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Toxicokinetics of pristine and aged silver nanoparticles in *Physa acuta*†

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Aquatic environments, particularly sediments, can be important final sinks for engineered nanoparticles (ENPs), with benthic biota being potentially exposed. There is an increasing need for hazard data to improve the environmental risk assessment of ENPs regarding aquatic systems. The aim of this study was to determine the toxicokinetics of several pristine (as manufactured) Ag-NPs, Ag₂S-NPs (used to simulate environmental aging of silver nanoparticles) and AgNO₃ as the ionic counterpart, in the freshwater snail *Physa acuta*. Snails were exposed through 1) contaminated water (without sediment), 2) contaminated water and clean sediment, and 3) contaminated sediment. Bioavailability of Ag to the snails was greatly influenced by Ag characteristics, as different uptake and elimination kinetics were found for the different Ag forms within the same exposure route. Snails exposed *via* water revealed, in general, similar uptake kinetics, differing from exposure *via* contaminated sediment, suggesting that exposure route also had a determining role in Ag bioavailability. The simulated aged form (Ag₂S-NPs) revealed fast uptake and depuration in snails from all experiments. When considering the double exposure route, which provides a more realistic contamination scenario, water was the main route, except for Ag₂S-NPs, for which sediment was more important. The remarkably low elimination and high stored fraction of Ag in some exposures emphasizes the bioaccumulation ability of *P. acuta* and may raise concerns about possible trophic transfer. Snail shells accumulated low amounts of Ag. The present study highlights the need for a proper examination of the overall exposure scenario of Ag-NPs to benthic organisms. Our results contribute to the environmental risk assessment of Ag-NPs in benthic environments.

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Environmental significance

Several studies have highlighted toxicokinetics, bioavailability and uptake routes of manufactured/aged nanoparticles as some of the key research topics to improve their environmental risk assessment in aquatic systems. This study aimed to address some of these topics, by studying the toxicokinetics of silver nanoparticles (Ag-NPs) in freshwater snails through different exposure routes. Key results showed that Ag-NP bioavailability was greatly determined by its characteristics and exposure route. Silver sulfide NPs were available for uptake and sediment seemed the most important route for this form. Furthermore, snails possibly have different mechanisms to deal with different Ag forms. This study contributes with important data, including models and parameters, to better understand the potential risk and exposure to NPs in benthic environments.

1. Introduction

The increasing demand for nano-containing products has led to the large-scale production and commercialisation of engineered nanoparticles (ENPs), which increases the risk of

their release into the environment.¹ Over the past two decades impressive progress has been made in understanding the potential hazards ENPs may pose to the environment and consequently to humans. While past studies focused on the ecotoxicity of ENPs in aquatic and terrestrial systems,^{2–5} subsequent works invested in studying the fate, kinetics and bioavailability of ENPs.^{6–9} Results suggest that the dynamic nature of ENPs can lead to continuous physicochemical transformations, during their entire life-cycle, which combined with their unique properties can trigger myriad biological and/or ecological responses with transformations continuing even after reaching biological receptors.^{10,11}

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Extensive research has been conducted on silver nanoparticles (Ag-NPs) as these are among the most widely produced ENPs and are applied in a broad variety of consumer products. This means that their discharge into aquatic environments is inevitable, with wastewater effluents being one of the main sources.¹² Studies have reported Ag-NP concentrations ranging from 0.3 to 2.5 ng L⁻¹ in Dutch surface waters¹³ and <12 ng L⁻¹ in wastewater effluents in Germany.¹⁴ A U.S. study from 1998 detected total silver concentrations between 0.028 and 5.56 µg L⁻¹ in wastewater effluent and between 1.78 and 105 µg L⁻¹ in wastewater influent.¹⁵ Another study measured total Ag concentrations of 0.21 µg L⁻¹ in wastewater influent during the summer of 2016 in Upper Austria.¹⁶ Once in the aquatic environment, the fate of Ag-NPs will be determined by transformation processes. These transformations are driven by the Ag-NP characteristics, the chemical properties of the aquatic systems, and the interaction of processes such as dissolution, agglomeration/heteroaggregation and sedimentation (among others) and are also seasonal variable.^{12,17,18} Aggregation and consequent sedimentation is a likely pathway for Ag-NPs thus, sediments have been suggested as ultimate sinks^{19,20} with predicted concentrations of Ag-NPs varying from 2.2 µg kg⁻¹ sediment²¹ to 30.1 µg kg⁻¹ sediment.²² Incomplete dissolution in natural waters has also been reported, which means that Ag-NPs can persist for long periods leading to long-term Ag⁺ release and subsequent mobility.^{18,23} In wastewater treatment plants, Ag-NPs are predominantly transformed into silver sulfide NPs (Ag₂S-NPs) and effluent discharges constitute entrance routes for this Ag species into surface waters. In aquatic systems, Ag₂S-NPs can be formed from Ag-NPs or Ag⁺ in the presence of naturally occurring sulfides, including metal sulfides.^{24,25} These transformations occur mainly at the bottom of the water column or in the sediment, although under certain conditions they can also occur higher in the water column.²⁶ Ag₂S-NPs have very low dissolution, leading to long-term exposure to these particles but also to low release of more toxic Ag⁺ ions. Due to reduced ion availability, these NPs are expected to be less toxic to organisms. However, these NPs have demonstrated some bioavailability and potential to cause hazardous effects.^{27–30}

