNO_3 and AgNP in a total of eight 96h acute toxicity tests, four for each length (2 snail stages x 2 silver forms x 2 media). Adult AgNO_3 exposures ranged from 500 to 2000 $\mu\text{g/L}$ for APW, and 40 to 250 $\mu\text{g/L}$ (nominal concentrations) for modified APW; AgNP exposures ranged from 2000 to 10000 $\mu\text{g/L}$ (nominal concentrations) for both media. Previously, range finding tests were performed for each silver form for each medium (data not shown), to find the range where effects were observable. An APW control was set up for each test, plus a modified APW control in the modified APW exposures, and also a control with the highest concentration of the AgNP protection layer present in each AgNP exposure (knowing that the protection layer makes up 12% (w/w) of the NPs). Tests were set up with 5 replicates per concentration/control and 3 snails per replicate, in 150 mL glass vials with 100 mL of the test solution. All tests were upheld under culture conditions, with one full media renewal performed at 48h (semi-static test). Snails were not fed during the experiments. Mortality was reported daily and snails were

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considered dead when, after a gentle agitation and stress induced with a dissecting needle to the foot of the snail, it showed no movement. The respective 96h LC50 (lethal concentration inducing 50% mortality) values were calculated (section 2.8). The same methodology was followed to perform the juvenile acute toxicity tests, with some adaptations: tests were set up in 50 mm diameter Petri dishes with ≈ 15 ml of test solution; snails ≤ 48 h old and never fed were used after confirmed to be alive by checking the heartbeat with a stereomicroscope prior to starting the test; mortality was recorded daily also based on the presence of heartbeat. Juvenile AgNO_3 exposures ranged from 12.5 to 100 $\mu\text{g/L}$ for APW medium and 7.5 to 45 $\mu\text{g/L}$ (nominal concentrations) for modified APW; juvenile AgNP exposures ranged from 12.5 to 400 $\mu\text{g/L}$ for APW medium and 12.5 to 100 $\mu\text{g/L}$ (nominal concentrations) for modified APW (values based on range finding tests performed prior to the acute exposures to find the range where effects were observable – data not shown).

Chronic toxicity tests

To assess chronic toxicity, two egg mass tests were performed: one with egg masses exposed to AgNO_3 , ranging from 0.94 to 15 $\mu\text{g/L}$, and another with egg masses exposed to AgNP ranging from 3.75 to 120 $\mu\text{g/L}$ (nominal concentrations). Each exposure was carried out until the control reached 90% hatching success (13 days). Each concentration range was based on range finding tests performed prior to the chronic exposures, to find the range within effects were observable (data not shown).

Chronic tests were only set up using APW medium, because calcium is crucial for the development of the snails' shell. A control with APW was set up for each test and also a control with the highest concentration of AgNP protection layer present for the AgNP test (as previously described in section 2.3). At the beginning of each test, the number of eggs (embryos) per egg mass (aged 2 to 4 days old) were counted using a stereomicroscope. Each test was set up in 50 mm diameter Petri dishes with ≈ 15 ml of test solution, with 5 replicates per concentration and 1 egg mass per replicate. Both tests were performed under the same conditions previously stated for the acute toxicity tests (section 2.3), but with aeration provided and full medium renewals every other day during the testing period (semi-static). This article is protected by copyright. All rights reserved

test), to decrease variability of the AgNP in terms of state and concentration. Mortality and hatching were reported daily. Four parameters were measured throughout each chronic test and checked daily: the number of alive and dead snails plus the number of alive and dead embryos. At the end of the test (day 13), the percentages of alive and dead snails and the percentage of alive and dead embryos were calculated. The LC50 values for cumulative mortality (snails and embryos included) and EC50 (effective concentration inducing 50% effect) values for hatching success (alive and dead snails included) were also calculated (section 2.8).

Characterization of the AgNP

The initial suspension of AgNP (1000 mg/L) provided by AMEPOX was dispersed in APW and modified APW to a concentration of 0.1 mg/mL. For *Transmission electron microscopy* (TEM) imaging samples were prepared by laying a drop of each dispersion medium on a punctured carbon coated Cu TEM grid and dried at room temperature for several hours before examination. Micrographs were carried out on a JEOL 2010 analytical TEM which has a resolution of 0.19 nm, an electron probe size down to 0.5 nm and a maximum specimen tilt of $\pm 10^\circ$ along both axes. The instrument is equipped with an Oxford Instruments LZ5 windowless energy dispersive X-ray spectrometer (EDS) controlled by INCA.

Aggregation/agglomeration and zeta average diameter of the AgNP in both media (concentrations ranging from 0.5 to 10 mg/L) were measured with Dynamic Light Scattering, DLS, (Malvern Zetasizer, Malvern instruments Ltd, Worcestershire, UK) using standard cuvettes.

Aggregation/agglomeration experiments were carried out for a short period of time (10 minutes) and samples were prepared immediately before the measurement was carried out. The stocks were mixed with each media to the desired concentrations in the cuvettes, shaken and immediately inserted in the instrument. The measurement started at a fixed attenuator and measurement position, obtained by standard measurement of the respective AgNP concentration in Milli-Q water to avoid the instrument-

optimization time. The correlation time was set to 2 seconds and 120 data points were generally obtained. All measurements were performed at 20 °C.

The zeta average diameters were followed for a period of 48h (sampling points 24 and 48h) in both media to simulate the time of exposure of the snails to the AgNP in the bioassays. The samples showed sedimentation and a discolored supernatant and therefore were measured before and after resuspended. These long term measurements were performed in triplicate allowing the instrument to set the attenuator and measurement position automatically. No differentiation between aggregates and agglomerates was made.

Size evolution experiments with AgNO₃ and silver speciation

To test if the potential formation of Ag precipitates (likely AgCl) observed in AgNO₃ treatments were due to the presence of chloride, size evolution experiments with DLS were performed in both media and also in a CaCl₂ solution, using concentrations that ranged between 4 and 10 mg/L AgNO₃. A solution, with the same concentration of CaCl₂ present in APW medium (294 mg/L), was prepared out of a 1M CaCl₂ stock solution (CAS 10043-52-4, Fluka Analytical). The size of the forming precipitates was also measured after 24 and 48 hours as described above (section 2.5).

Thermodynamic speciation of silver was estimated for both media, whereas the different species of silver (Ag⁺, AgCl (aq), AgCl₂⁻ and other dissolved species) were calculated (check the statistical analysis section 2.8 for more details).