It is fundamental for risk assessment to understand and evaluate the bioavailability and exposure of ENPs to organisms. Therefore, emphasis should be given to the determination of kinetic processes such as uptake, internal distribution and depuration of ENPs. Moreover, studies on the exposure and bioaccumulation in benthic organisms, especially for aged/modified ENPs, are needed.^{19,31} Some studies have evaluated the uptake and bioavailability of Ag-NPs towards benthic organisms. The results varied according to the nature of the Ag-NPs, exposure medium and organism used, highlighting that the interplay of these factors largely determines exposure and bioaccumulation.^{32–38} Benthic ecosystems are essential components of aquatic environments, and benthic organisms may potentially be the biota most affected by ENPs as they can be exposed through both water and sediments. Exposure can potentially happen *via* ingestion of sediment or food and through direct contact

of their body surfaces with both phases (*e.g.* transdermal uptake).^{20,39,40} Addressing the need for kinetic studies to improve the environmental risk assessment of ENPs, the present study aimed at determining the toxicokinetics of Ag-NPs in the freshwater snail *Physa acuta*. Several pristine (as manufactured) Ag-NPs with different characteristics, Ag₂S-NPs (simulating an environmentally aged Ag-NP form) and AgNO₃ (ionic control) were tested through different exposure routes to *P. acuta*: 1) contaminated water (without sediment), 2) contaminated water and clean sediment, and 3) contaminated sediment (clean water). *P. acuta* is an invasive snail found in lotic (riverine) and lentic (still water) ecosystems, representing a link between primary producers and predators in freshwater food chains, and can be vulnerable to contaminants in both sediment and water phases.⁴¹ The toxicity of Ag-NPs to *P. acuta* has been previously studied.^{41–43} Studies often evaluate accumulation only in soft tissues. However, shells can also accumulate contaminants, which can continue over time and possibly lead to accumulation by food-chain transfer.^{44,45} Considering this, shell fractions were separately analysed in this study.

2. Material and methods

2.1 Test species and breeding

Adult *Physa acuta* were collected from a pond at the Campus of the University of Aveiro, Portugal, and acclimated under laboratory conditions for a month before using egg masses deposited by adult snails to increase the cultures. Cultures were maintained in 6 L glass aquariums filled with 3 L of artificial pond water (APW) medium and with approximately 50 individuals per aquarium. For the APW medium, the following stock solutions were prepared using ultrapure water (UPW): calcium chloride (58.8 g L⁻¹ CaCl₂·2H₂O), potassium chloride (1.15 g L⁻¹ KCl), magnesium sulphate (24.65 g L⁻¹ MgSO₄·7H₂O) and sodium hydrogen carbonate (12.95 g L⁻¹ NaHCO₃). Then, 100 mL of each stock solution were added to a 20 L carboy filled with UPW.⁴⁶ APW medium was fully renewed once a week and partially renewed every other day. Medium pH was kept to basic levels (7.9 ± 0.3) to avoid shell fracturing, and continuous aeration was applied, maintaining levels of dissolved oxygen above 8 mg L⁻¹. Snails were fed *ad libitum* with ground TetraMin® (fish food) every other day and kept under controlled conditions of 20 ± 1 °C and 16 : 8 h light:dark photoperiod.⁴¹

2.2 Nanoparticles

NP batches were supplied by partners of the EU H2020 NanoFASE project (<http://www.nanofase.eu/>). Three pristine Ag-NPs with different sizes (nominal sizes of 3–8 nm, 50 nm and 60 nm) and silver sulfide NPs (Ag₂S-NPs, nominal size of 20 nm) were tested. The soluble salt silver nitrate (AgNO₃; Sigma Aldrich; CAS number 7761-88-8; 99% purity; crystalline powder) was used as the ionic control. Ag-NPs of 3–8 nm (alkane coated) and 60 nm (polyvinylpyrrolidone (PVP)) were supplied by AMEPOX Enterprise (Poland), dispersed in UPW

at a concentration of 1000 mg Ag L⁻¹. Dispersed solutions of Ag-NPs of 50 nm (5.5 mM sodium citrate, 25 µM tannic acid, 47.3 ± 5.3 nm, 12.3 g Ag L⁻¹) and Ag₂S-NPs (PVP; with two stock suspensions of 3.6 g Ag L⁻¹ and 6.56 g Ag L⁻¹) were supplied by Applied Nanoparticles (Barcelona, Spain). Ag₂S-NPs were used as a simulated aged Ag-NP form, in order to increase the environmental relevance of the exposures and to compare it with the pristine Ag-NPs.

2.3 Nanoparticle characterization

Size and morphology of the pristine 3–8 nm Ag-NPs have been reported in Ribeiro *et al.*⁵ Details on the TEM data for the 50 nm Ag-NPs and Ag₂S-NPs have been reported previously in Baccaro *et al.*⁷ The stability of all NPs was monitored by measuring the hydrodynamic size, zeta potential and dissolution at different time points matched to the periods between water changes. The hydrodynamic size, polydispersity index (PDI) and Z-potential were measured in UPW (pH: 7.00) and APW (pH: 8.00) suspensions prior to exposure and at 0, 2, 4, 8, 24 and 48 hours post-exposure using a Malvern Zetasizer (Nano ZS) equipped with a LASER of 632.8 nm and a scattering angle of 173°. All measurements were performed at 20 °C following a 2 minute equilibration, using Sarstedt polystyrene (ref. 67.742, 10 × 4 × 45 mm) cuvettes and Malvern Zetasizer DTS0170 disposable folded capillary cells, respectively. In both cases, the concentration of the suspensions was 1 mg L⁻¹. The operation procedures used the built-in values for the refractive indices (η) and absorption coefficients (α) for Ag and Ag₂S of the Malvern Zetasizer Software (version 7.13). The acquired results are the average of 5 consecutive measurements (14 replicates per measurement).

NP dissolution monitoring was performed using the protocol of Avramescu *et al.*,⁴⁷ based on the European Committee for Standardisation (CEN) guidance EN 71-3:2019 on “Safety of toys – Part 3: Migration of certain elements”⁴⁸ and is commonly used in metal bioaccessibility assays.⁴⁹ Ag-NP and Ag₂S-NP dissolution was measured in 1 mg L⁻¹ suspensions of UPW and APW at 0, 2, 4, 8, 24 and 48 hours. At each time point, part of each sample was filtered to separate the dissolved from the particulate material using 0.02 µm pore-diameter syringe filters (Anotop™, Whatman) and directly acidified for analysis using pure ICP-grade HNO₃ (Sigma Aldrich; CAS Number 7697-37-2) to a final concentration of 2% HNO₃. Syringe filtering was chosen over centrifugation, so that the standard 30 minute centrifugation delay needed in the latter case, would not affect the results. The dissolved Ag⁺ concentration was measured using an inductively coupled plasma mass spectrometer (ICP-MS, PerkinElmer, Nexion 3000). Appropriate calibration curves were created using ICP-grade standards and were considered acceptable when the correlation coefficient was greater than 0.999. All samples were spiked with ¹⁰³Rh as internal standard for QC purposes. Failed QCs resulted in recalibration and reanalysis of the respective samples. Results were converted and reported as percentage dissolution (% Diss), determined as the amount of dissolved Ag ([DAg⁺]) divided by the original Ag concentration ([Ag⁺]) used for the experiment (% Diss = ([DAg⁺]/[Ag⁺]) × 100%) to correspond

with that used in the OECD classification scheme.⁵⁰ The rest of the sample was, simultaneously, analysed to measure the hydrodynamic size and Z-potential as described above.