Chemical analysis

Total Ag measurements were performed for both silver forms, ion and particulate, in the lowest and highest concentration samples (in triplicate) of each toxicity test (acute and chronic), at time 0h (beginning of the test) and 48h (before full renewal) using a digestion method. Prior to digestion (24h), concentrated H₂O₂ and HCl were added to 10 mL of each sample, bringing its concentration to 5% and 1% (v/v) in the sample, respectively. Samples were then transferred to Teflon beakers and allowed to evaporate (without boiling) on a hot plate until reaching a volume between 0.5 – 1.5 mL. After cooling, This article is protected by copyright. All rights reserved

aqua-regia 1 HNO₃: 3 HCl (25% v/v: 56%v/v) was added to each sample and heated again for 30 minutes. All samples were allowed to cool to room temperature and then transferred to 50 mL falcon tubes, diluted to 45 mL with 1% HCl solution for concentrations ≤ 0.5 mg/L and with 5% HCl solution for concentrations > 0.5 mg/L. Total Ag was measured by Graphite-Furnace Atomic Absorption Spectroscopy (GF-AAS) (detection limit 0.318 $\mu\text{g/L}$). To measure the recovery efficiency of the method, concentrations of Ag in the same order magnitude of the concentration used for AgNO₃ and AgNP in the toxicity tests were prepared with each test media (APW and modified APW).

Dissolved silver was also measured in the lowest and highest concentration samples (in triplicate) of each toxicity test (acute and chronic) performed with AgNP at time 48h (before renewal), without pre-digestion. Amicon Ultra-15 Centrifugal Filter Units (pore size 10 kDa) were used to filter 10 mL of each sample. Prior to filtration, the centrifugal filter units were saturated with a 0.1M copper nitrate solution, by centrifuging 2 mL of the solution for 30 minutes at 4000 rpm. Afterwards the filter was removed, rinsed with Milli-Q water and allowed to dry for 30 minutes. Samples were then centrifuged for 30 minutes at 4000 rpm and transferred to 15 mL falcon tubes and HNO₃ and HCl added in the same proportions as for the total Ag measurement samples (HNO₃ 25% v/v: HCl 56%v/v).

Statistical analysis

For acute toxicity tests, data was statistically analysed by probit analysis using the PriProbit software, version 1.63 [33], and 96h LC50 values calculated.

Chronic toxicity results were analysed by one way analyses of variance (ANOVA) for each of the four parameters measured per treatment/concentration in each exposure (AgNO₃ and AgNP): alive and dead snails and alive and dead embryos. Data from each parameter measured were transformed in percentage before statistical analysis. Whenever differences were attained ($p < 0.05$) in the ANOVA analysis, a post-hoc multiple comparison Dunnett's method was used to compare each treatment with the control [34]. Whenever data failed normality distribution and/or homoscedasticity, and no data transformation was successful, a Kruskal–Wallis ANOVA on Ranks was performed [34], followed by

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post-hoc multiple comparison Dunn's method when significant differences were found ($p < 0.05$). Results and discussion are based on the parameters where significant differences relatively to the control were obtained.

EC50 and LC50 values were also calculated for each chronic toxicity test using the equation/model that best fitted the data. A 4 parameter logistic model was applied for AgNO₃ hatching success; for the AgNP exposure a sigmoidal 3 parameter model and a Weibull 5 parameter model were applied for hatching success and mortality, respectively. All data treatment was performed using the SigmaPlot software for windows, version 11 [35].

To check the absence/presence of statistical significant differences between control medium and the protecting layer (coating), a t-test for each control medium (APW and modified APW) was performed or the respective non parametric test (Mann-Whitney Rank Sum Test) for all AgNP assays (Supplemental Data, tables 3S and 4S).

Thermodynamic speciation of silver was calculated using the Visual MINTEQ version 3.0 at 20°C [36], with activity coefficients calculated using the Davies equation (valid for ionic strengths smaller than 0.3 M) and the thermodynamic database included in the software.

RESULTS

Characterization of the AgNP

TEM analysis showed no major differences between AgNP in APW and modified APW media (Figure 2). In both media, single particles and aggregates/agglomerates were present and an organic layer was visible on the outside of the particles.

The results of the aggregation/agglomeration experiments for AgNP in both media are shown in Figure 3. In APW, an increase in zeta average diameter was observed over time and the highest concentration (10 mg/L) showed slightly higher aggregation/agglomeration rates, increasing from an initial size of 100 nm to nearly 140 nm within minutes. As for the modified APW, the aggregation/agglomeration of AgNP was reduced compared to the APW medium, varying from an

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initial size of 100 to 120 nm. The quality of the data decreased for lower concentrations (<1 mg/L), which was reflected in the unstable size measurements for AgNP, majorly in the APW media.

The size measurements of the AgNP after 24 and 48 hours with APW and modified APW showed an increase in time (Figure 4), which was more pronounced in the first 24 h. In both media the initial size of the particles was around 100 nm. However, the increase in time was more pronounced in the APW medium, where at lower concentrations sizes were under 250 nm, but at higher concentrations sizes reached 350 nm after 48 h. The AgNP sizes measured in the modified APW medium after 48 h were mostly between 120 and 200 nm, much lower than observed for the APW medium. The increase in size after 48 hours was ambiguous for 2 mg/L AgNP in APW. The resuspended samples showed in general zeta average diameters greater than the ones found in the supernatant due to the resuspension of larger particles from the bottom of the cuvette.

Size evolution experiments with AgNO_3 and silver speciation

The size evolution experiments with AgNO_3 in APW, modified APW and CaCl_2 showed the occurrence of particles (Supplemental Data, Figure 1S). All media solutions and the AgNO_3 stock solution (in Milli-Q water) were particle free but, after mixing each medium with the AgNO_3 stock solution, white precipitates were formed. The experiment with AgNO_3 in CaCl_2 solution also showed precipitation. The measured zeta average diameters in modified APW medium were in general higher compared to APW or to the CaCl_2 solution. The thermodynamic speciation of silver (Figure 5) showed a higher complexation between Ag^+ and Cl^- in APW compared to modified APW. The dominant form of silver in modified APW was the ionic form. Therefore, the white precipitates observed in the size evolution experiments were most probably cerargyrite (AgCl(s)).

Chemical analysis

The average percentage recovery of Ag (percentage of Ag actually measured comparatively to the expected nominal value) in AgNO_3 samples measured at time 0 h ranged between 76 and 100%, with a few samples showing recovery rates higher than 100%. For the AgNO_3 samples measured at

time 48h, the average percentage recovery ranged between 31 and 116% (Supplemental Data, Table 1S). As for AgNP samples, the average percentage recovery ranged between 91 and 131% at time 0h and between 91 and 146% at time 48h. The recovery efficiency of the method was above 95%. The 48h dissolved silver samples from AgNP exposures showed that less than 11% of total silver was in the dissolved form in the modified APW medium and less than 2.2% in the APW medium (Supplemental Data, Table 2S). Results and discussion are based on nominal concentrations, since only the lowest and highest concentrations of each toxicity test were analysed for total and dissolved silver.