2.4 Experimental set up and sampling procedure

Experiments considering different routes of exposure were performed: 1) contaminated APW medium at a nominal concentration of 10 µg Ag L⁻¹ (no sediment), 2) contaminated APW medium at a nominal concentration of 10 µg Ag L⁻¹ and clean sediment, 3) contaminated sediment at a nominal concentration of 10 mg Ag kg⁻¹ and clean APW medium. The different experiments will be referred to as 1) Ag-spiked water test, 2) Ag-spiked water and clean sediment test, 3) Ag-spiked sediment test. Both tests with spiked water used a low nominal concentration that allowed reliable analytical detection and could still be close to environmentally relevant concentrations of Ag-NPs and total Ag. In the Ag-spiked water and clean sediment test, it was found that snails were also exposed to sediment concentrations of environmental relevance (explained in section 2.6). Therefore, in the Ag-spiked sediment test a higher concentration was used to increase the Ag availability to the snails in order to better understand the toxicokinetic processes.

All NP stock solutions were prepared according to the OECD guideline 318.⁵¹ Tests consisted of an uptake phase and an elimination phase. Snails (around 2 months old, shell size between 4–7 mm) were individually exposed to contaminated medium during the uptake phase for 7 days, and then transferred to clean medium for a 7 day elimination phase. Tests were maintained under controlled conditions, at 20 ± 1 °C and 16:8 h light:dark photoperiod. Temperature, dissolved oxygen (DO) concentration and pH were recorded during all experiments to ensure parameter quality, according to the OECD guideline 243.⁵²

2.4.1 Ag-spiked water test. Snails were exposed in 6-well microplates, with each well filled with 12.5 mL of test medium. The test medium was renewed every other day in the uptake and elimination phases, therefore no aeration was provided. For spiking, stock solutions/suspensions were prepared in UPW, then diluted in APW medium to the desired concentration and poured into the microplates of the respective treatment. Snails were inspected daily and not fed during the experiment. At 1, 2, 5, 7, 8, 9, 12 and 14 days, snails from three replicates per treatment were sampled, washed three times in UPW and frozen at -80 °C to allow dissecting into soft body and shell. Partially thawed snails were dissected⁵³ under a magnifying glass, with the help of two tweezers, one tweezer carefully held the shell, and the other pulled the soft body. Shell and soft body were oven-dried at 60 °C for 48 h and individually weighted. In addition, water was sampled at day 0 ($n = 3$ per treatment) for total Ag analysis.

2.4.2 Ag-spiked water and clean sediment test. Snails were exposed in 50 mL glass vials, with a depth ratio of 4:1 of APW: sediment (inorganic fine sediment (<1 mm), previously burnt for 4 h at 500 °C), prepared 24 h prior to spiking. The spiking of APW was performed with adaptations of the OECD protocol 233.⁵⁴ Briefly, the required amount of the stock solution/

suspension (in UPW) was applied to each test vial, below the water surface and then gently stirred with a needle to ensure homogenization while not disturbing the sediment. Each vial was covered with a lid to avoid evaporation. Low aeration was provided 24 h after water spiking by needles connected to an air pump, to avoid disturbing the sediment.⁵² No food was provided to the organisms during the experiment and vials were inspected daily. At 1, 2, 5, 7, 8, 9, 12 and 14 days, snails from three replicates per treatment were sampled and left to depurate in clean APW medium for 24 h to empty their gut, since benthic snails can ingest sediment particles.^{35,55} After depuration, the same procedure as described in section 2.4.1 was followed. In addition, water and sediment were sampled at days 0, 3 and 7 ($n = 3$ per treatment) for total Ag analysis (sediment was stored frozen until analysis).

2.4.3 Ag-spiked sediment test. Snails were exposed in 50 mL glass vials, with the APW:sediment ratio as for the previous test (section 2.4.2). The spiking of sediment followed adaptations of OECD protocol 218.⁵⁶ Briefly, 10 g of sediment were spiked in the individual test vials with the required amount (volume corresponding to 20% of the sediment amount) of the stock solutions (in UPW) and homogenised by hand, making sure the solution was evenly distributed. Then, APW medium was very carefully added, avoiding disturbance of the sediment as much as possible, and then left to equilibrate for 48 h prior to the start of the exposures. Lid covering and low aeration was provided to each vial after 48 h of equilibration, as in section 2.4.2. Incubations and sampling (organisms and sediment) were performed as described in section 2.4.2.

2.4.4 Ultrafiltration of water samples. In separate experiments, water samples were collected ($n = 3$ per treatment) during the uptake phase (nominal concentration of $10 \mu\text{g Ag L}^{-1}$) of the different Ag exposures at days 0 (meaning at 0 h), 1 and 2 of the Ag-spiked water test, and at days 0 (0 h), 1, 2 and 7 of the Ag-spiked water and clean sediment test, for ultrafiltration. Additionally, water samples were also collected at day 0 (0 h) for both experiments and digested for total Ag determination. For ultrafiltration, 3 mL of water collected directly from the test vials was filtered using a syringe filter with a membrane pore size of $0.02 \mu\text{m}$ (AnotopTM, Whatman). To prevent losses of Ag to the filter, filters were previously treated with a solution of 0.1 M CuSO_4 .⁵⁷ Following filtration, samples were immediately acidified with concentrated HNO_3 (65% purity, PanReac AppliChem, trace analysis) to a final acid concentration of 2%.