Bioassays

The 96h LC50 values calculated for all acute toxicity tests are presented in Table 1. Silver was more toxic to both juvenile and adult snails in the ionic form than in the nanoparticle form, except in the modified APW exposures where both forms of silver showed similar toxicity. However, the juvenile snails were more sensitive to both silver forms, showing much lower 96h LC50 values than adults. The 96h LC50 values for adult snails in the AgNO₃ exposure showed that silver in the ionic form was around 6 times more toxic in modified APW medium than in APW medium, 116 µg/L vs 740 µg/L, respectively. As for the juvenile snails, this difference was much lower, around 1.7 times, 27.4 µg/L vs 45.8 µg/L, respectively. The 96h LC50 values for adult snails exposed to the AgNP exposure was approximately 1.7 times more toxic in the modified APW medium than in the APW medium, 4110 µg/L vs 7050 µg/L, respectively. For the juvenile snails, the difference between media increased around 5 times, 48.5 µg/L for modified APW medium vs 258.7 µg/L for APW medium.

The results obtained in the chronic egg mass tests with AgNO₃ and AgNP are shown in Figures 6 and 7, respectively. The percentage of alive snails in the AgNO₃ exposure decreased with increasing concentration, where the highest concentration, 15 µg/L, was significantly different from the control ($p < 0.05$ Dunnett's Method), showing the lowest percentage of alive snails. The percentage of dead embryos increased with concentration, where once again the highest concentration, 15 µg/L, was

significantly different from the control ($p < 0.05$ Dunnett's Method), showing the highest percentage of dead embryos. In the AgNP exposure, the percentage of alive snails fluctuated, where the highest concentration, 120 $\mu\text{g/L}$, was significantly different from the control ($p < 0.05$ Dunnett's Method), showing the lowest percentage of alive snails. The percentage of dead embryos increased with increasing concentration, being significantly different from the control at the highest concentration ($p < 0.05$ Dunnett's Method), 120 $\mu\text{g/L}$, with the highest percentage of dead embryos.

Table 2 illustrates the EC50 values calculated for hatching success and LC50 values for mortality. For hatching success, the EC50 was higher for AgNP (95.7 $\mu\text{g/L}$), revealing a lower toxicity compared to AgNO₃ (1.03 $\mu\text{g/L}$). Regarding mortality, there was a significant increase in the percentage of the embryos' mortality when concentrations increased for both silver forms with an LC50 value of 81.6 $\mu\text{g/L}$ for AgNP. No LC50 value for mortality was retrieved for AgNO₃ meaning that it is greater than the highest concentration here tested (LC50 > 15 $\mu\text{g/L}$).

No significant differences were observed between the control media treatments (APW and modified APW) and the AgNP protection layer treatments for all AgNP bioassays (t-test/Mann-Whitney Rank Sum Test, $p > 0.05$; Supplemental Data, Table 3S and 4S).

DISCUSSION

Several studies have suggested that AgNPs' toxicity is mainly due to the release of silver ions and depends on rates of release in time; others suggest that the intrinsic properties of AgNP are responsible, and still others suggest that both factors are responsible [15-17, 20]. The present approach allowed us to compare the acute toxicity of AgNP in two different media, differing in chloride concentrations, in adult vs juvenile snail stages. Physical-chemical parameters vary greatly between streams, rivers, lagoons and other aquatic systems, leading to different levels of AgNP toxicity. The stability of AgNP and bioavailability of silver depends on the amount of organic matter, electrolytes and many abiotic factors like pH and ionic strength. Chloride concentration also varies widely between

freshwater environments, with values varying from as low as 0.2 mg/L up to 1553 mg/L [37-40]. Thus, each tested media is representative of a different chloride content: the modified APW medium, with a chloride concentration of 2.7 mg/L, is representative of freshwater environments with low chloride concentration; and the APW medium, with a chloride concentration of 73.7 mg/L, is representative of a medium level of chloride.

Acute exposures and medium

Chloride can impact the toxicity of Ag, by influencing its bioavailability, since it is an inorganic ligand that can bind to Ag. Looking at the 96h LC50 values for adult snails exposed to AgNO₃ (shell length > 5mm), Ag was around 6 times more toxic in the modified APW than in APW. This higher toxicity in the modified APW medium correlates with the results of the thermodynamic speciation of silver (figure 5), where the dominant form of silver was the ionic form, which is considered the most toxic form. The most dominant form of silver in APW medium was AgCl (aq). Besides Ag⁺, AgCl (aq) is also bioavailable and its direct uptake by aquatic invertebrates is overall important in the bioaccumulation of Ag [20]. Ag⁺ led to a higher toxicity of silver in the modified APW medium comparatively to the toxicity of silver in the APW medium mediated by AgCl (aq). Contrastingly, for the juvenile snails (shell length < 1mm), the difference was much lower. Lower AgNO₃ concentrations were used in the juvenile exposures. As shown in the thermodynamic speciation of silver (figure 5), smaller concentrations of total Ag revealed comparative total dissolved Ag concentrations of Ag⁺ in the modified APW as AgCl (aq) in the APW media. Each form of dissolved Ag represents the major form of Ag responsible for the toxicity in each respective medium. Thus, as juvenile snails are much more sensitive than adult snails, both forms were equally toxic, Ag⁺ in the modified APW and AgCl (aq) in the APW medium. For the adult snails exposed to AgNP (shell length > 5mm), the 96h LC50 between media differed only approximately 1.7 times, with the modified APW medium showing a higher toxicity. This difference in toxicity can be due to a combination of the higher aggregation/agglomeration of AgNP observed in the APW medium (figure 4) and consequently lower

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surface area (decreasing the rate of Ag^+ dissolution), leading to a reduced uptake of particles by the snails due to their larger agglomerate size. The formation of AgCl(s) upon release of Ag^+ into the medium was consequently lower in the APW medium comparatively to the modified APW medium.

The juvenile snails (shell length < 1mm) showed an increased difference, around 5 times more toxic in modified APW than APW, which emphasizes that the behavior of AgNP varies with concentration and medium.

The size evolution experiments with AgNO_3 and CaCl_2 solution showed precipitation, very likely to be AgCl(s) due to its low solubility product ($1.8 \times 10^{-10} K_{\text{sp}}$). This may have influenced the different LC50 values obtained, since different patterns of complexation between Ag^+ and chloride occurred between the APW and modified APW media, thus influencing the toxicity of Ag in each medium. A higher toxicity of silver in the modified APW medium was observed for both forms (ion and particulate), although at a higher extent for the adults comparatively to the juvenile snails. This may be a reflection of the higher sensitivity of the juveniles. . The present results allowed us to conclude that the medium influenced the toxicity of waterborne AgNP, as previously described by other authors [41, 42].

Chronic exposures

Hatching success was much more sensitive to the AgNO_3 than AgNP exposure, with EC50 values of 1.03 $\mu\text{g/L}$ and 95.7 $\mu\text{g/L}$ in the APW medium, respectively. In the case of mortality, there was a significant increase in the % of dead embryos and a significant decrease in the % of alive snails at a concentration of 15 $\mu\text{g/L}$ for the AgNO_3 exposure, while for the AgNP exposure, a similar effect wasn't achieved until a much higher concentration, 120 $\mu\text{g/L}$. Since the % of dissolved silver was very low, < 2.2 %, in the AgNP exposure (Supplemental Data, Table 2S), the toxicity observed was probably mostly due to intrinsic properties of the AgNP and less related to Ag^+ released. Moreover, no LC50 was retrieved for the AgNO_3 exposure, whereas the highest concentration used was 15 $\mu\text{g/L}$.

This demonstrates that the % of dissolved silver (corresponding to a concentration of approximately

2.64 $\mu\text{g Ag/L}$) measured in the highest concentration of the AgNP exposure had little influence on the LC50 retrieved for the AgNP exposure. Focusing on the toxicity due to intrinsic properties of AgNP, recent efforts to understand their toxic mechanism effects have been undergoing. Griffitt et al. [43] argues that the toxicity of AgNP is far from being simply due to the release of Ag^+ , but also greatly dependent on coating, size, surface, surface charge and other particular properties of the NPs. Coating greatly influences the uptake of AgNP, as demonstrated by Farkas et al [44] when studying the uptake and effect of citrate (AgNP_{CIT}) and polyvinylpyrrolidone (AgNP_{PVP}) coated manufactured AgNP using primary gill cells of the rainbow trout *Oncorhynchus mykiss*. Uptake was higher for AgNP_{CIT} comparatively to AgNP_{PVP} . The results of the chronic exposures may also reflect different patterns of penetration through the egg mass membrane by the ions relatively to the NPs. Possibly the ions penetrate easier and the aggregation of the AgNP prevents their penetration leading to a very low hatching success in the AgNO_3 exposure in comparison. Ma and Lin [45], studied the complex nano-bio interactions at the outer surfaces of aquatic organisms and argued that the mucus secreted by the fish epidermis may embed NPs. In analogy, the egg masses of the snails are also mucous, which may have embedded the NPs on the surface of the egg membrane preventing a massive penetration of the NPs. Ribeiro et al [46] studied the uptake and elimination kinetics of AgNP (the same used in the present study) comparatively to AgNO_3 in the micro green algae *Raphidocelis subcapitata*. Results showed that the algae internalized Ag mostly in the ionic form when looking at the AgNP exposures. In addition a higher bioconcentration factor (BCF) was obtained for the dissolved form of Ag rather than other Ag-sized fractions.

Interestingly, the sensitivity of the snails in the AgNP exposure was very similar for mortality and hatching success, presenting close EC50 and LC50 values, while for the AgNO_3 exposure this phenomenon was not observed, meaning the sensitivity of both parameters was dissimilar. This may reflect the influence of the silver form snails were exposed too: the particulate form of silver in the AgNP exposure lead to similar values between the EC50 and LC50 and the ionic form in the AgNO_3

exposure lead to dissimilar values. Thus, as stated above, the toxicity in the AgNP exposure was probably mainly due to intrinsic properties and less related to Ag^+ .

AgNP toxicity

Overall, the chronic and acute assays suggest that the toxicity of AgNP was not solely due to the release of Ag^+ but also influenced by transformation processes of the AgNP (aggregation/agglomeration, sedimentation, dissolution, surface modification). However, to confirm this hypothesis further investigation should be carried out, focusing on the transformation processes of the AgNP. The EC50 and LC50 values between AgNP and Ag^+ were very different, with one exception: the LC50 values between the juveniles exposed to AgNO_3 and AgNP in modified APW medium, with overlapping CIs values. Thus, besides bioavailability (related to different silver forms in each exposure), different modes of action seemed to be involved, depending on which form of silver the snails were exposed to, and also different toxic mechanisms. In addition, we were handling exposures with multiple Ag types (AgNP, Ag^+ , AgCl (aq), AgCl_2^-), which could lead to additive effects or even an interaction between Ag forms. Hoheisel et al. [47] exposed *Daphnia magna* to AgNP of different sizes and concluded that toxicity increased with decreasing particle size. To some extent, size may have also influenced toxicity results in the present study, since different aggregation/agglomeration states were obtained, influencing the size of the AgNP snails were exposed to in each medium. Aggregation/agglomeration increased with increasing concentrations, especially in the APW medium, consequently reducing the release of free Ag^+ . Also, since semi-static tests were performed and AgNP concentrations were renewed after 48h, this represents a small period of time for dissolution of Ag^+ to occur. Chemical analysis performed did show that a low percentage of total silver was in the dissolved form after 48h of exposure: less than 2.2% for APW medium and 11% for modified APW medium. Nevertheless, this low percentage can be related to the formation of AgCl (s) and other compounds of Ag with organic ligands or/and sorption to the vessels walls. Liu and Hurt [48]

studied the dissolution rate of citrated capped AgNP and found that the oxidation occurs at a very low rate (6 to 125 days to completion), meaning the particle form persists for some time.

AgNP in freshwater environments

The sediment compartment is also an important element that greatly influences the toxicity of silver in freshwater environments. Bernot and Brandenburg [27] exposed *Physa acuta* to AgNP in the presence (whole sediment exposure, with uncontaminated medium and contaminated sediment) and absence of sediment (water-only exposure) and observed that more snails survived the 4-day bioassays in the presence of sediment. This suggests that sediment in natural systems may reduce exposure through the water column and therefore toxicity of silver possibly by acting as a sinking agent, immobilizing the particles through sedimentation or deposition and ions via sorption. Nevertheless, organisms living in the sediment will experience increased exposure concentration to AgNP, which also is of great concern regarding environmental assessment risk.

Predicted environmental concentrations (PEC) for Ag have been modelled in some studies for NPs and for total Ag. Concentrations of 0.18 ng/L [7], 0.70 ng/L [8], and 40 ng/L have been reported for total Ag [9]. Considering that a continuous and potentially increasing input of Ag is predicted for the environment, PEC values may tend to approach PNEC values in the near future.

CONCLUSION

In the present study the deleterious effects of silver nanoparticles and ions were compared using the freshwater snail *Physa acuta* as aquatic model. AgNP were not acutely toxic at environmental realistic concentrations. Sensitivity increased as shell length decreased both for silver nanoparticles and ions, although toxicity was overall higher for silver ions exposures; the embryonic phase was the most sensitive for AgNO₃ exposures, followed by the juvenile stage (< 1mm shell length) and the adult stage (> 5mm shell length); for the AgNP exposures both embryonic and juvenile stages had similar sensitivity and the adult stage was the less sensitive. Silver in the ionic form was chronically toxic at low concentrations (~1 µg/L). Although AgNP were not chronically toxic at such low concentrations, This article is protected by copyright. All rights reserved

engineered AgNP represent an additional source of silver ions into the environment, which should be taken under consideration since the embryonic phase is the most important stage of the snails' life cycle and insures the continuity of the species and population stability.