2.5 Sample digestion and total Ag analysis

Water samples were digested following the approach of Ribeiro *et al.*^{5,58} Briefly, samples were placed in Teflon beakers on a hotplate and left to evaporate until 1 mL volume was reached. Then, a 4 mL mixture of concentrated HCl (37%): HNO_3 (65%) (*aqua regia*) (3:1. v/v, PanReac AppliChem, trace analysis) was added and left to evaporate until approximately 1 mL. Afterwards, samples were diluted to a final volume of 45 mL with a solution of 1% HCl (37% purity, PanReac AppliChem,

trace analysis). In each digestion run, three blanks (APW medium) and three recovery controls (with known Ag concentration) were analysed jointly with the samples to evaluate the accuracy and recovery of the procedure. The average recovery of Ag was of $101 \pm 29.8\%$ (mean $\pm$ SD; $n = 66$).

Snail soft body and shell samples were digested according to Ribeiro *et al.*⁵⁹ Firstly, 3 mL of concentrated HNO_3 (65% purity, PanReac AppliChem, trace analysis) was added to Teflon beakers and samples were heated on a hotplate with the lids on. After breaking down the tissue, 1 mL of concentrated HCl (37% purity, PanReac AppliChem, trace analysis) was added. Lids were removed after 30 min, and samples were left to evaporate until approximately 1 mL. After evaporation, samples were diluted to a final volume of 45 mL with a solution of 1% HCl (37% purity, PanReac AppliChem, trace analysis). In the individual digestion runs, blanks and reference material were analysed in triplicate to assess method accuracy and recovery. The certified reference materials DOLT-3 and DOLT-5 (Dogfish liver) were used, giving an average Ag recovery of $102 \pm 31.6\%$ (mean $\pm$ SD; $n = 30$).

Sediment samples were oven dried at 50°C before digestion. Approximately 130 mg of sediment was digested for 7 h in closed Teflon containers using a 2 mL mixture of concentrated HCl (37%): HNO_3 (65%) (4:1. v/v, J. T. Baker, trace analysis), in an oven (BINDER ED53) at 140°C , after which samples were diluted to a final volume of 10 mL with demineralized water. DOLT-4 was used as the certified reference material, giving $103 \pm 6.1\%$ (mean $\pm$ SD; $n = 2$) of Ag recovery.

Total Ag concentrations in soft body, shell, water and sediment samples were measured by Graphite furnace Atomic Absorption Spectrometry (AAS; PinAAcle 900Z, PerkinElmer, Singapore). The limit of detection (LOD) ranged between $0.035\text{--}0.198 \mu\text{g Ag L}^{-1}$ in the different runs.

2.6 Toxicokinetics modelling

First order one-compartment models, adapted from van den Brink *et al.*⁶⁰ were fit to the data to describe the toxicokinetics of the different Ag forms in the snails. These models considered a stored fraction (SF) in which metals are stored and not eliminated from the body during uptake and elimination phases.⁶⁰

Model 1 considers only one exposure route and was used to fit the data of the Ag-spiked water and Ag-spiked sediment tests. Sediment analysis of the Ag-spiked water and clean sediment test revealed Ag background concentrations at day 0. Since Ag concentrations increased over time in the sediment while decreasing in the water (see section 3.3), a model including both exposures (model 2) was applied to Ag-spiked water and clean sediment test data. Ag concentration decrease rate constant in water ($k_{\text{d,water}}$) and increase rate constant in sediment ($k_{\text{i,sed}}$) were calculated using an exponential equation of Ag concentration vs. time. To check whether the kinetics were better explained by single or double exposures, further modelling was performed considering only exposure through water (model 3) and exposure only through sediment (model 4). Since the snails

lost weight in all tests (due to lack of feeding), a growth decrease rate constant was calculated and included in all models, by fitting an exponential curve to the individual snail dry weights (dw) during the 14 day test duration.

In model 1, uptake and elimination phases were modelled applying eqn (1) and (2), respectively:

$$Q(t) = C_0 + (C_{\text{exp}} \times k_1 \times \text{SF} \times t) + \left(C_{\text{exp}} \times \left(\frac{k_1}{(k_2 + k_{\text{growth}})} \right) \times \left(1 - e^{-(k_2 + k_{\text{growth}}) \times t} \right) \times (1 - \text{SF}) \right) \quad (1)$$

$$Q(t) = C_0 + (C_{\text{exp}} \times k_1 \times \text{SF} \times t_c) + \left(C_{\text{exp}} \times \left(\frac{k_1}{(k_2 + k_{\text{growth}})} \right) \times \left(1 - e^{-(k_2 + k_{\text{growth}}) \times t_c} \right) \times e^{-(k_2 + k_{\text{growth}}) \times (t - t_c)} \times (1 - \text{SF}) \right) \quad (2)$$

eqn (3) and (4) were used in model 2 to fit the data from the uptake and elimination phases:

$$Q(t) = C_0 + ((C_{\text{expwater}} \times k_w + C_{\text{expsed}} \times k_s) \times \text{SF} \times t) + \left(\frac{(C_{\text{expwater}} \times k_w + C_{\text{expsed}} \times k_s)}{(k_2 + k_{\text{growth}} - k_{\text{dwater}})} \times \left(e^{(-k_{\text{dwater}} \times t)} - e^{-(k_2 + k_{\text{growth}}) \times t} \right) \times (1 - \text{SF}) \right) \quad (3)$$

$$Q(t) = C_0 + ((C_{\text{expwater}} \times k_w + C_{\text{expsed}} \times k_s) \times \text{SF} \times t_c) + \left(\frac{(C_{\text{expwater}} \times k_w + C_{\text{expsed}} \times k_s)}{(k_2 + k_{\text{growth}} - k_{\text{dwater}})} \times \left(e^{(-k_{\text{dwater}} \times t)} - e^{-(k_2 + k_{\text{growth}}) \times t} \right) \times \left(e^{-(k_2 + k_{\text{growth}}) \times (t - t_c)} \right) \times (1 - \text{SF}) \right) \quad (4)$$