The chemical composition of the medium, and equivalently natural aquatic environments, play a key role when it comes to assessing the deleterious effects of silver (NPs and Ag⁺). Chloride concentration greatly influenced not only the bioavailability of silver but also its speciation and toxicity. Aggregation/agglomeration states of the AgNP were also greatly influenced by the chloride concentration in each medium, whereas the higher concentration (APW medium) lead to a higher aggregation/agglomeration state.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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Data availability—Data are available upon request from the authors at sgoncalves@ua.pt or sloureiro@ua.pt

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Figure 1. *Physa acuta* in different stages of development: A) day 3 of embryonic development (phase when the chronic egg mass assays were started); B) day 5 of embryonic development; C) juvenile stage; D) adult stage.

Figure 2. TEM images of AgNP dispersed in the APW medium (A and B) and in the modified APW medium (C and D), showing the AgNPs' morphology, aggregation/agglomeration and shape in each medium.

Figure 3. Aggregation/agglomeration experiments of AgNP in: A) APW medium and B) modified APW medium during 10 minutes (600 s). The y axis shows the zeta average (Z-ave) diameter in nm, obtained from the Dynamic Light Scattering (DLS) measurements with a Malvern Zetasizer. The x axis gives the time in seconds (s).

Figure 4. Zeta average diameter of AgNP measured before and after resuspended in: A) APW medium and B) modified APW medium, at 24h and 48h. The y axis shows the zeta average (Z-ave) diameter in nm, obtained from the Dynamic Light Scattering (DLS) measurements with a Malvern Zetasizer. The x axis gives the time in hours (h).

Figure 5. Thermodynamic speciation of silver (as AgNO₃), calculated using the Visual MINTEQ, in: A) APW medium and B) modified APW medium.

Figure 6. Embryo development of *Physa acuta* exposed to AgNO₃ for 13 days in APW medium: A) percentage of alive snails; B) percentage of dead snails; C) percentage of alive embryos; D) percentage of dead embryos. Data was expressed as mean values (bars represent standard error); concentrations are presented as nominal values. Asterisks denote statistical significant differences (Dunn's Method $P < 0.05$).

Figure 7. Embryo development of *Physa acuta* exposed to AgNP for 13 days in APW medium: A) percentage of alive snails; B) percentage of dead snails; C) percentage of alive embryos; D) percentage of dead embryos. Data was expressed as mean values (bars represent standard error); concentrations are

presented as nominal values. Asterisks denote statistical significant differences (Dunn's Method $P < 0.05$). Legend: P – AgNP protection layer.

Table 1. 96h LC50 values for *Physa acuta* with different shell lengths exposed to AgNO₃ and AgNP in each medium (APW and modified APW). Data is expressed as mean with 95% CI in brackets.

96h LC50 (µg/L)				
Snail	Juveniles (> 1mm)		Adults (> 5mm)	
Size				
Media	APW	Modified APW	APW	Modified APW
AgNO ₃	45.8 (20.1 - 78.4)	27.4 (20.1 - 44.9)	740 (550-920)	116 (95 - 143)
AgNP	258.7 (158.1 - 458.1)	48.5 (35.6 - 72)	7050 (5360-9560)	4110 (3300 - 5010)

Table 2 - EC50 and LC50 values obtained in the chronic egg mass (embryo) test with *Physa acuta* exposed to AgNO₃ and AgNP in APW medium for 13 days (nominal values). Data is expressed as mean with standard error in brackets.

Exposure	Hatching Success EC50 (µg/L)	Mortality LC50 (µg/L)
AgNO ₃	1.03 (0.24)	> 15
AgNP	95.7 (38.5)	81.6 (2.6)

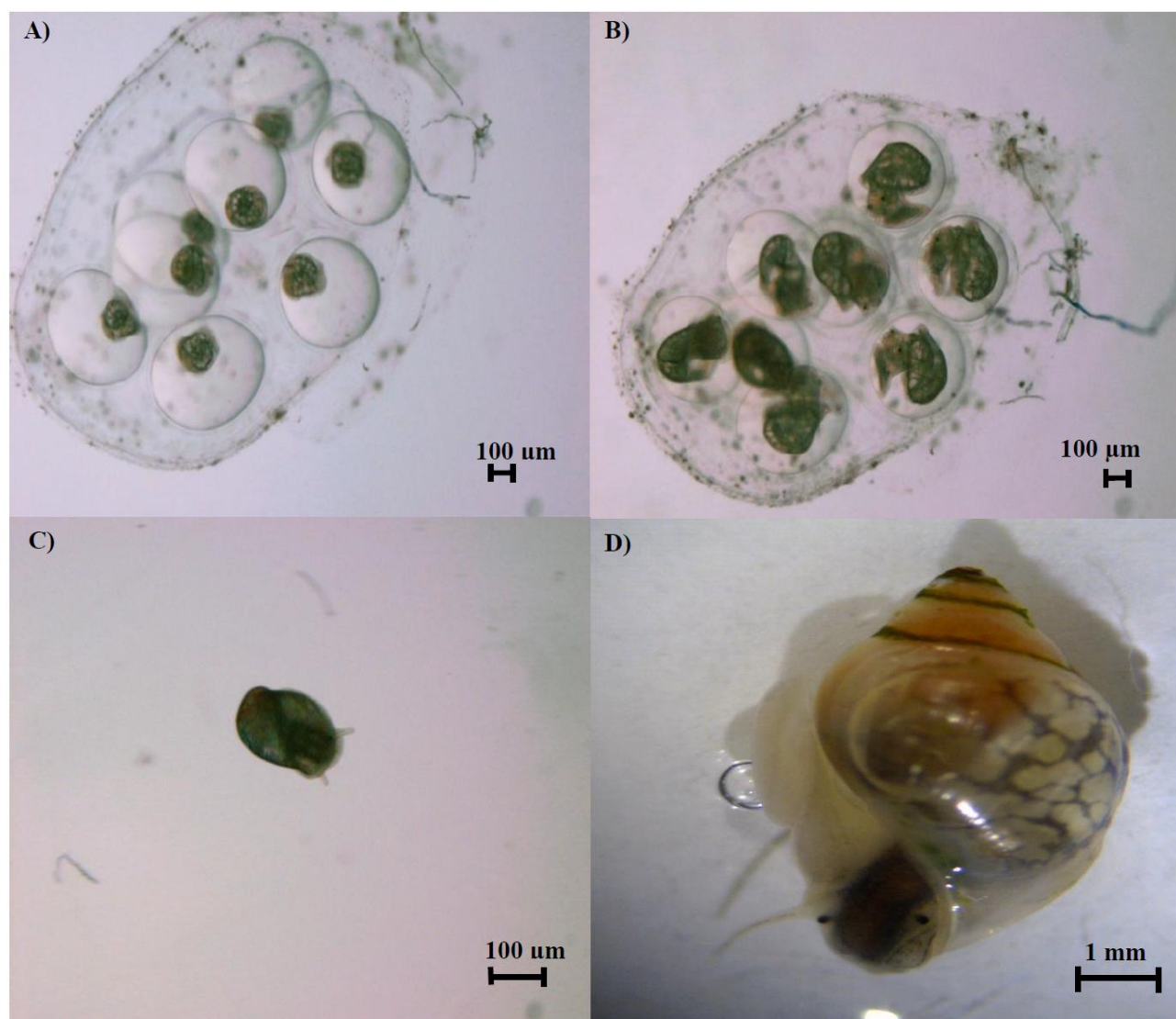
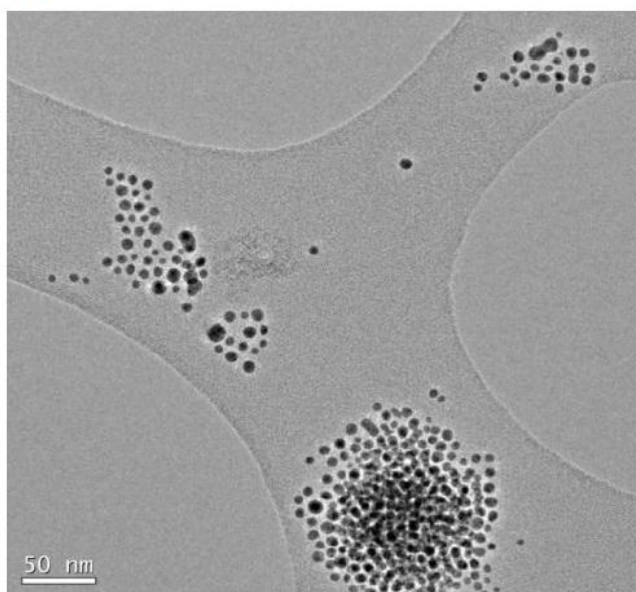
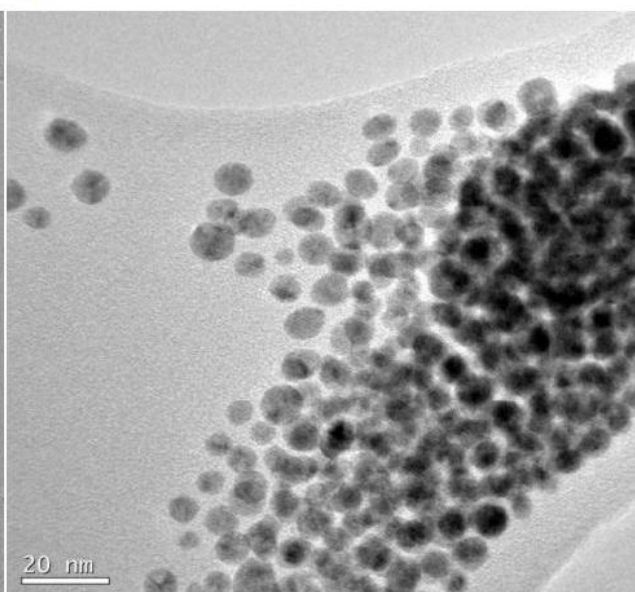
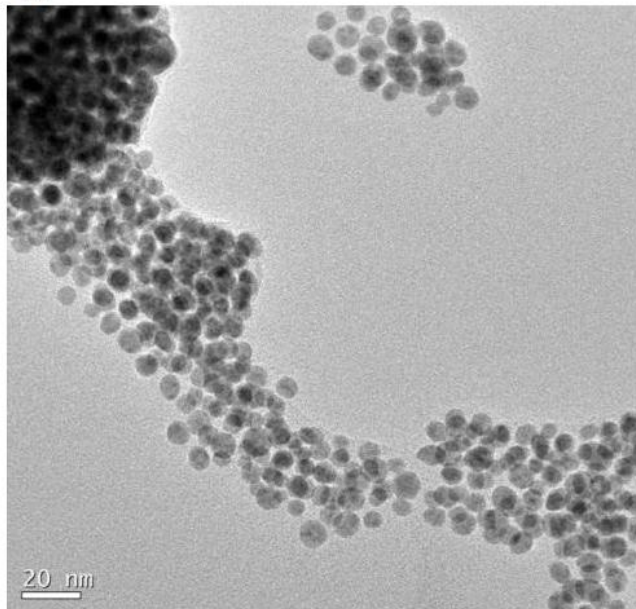
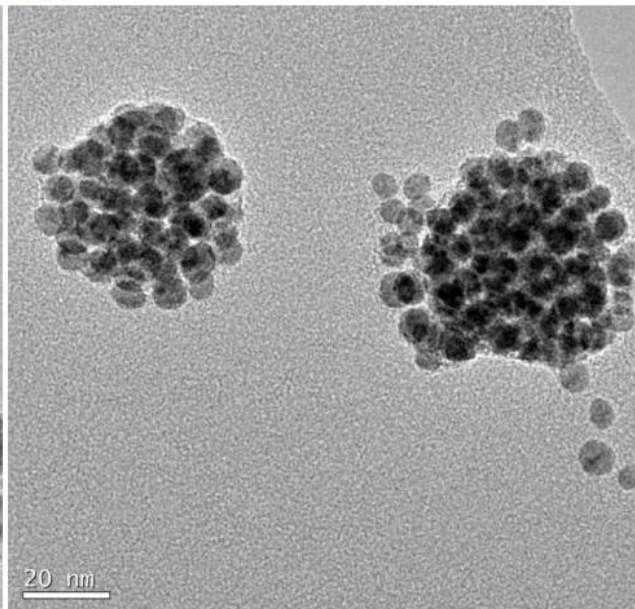