For the individual exposures of the Ag-spiked water and clean sediment test, model 3 (eqn (5) and (6)) was applied to water exposure and model 4 (eqn (7) and (8)) to sediment exposure:

where $Q(t)$ = Ag internal concentration in the snails at time t days ($\mu\text{g Ag g}^{-1}\text{organism dw}$); k_1 = uptake rate constant ($\text{L g}^{-1}\text{organism per day}$ or $\text{g}_{\text{sediment}} \text{g}^{-1}\text{organism per day}$); k_2 = elimination rate constant (day^{-1}); C_0 = background internal concentration measured at day 0 ($\mu\text{g Ag g}^{-1}\text{organism dw}$), C_{exp} = Ag exposure concentration in water ($\mu\text{g Ag L}^{-1}$) or sediment (mg Ag kg^{-1}); t = time in days and t_c = time at which snails were transferred from contaminated to clean medium (day 7); SF = stored fraction (ranging from 0 to 1; unit less); k_{growth} = growth decrease rate constant (day^{-1}); k_w = uptake rate constant from water ($\text{L g}^{-1}\text{organism per day}$); k_s = uptake rate

constant from sediment ($\text{g}_{\text{sediment}} \text{g}^{-1}\text{organism per day}$); $C_{\text{exp water}}$ = Ag exposure concentration in water ($\mu\text{g Ag L}^{-1}$); $C_{\text{exp sed}}$

= Ag exposure concentration in sediment ($\mu\text{g Ag kg}^{-1}$); k_{dwater} = Ag water concentration decrease rate constant (day^{-1}); k_{ised} = Ag sediment concentration increase rate constant (day^{-1}).

$$Q(t) = C_0 + (C_{\text{expwater}} \times k_1 \times \text{SF} \times t) + \left(C_{\text{expwater}} \times \left(\frac{k_1}{(k_2 + k_{\text{growth}} - k_{\text{dwater}})} \right) \times \left(e^{(-k_{\text{dwater}} \times t)} - e^{-(k_2 + k_{\text{growth}}) \times t} \right) \times (1 - \text{SF}) \right) \quad (5)$$

$$Q(t) = C_0 + (C_{\text{expwater}} \times k_1 \times \text{SF} \times t_c) + \left(C_{\text{expwater}} \times \left(\frac{k_1}{(k_2 + k_{\text{growth}} - k_{\text{dwater}})} \right) \times \left(e^{(-k_{\text{dwater}} \times t)} - e^{-(k_2 + k_{\text{growth}}) \times t} \right) \times \left(e^{-(k_2 + k_{\text{growth}}) \times (t - t_c)} \right) \times (1 - \text{SF}) \right) \quad (6)$$

$$Q(t) = C_0 + (C_{\text{expsed}} \times k_1 \times \text{SF} \times t) + \left(C_{\text{expsed}} \times \left(\frac{k_1}{(k_2 + k_{\text{growth}} - k_{\text{ised}})} \right) \times \left(e^{(-k_{\text{ised}} \times t)} - e^{-(k_2 + k_{\text{growth}}) \times t} \right) \times (1 - \text{SF}) \right) \quad (7)$$

$$Q(t) = C_0 + (C_{\text{expsed}} \times k_1 \times \text{SF} \times t_c) + \left(C_{\text{expsed}} \times \left(\frac{k_1}{(k_2 + k_{\text{growth}} - k_{\text{ised}})} \right) \times \left(e^{(-k_{\text{ised}} \times t)} - e^{-(k_2 + k_{\text{growth}}) \times t} \right) \times \left(e^{-(k_2 + k_{\text{growth}}) \times (t - t_c)} \right) \times (1 - \text{SF}) \right) \quad (8)$$

For the double exposure, further calculations (eqn (9) and (10)) were performed in order to determine the relative contribution of each uptake route to the total uptake.

Water uptake route:

$$\left(\frac{C_{\text{expwater}} \times k_w}{(C_{\text{expwater}} \times k_w + C_{\text{expsed}} \times k_s)} \right) \times 100 \quad (9)$$

Sediment uptake route:

$$\left(\frac{C_{\text{expsed}} \times k_s}{(C_{\text{expwater}} \times k_w + C_{\text{expsed}} \times k_s)} \right) \times 100 \quad (10)$$

2.7 Statistical analysis

The described models were fitted to the data and toxicokinetics parameters were determined by non-linear regression in SPSS (version 25). Several models were used to fit the data (data not shown) and the selection of the best fitting ones was performed by applying the Akaike Information Criteria tests (AIC and AICc). Differences between k_1 and k_2 of each Ag form were tested using Generalised Likelihood Ratio Tests (GLR) in SPSS. Using the SigmaPlot 12.5 software, one-way analysis of variance (ANOVA) followed by the Holm-Sidak method ($p < 0.05$) was conducted to determine statistically significant differences in exposure medium concentrations (water and sediment; total and dissolved Ag) between Ag treatments of each experiment, and percentage of dissolved Ag between UPW and APW medium. Data transformation was performed when ANOVA assumptions were not verified.

3. Results

3.1 Nanoparticle stability

NP stability was evaluated by monitoring the hydrodynamic size, Z-potential and dissolution in UPW and APW at 0, 2, 4, 8, 24 and 48 hours. Detailed results can be found in Tables S1 and S2† for UPW and APW, respectively. Dissolution results are presented graphically in Fig. S1 and S2.† In APW medium, the Z-potential for the 3–8 nm and 50 nm Ag-NPs

and Ag₂S-NPs decreased when compared to UPW (Tables S1 and S2).† Strong agglomeration was observed for 3–8 nm Ag-NPs in both APW and UPW, being higher in APW with hydrodynamic size ranging from 131–270 nm. While in UPW the hydrodynamic diameter of the 50 nm Ag-NPs remained close to their nominal size (~58 nm) (Table S1†), in APW agglomeration led to a substantial increase of sizes, ranging from 75.6–1840 nm (Table S2†). The Z-potential of the 60 nm Ag-NPs substantially increased in APW (from –16 to –24.8 mV). This affected their hydrodynamic size, which was substantially lower (79.2–174 nm), with respective reductions in the PDI values (Table S2†). While 60 nm Ag-NPs in UPW revealed high dissolution (stable around 27–29%), much lower dissolution was observed for these Ag-NPs in APW, and decreasing from 2.3% at 2 h to 0.82% at 48 h (Table S2†). This was significantly lower (Holm-Sidak method, $p < 0.05$) than dissolution in APW observed for the 3–8 nm and 50 nm Ag-NPs and Ag₂S-NPs (Table S2†). The hydrodynamic diameter of Ag₂S-NPs was relatively stable over time in APW and minimal dissolution was observed (<0.2%) (Table S2†).