Fig. 1

A**B****C****D****Fig. 2**

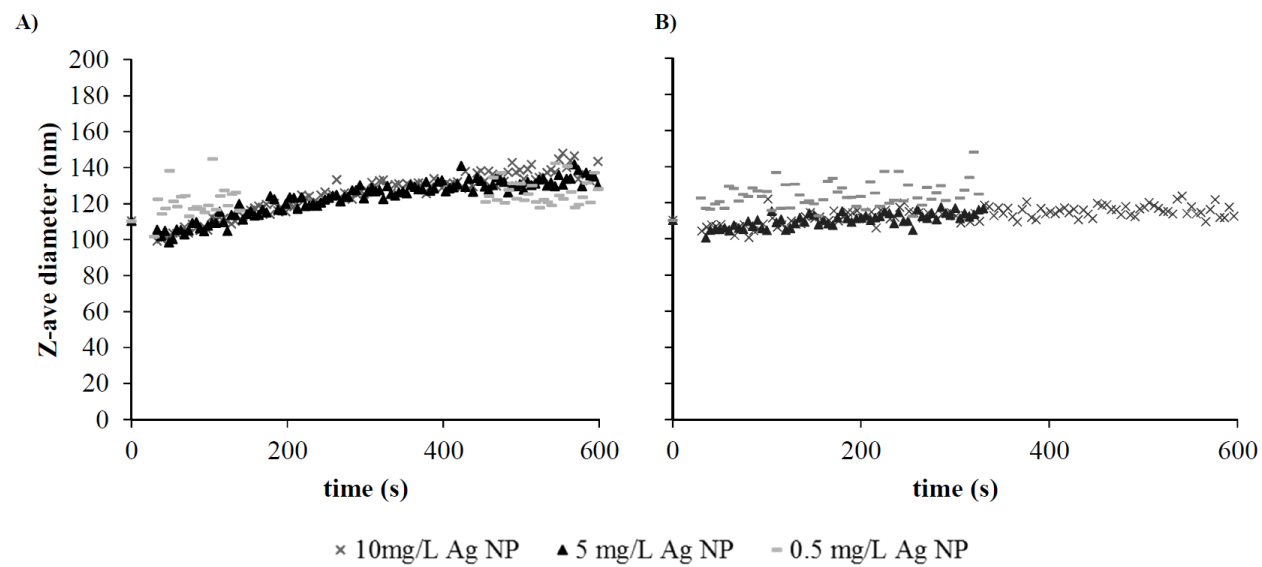


Fig. 3

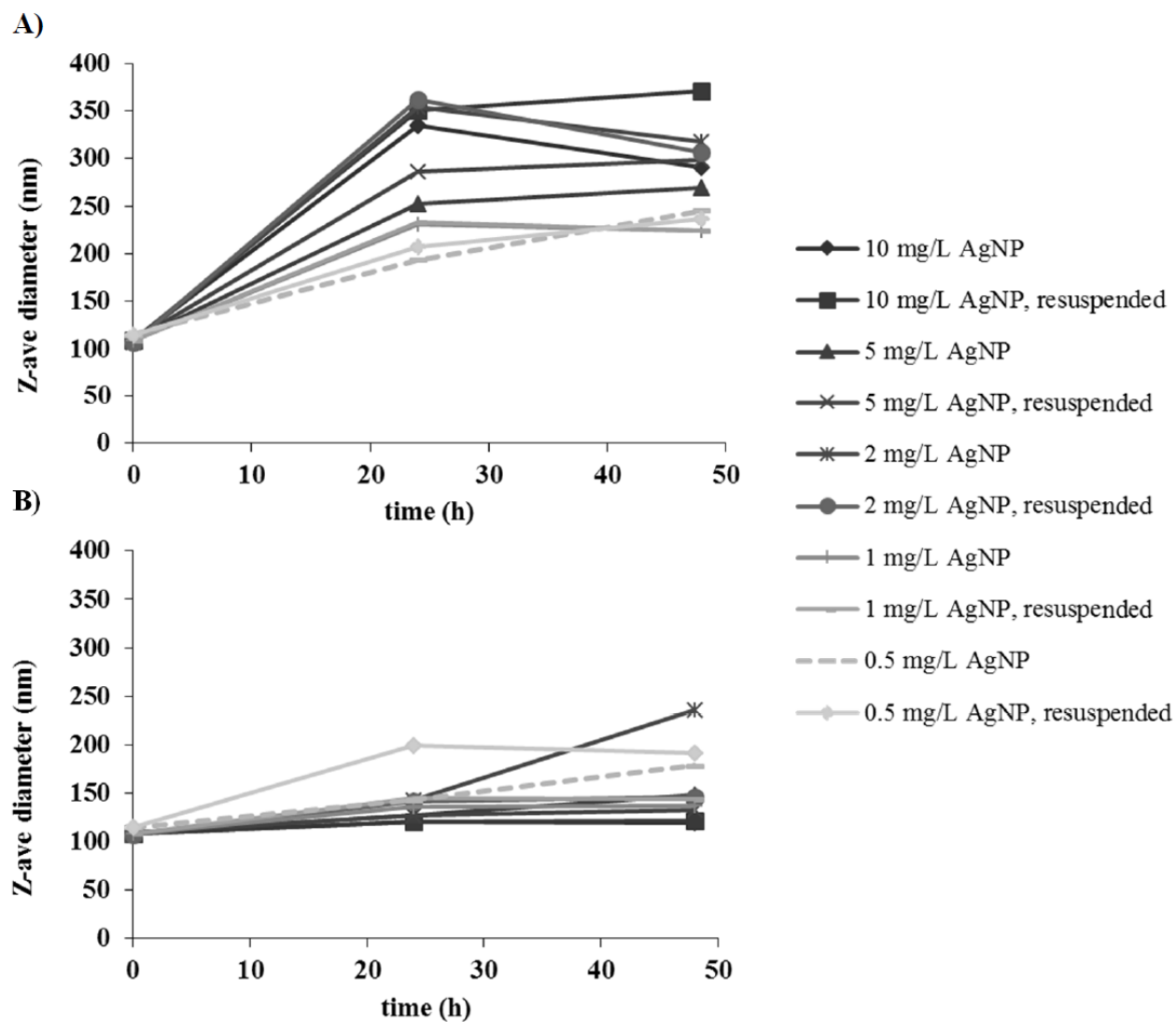


Fig. 4

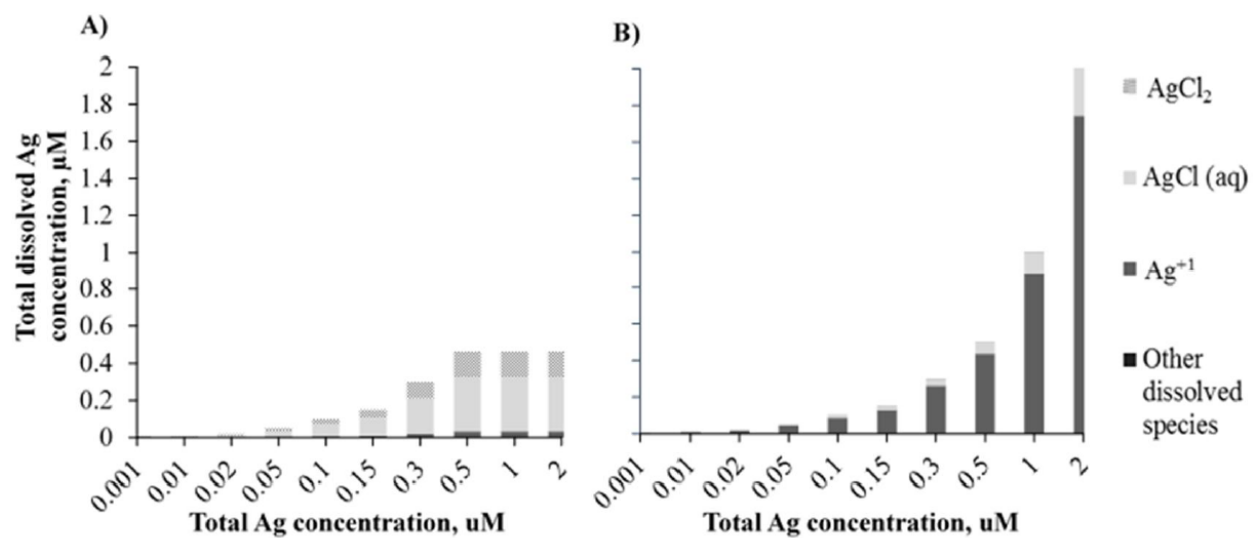


Fig. 5

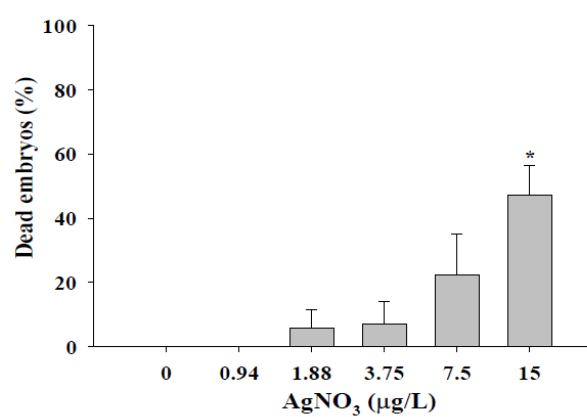
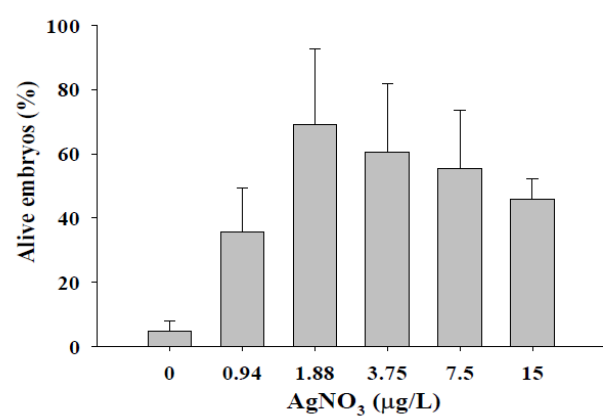