3.2 Ag-spiked water test

Concentrations of Ag in APW were determined at time 0, and since the medium was renewed every 48 h, concentrations in the water column were assumed to be constant during the uptake phase. Still, some adsorption of Ag to the microplate walls could have occurred, contributing to higher exposure to snails, for instance, by adsorption to the foot/shell. Measured Ag concentrations were close to the nominal ones for all Ag forms except Ag₂S-NPs, which revealed a value 4 times lower than the nominal 10 µg Ag L^{–1} concentration and significantly lower (Holm-Sidak method, $p < 0.05$) than concentrations measured in the other NP treatments (Table 1).

Low mortality (<5%) was observed during the experiment (data not shown). Distinct uptake patterns were seen for 60 nm Ag-NPs and AgNO₃ compared to the remaining NPs (Fig. 1). On the last day of the uptake phase, mean Ag internal concentrations in snails exposed to 60 nm Ag-NPs and AgNO₃ were 70.3 and 80.8 µg Ag g^{–1} dw, respectively. For 3–8 nm and 50 nm Ag-NPs and Ag₂S-NPs body concentrations were 12.0, 19.4 and 7.70 µg Ag g^{–1} dw, respectively. Although

Table 1 Measured concentrations (µg Ag L^{–1}) given as mean and standard deviation (mean ± SD; $n = 3$) and toxicokinetic parameters for 3–8 nm, 50 nm and 60 nm Ag-NPs, Ag₂S-NPs and AgNO₃ in *Physa acuta* exposed to water spiked at a nominal concentration of 10 µg Ag L^{–1}, in the Ag-spiked water test. k_1 is the uptake rate constant, k_2 the elimination rate constant, k_g the growth rate constant and SF the stored fraction. 95% confidence intervals (CI) are given in brackets. Different capital letters in bold within a column indicate statistically significant differences ($\chi^2_{(n)} > 3.84$; $p < 0.05$). Different small letters in italics within a column indicate statistically significant differences (one-way ANOVA followed by Holm-Sidak method ($p < 0.05$))

Exposure route	Ag form	Measured concentration (µg Ag L ^{–1})	k_1 (L _{water} g ^{–1} organism per day)	k_2 (day ^{–1})	SF	k_g (day ^{–1})
Water	3–8 nm	9.85 ± 0.40 <i>a</i>	0.18(0.08–0.29) A	0.03(n.d.)	1(—)	–0.03
	50 nm	8.32 ± 0.30 <i>b</i>	0.22(–0.04–0.48) A,C	0.12(–1.35–1.58) A	0(—)	–0.06
	60 nm	10.3 ± 0.26 <i>a</i>	1.43(0.32–2.54) B	0.11(–0.63–0.85) A	0(–5.22–5.22)	–0.03
	Ag ₂ S-NPs	2.46 ± 0.48 <i>c</i>	1.07(–0.97–3.10) B	0.44(–0.79–1.67) A	0(–0.52–0.52)	–0.07
	AgNO ₃	9.46 ± 0.59 ^a <i>a</i>	1.20(0.69–1.70) B,C	0.02(n.d.)	1(—)	–0.02

(—) very wide 95% confidence intervals. (n.d.) not possible to determine 95% confidence intervals. ^a $n = 2$.

Fig. 1 Uptake and elimination kinetics of 3–8 nm, 50 nm and 60 nm Ag-NPs, Ag₂S-NPs and AgNO₃ in the freshwater snail *Physa acuta* exposed for 7 days to water spiked at a nominal concentration of 10 µg Ag L⁻¹ and then transferred to clean water for 7 days, in the Ag-spiked water test. Lines represent the fit of a one-compartment model to the data, which represent Ag concentrations measured in individual snail soft bodies.

the uptake curves were very similar for 60 nm Ag-NPs and AgNO₃, the elimination curves differed (Fig. 1).

The Ag toxicokinetic parameters are shown in Table 1. Values of k_1 were lowest for snails exposed to 3–8 nm (0.18 L_{water} g⁻¹ organism per day) and 50 nm (0.22 L_{water} g⁻¹ organism per day) Ag-NPs and did not significantly differ ($X^2_{(1)} < 3.84$; $p > 0.05$). The k_1 values of 3–8 nm Ag-NPs were significantly lower ($X^2_{(1)} > 3.84$; $p < 0.05$) compared to 60 nm Ag-NPs, Ag₂S-NPs and AgNO₃, and k_1 values of 50 nm Ag-NPs significantly differed ($X^2_{(1)} > 3.84$; $p < 0.05$) from 60 nm Ag-NPs and Ag₂S-NPs. No significant differences ($X^2_{(1)} < 3.84$; $p > 0.05$) in k_2 values were found.

Low k_2 values were registered for 3–8 nm Ag-NPs (0.03 day⁻¹) and AgNO₃ (0.02 day⁻¹), with SF = 1 for both suggesting limited elimination. The remaining Ag-NPs revealed higher k_2 values and SF of 0, with curves indicating slow elimination. Ag₂S-NPs showed the highest k_2 (0.44 day⁻¹) and a steeper elimination curve (Fig. 1, Table 1).

Table 1 also displays the constants (k_g) for snail growth rate of each treatment. Snails showed some weight loss during all tests. Total and dissolved Ag concentrations measured in samples collected for ultrafiltration from all exposures are shown in Table S3†. Since the filters used for ultrafiltration had a pore size of 0.02 µm, possibly other Ag forms (<20 nm) were measured besides ionic Ag. Mean % of dissolved Ag compared with the total Ag determined was 1.87 ± 0.69%, 2.52 ± 1.87%, 33.8 ± 2.04%, 0.59 ± 0.4% and 34.7 ± 4.47% for 3–8 nm, 50 nm, 60 nm Ag-NPs, Ag₂S-NPs and AgNO₃, respectively (data not shown). Dissolved Ag concentrations were significantly higher (Holm–Sidak method, $p < 0.05$) for 60 nm Ag-NPs and AgNO₃ compared to the remaining Ag forms from day 0 to day 2, and only differed significantly (Holm–Sidak method, $p < 0.05$) between sampling days for AgNO₃ (Table S3†).