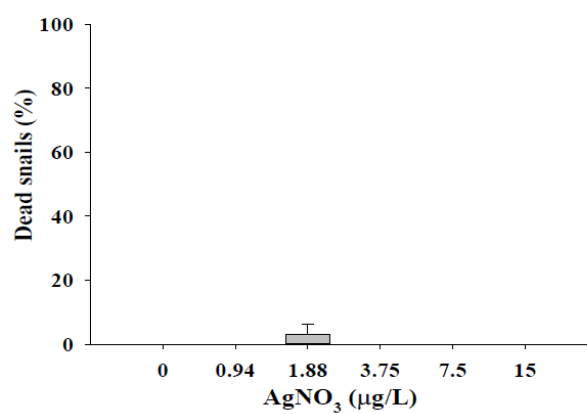
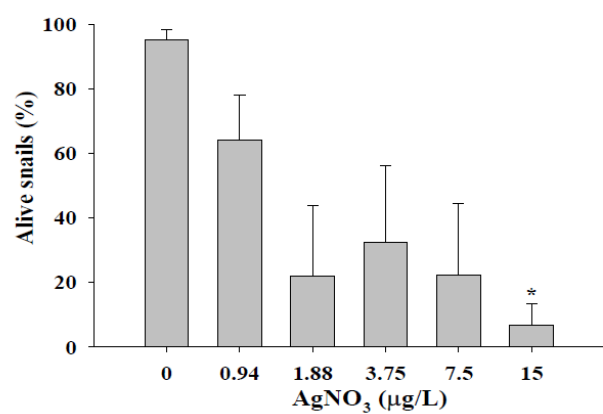


Fig. 6

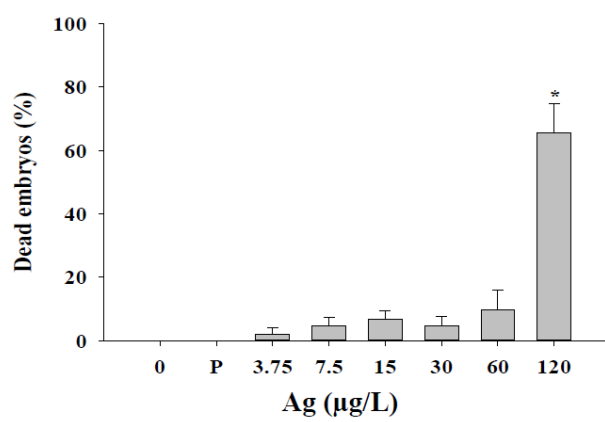
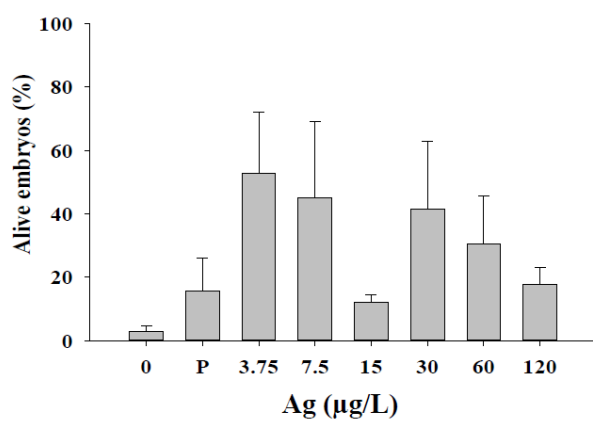
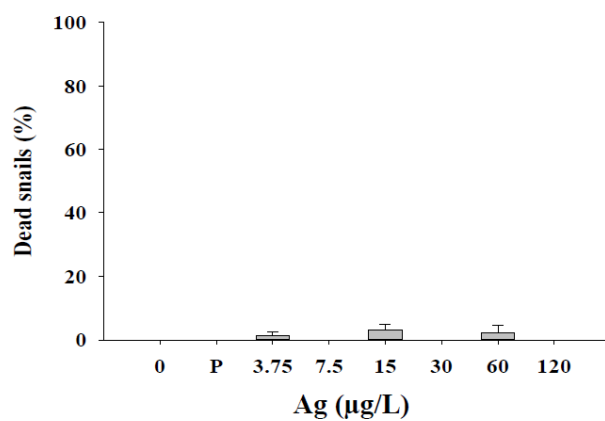
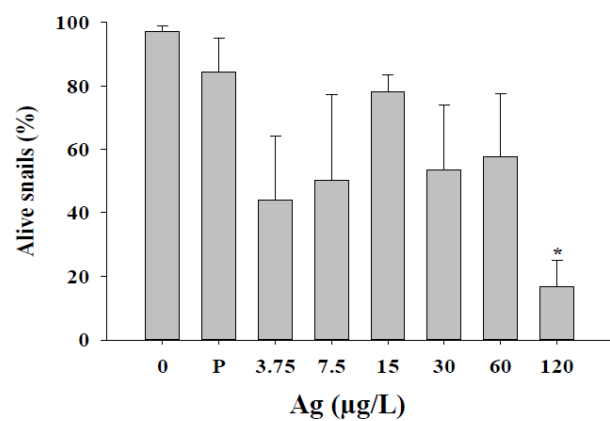


Fig. 7