3.3 Ag-spiked water and clean sediment test

Measured Ag concentrations in water and sediment are displayed in Table S4†. A low background Ag concentration of

10.1 ± 2.95 µg Ag kg⁻¹ was measured in sediments sampled at day 0 from the initial batch used in all treatments, therefore the snails were exposed to Ag through water and sediment from the beginning of the exposures. Concentrations of Ag in water at day 0 were close to the nominal 10 µg Ag L⁻¹, and showed a steep decrease in the first three days of the uptake phase, which was significant (Holm–Sidak method, $p < 0.05$) for all treatments. Only at day 0 significant differences (Holm–Sidak method, $p < 0.05$) were detected in water concentrations between treatments, being highest for Ag₂S-NPs. Values of $k_{d,water}$ and $k_{i,sed}$ were lower for 3–8 nm and 50 nm Ag-NPs. The increase in Ag concentration in sediments from day 0 to 7 was not significant (Holm–Sidak method, $p > 0.05$) for exposures to 3–8 nm Ag-NPs and AgNO₃ (Table S4†).

Total and dissolved Ag concentrations measured in water samples collected for ultrafiltration from all exposures are presented in Table S5†. Mean% of dissolved Ag compared with the total Ag determined was 0.72 ± 0.6%, 1.61 ± 1.86%, 4.23 ± 5.14%, 0.2 ± 0.22% and 3.6 ± 4.24% for 3–8 nm, 50 nm, 60 nm Ag-NPs, Ag₂S-NPs and AgNO₃, respectively (data not shown). No dissolved Ag was detected at day 0 in any exposure, including AgNO₃. However, an increase in dissolved Ag was seen from day 0 to day 1. The 60 nm Ag-NPs and AgNO₃ had higher dissolved Ag concentrations at days 1 and 2, but only at day 2 significant differences (Holm–Sidak method, $p < 0.05$) were detected between treatments. From day 1 a decrease in concentration was visible, except for 50 nm Ag-NPs which showed a low increase (not significant, Holm–Sidak method, $p > 0.05$). However, from days 2 to 7 Ag concentrations revealed a significant decrease (Holm–Sidak method, $p < 0.05$) to zero, except for 3–8 nm Ag-NPs (Table S5†).

No mortality was observed (data not shown). Uptake and elimination curves and kinetics parameters are presented in Fig. 2 and Table 2, respectively. Higher internal Ag concentrations were measured after 7 days in snails exposed to 60 nm Ag-NPs (31.3 µg Ag g⁻¹ dw) and AgNO₃ (53.6 µg Ag g⁻¹ dw) than in snails exposed to 3–8 nm (15.6 µg Ag g⁻¹ dw)

Fig. 2 Uptake and elimination kinetics of 3–8 nm, 50 nm and 60 nm Ag-NPs, Ag₂S-NPs and AgNO₃ in the freshwater snail *Physa acuta* exposed for 7 days to water spiked at a nominal concentration of 10 μg Ag L⁻¹ and then transferred to clean water for 7 days, in the Ag-spiked water and clean sediment test. Lines represent the fit of a one-compartment model to the data, which represent Ag concentrations measured in individual snail soft bodies.

and 50 nm (9.22 μg Ag g⁻¹ dw) Ag-NPs and Ag₂S-NPs (2.98 μg Ag g⁻¹ dw). Highest uptake rates were determined for 60 nm Ag-NPs and AgNO₃ (Fig. 2). Ag body concentrations for snails exposed to Ag₂S-NPs reached a peak at day 2, followed by a considerable decrease afterwards. The kinetic curve follows this pattern, with a fast increase of the uptake rate during the first day, and a steep decline after day 2 (Fig. 2).

Highest k_w (uptake from water) values were calculated upon exposure to 60 nm Ag-NPs (1.37 L_{water} g⁻¹ organism per day) and AgNO₃ (1.15 L_{water} g⁻¹ organism per day). For uptake from sediment, Ag₂S-NPs presented the highest k_s value, of 1.89 g_{sediment} g⁻¹ organism per day. It was not possible to evaluate differences between k_w or k_s of the different Ag forms as no 95% CI could be determined (Table 2).

Snails exposed to 60 nm Ag-NPs did not eliminate Ag during the 7 day depuration period, showing a steady curve and a k_2 of 0 day⁻¹, while snails exposed to Ag₂S-NPs presented the highest k_2 value (0.74 day⁻¹). This k_2 value was significantly different ($X^2_{(1)} > 3.84$; $p < 0.05$) from all treatments, except for 50 nm Ag-NPs. In general, very low SF were calculated, with the highest value of 0.33 determined for AgNO₃ (Table 2).

The percentage of the relative contribution of each uptake route was calculated (Table 3). In these treatments, uptake from water was higher than from sediment, revealing very close percentage values between the different Ag forms. In Ag₂S-NP exposure, uptake from sediment was more important (74.2%) than uptake from water (Table 3).

When considering a single exposure route (models 3 and 4), it was possible to determine 95% CI for the kinetic parameters (Table 2). When accounting only for water exposure (model 3), the lowest k_1 value was determined for 3–8 nm Ag-NPs, similar to model 2. Values of k_2 and SF determined by model 3 were identical to those determined by model 2, with the same statistical differences between k_2 values. Statistically significant differences ($X^2_{(1)} > 3.84$; $p < 0.05$) were found for k_1 values of 3–8 nm and 50 nm Ag-NPs in comparison with 60 nm Ag-NPs, Ag₂S-NPs and AgNO₃, and also between Ag₂S-NPs and AgNO₃ (Table 2).

Considering sediment as single exposure route (model 4), Ag₂S-NPs and AgNO₃ presented the highest k_1 (1.88 g_{sediment} g⁻¹ organism per day and 2.05 g_{sediment} g⁻¹ organism per day, respectively) and k_2 values (2.68 day⁻¹ and 2.18 day⁻¹, respectively) (Table 2), probably explained by the fast drop of the curve from day 7 to 8 (Fig. S3†). A SF of zero was determined for all treatments, except for 60 nm Ag-NPs, which presented a SF of 1. This SF contrasts with the values very close to zero determined for this Ag form by models 2 and 3 (Table 2). Although the general patterns of the curves share similarities, some differences were observed between kinetic curves determined by model 2 and model 4 (Fig. 2 and S3†). This is especially notable for the Ag₂S-NP uptake curve, which increases gradually after the first day of the uptake phase (Fig. S3†). Kinetic curves determined by model 3 are the same as those observed in Fig. 2.

3.4 Ag-spiked sediment test

Kinetics curves determined for snails exposed to Ag-spiked sediment are presented in Fig. 3, and corresponding kinetic parameters are given in Table 4. Total Ag concentrations in sediment were similar to the nominal 10 mg Ag kg⁻¹ (Tables 4 and S6†). Significant differences (Holm–Sidak method, $p < 0.05$) in Ag concentrations were only detected between day 0 and days 3/7 for 3–8 nm Ag-NPs and between day 3 and 7 for Ag₂S-NPs (Table S6†). Nevertheless, concentrations were very similar and for the sake of comparison between Ag forms, the mean Ag concentration at days 0, 3 and 7 were used as the exposure concentration for each treatment, instead of calculating rate constants for concentration changes (Table 4). Very low mortality (3%) occurred (data not shown) although much higher internal Ag concentrations were measured in the snails at the end of the uptake phase, increasing in the order: 50 nm Ag-NPs (111 μg Ag g⁻¹ dw), 60 nm Ag-NPs (133 μg Ag g⁻¹ dw), AgNO₃ (135 μg Ag g⁻¹ dw), Ag₂S-NPs (153 μg Ag g⁻¹ dw) and 3–8 nm Ag-NPs (176 μg Ag g⁻¹ dw).

Table 2 Toxicokinetic parameters for 3–8 nm, 50 nm and 60 nm Ag-NPs, Ag₂S-NPs and AgNO₃ in *Physa acuta* exposed to water spiked at nominal concentration of 10 µg Ag L⁻¹, in the Ag-spiked water and clean sediment test. k_1 is the uptake rate constant, k_w the elimination rate constant, k_s the uptake rate constant from water, k_g the uptake rate constant from sediment, k_g the growth rate constant and SF the stored fraction. 95% confidence intervals (CI) are given in brackets. Different letters within a column indicate statistically significant differences ($X^2_{(1)} > 3.84$; $p < 0.05$)

Exposure route	Ag form	k_w (L _{water} g ⁻¹ organism per day)	k_s (g _{sediment} g ⁻¹ organism per day)	k_1 (L _{water} g ⁻¹ organism per day; g _{sediment} g ⁻¹ organism per day)	k_2 (day ⁻¹)	SF	k_g (day ⁻¹)
Water and sediment (model 2)	3–8 nm	0.57(n.d.)	0.25(n.d.)		0.05(–0.02–0.11) A	0(–0.60–0.60)	–0.02
	50 nm	0.69(n.d.)	0.30(n.d.)		0.65(–0.23–1.53) A,B,C	0.13(0.04–0.22)	–0.01
	60 nm	1.37(n.d.)	0.50(n.d.)		0(–0.04–0.02) B	0.01(–0.33–0.33)	0
Water (model 3)	Ag ₂ S-NPs	0.82(n.d.)	1.89(n.d.)		0.74(–0.001–1.48) C	0.001(–0.02–0.02)	0
	AgNO ₃	1.15(n.d.)	0.40(n.d.)		0.16(–0.42–0.75) A,B	0.33(0.05–0.61)	–0.01
	3–8 nm			0.88(0.35–1.40) A	0.05(–0.02–0.11) A	0(–0.57–0.57)	
Sediment (model 4)	50 nm			1.11(0.27–1.95) A	0.65(–0.22–1.51) A,B,C	0.13(0.04–0.21)	
	60 nm			2.19(0.79–3.36) B,C	0(–0.03–0.02) B	0.01(–0.32–0.32)	
	Ag ₂ S-NPs			3.16(1.36–4.95) B	0.74(0.12–1.36) C	0.001(–0.02–0.02)	
	AgNO ₃			1.88(0.59–3.17) C	0.16(–0.41–0.73) A,B	0.33(0.14–0.53)	
	3–8 nm			0.42(0.28–0.56) A	0.18(–0.17–0.52) A	0(–5.24–5.24)	
	50 nm			0.61(–0.62–1.83) A,B	1.76(–3.27–6.80) A,B	0.19(–0.17–0.56)	
Ag ₂ S-NPs	60 nm			0.64(0.30–0.99) A,B	0.16(n.d.)	1(–4.10–6.10)	
	AgNO ₃			1.88(–6.14–9.91) B,C	2.68(–9.86–15.2) B	0(–0.06–0.06)	
	AgNO ₃			2.05(–1.52–5.61) C	2.18(–3.65–8.00) B	0.27(–0.18–0.72)	

(n.d.) not possible to determine 95% confidence intervals.

Uptake curves were similar between Ag forms except for Ag₂S-NPs (Fig. 3). Higher k_1 values were calculated for all Ag treatments compared to the previous experiment, probably due to the much higher Ag exposure concentrations. The k_1 value was highest for Ag₂S-NPs (16.5 g_{sediment} g⁻¹ organism per day) followed by 3–8 nm Ag-NPs (7.24 g_{sediment} g⁻¹ organism per day) (Table 4). Values of k_2 were also higher for Ag₂S-NPs and especially for 3–8 nm Ag-NPs. Although 3–8 nm Ag-NPs presented a SF of 0.22 and the elimination curve remained relatively constant, this k_2 value may be explained by the decrease in Ag body mean concentrations at the first day of elimination (95.1 µg Ag g⁻¹ dw) and consequently forming the slope in the curve during the beginning of the elimination phase.

SF was 1 for 50 nm and 60 nm Ag-NPs and SF = 0 for AgNO₃, while Ag₂S-NPs had a SF of almost 0. No statistically significant differences ($X^2_{(1)} < 3.84$; $p > 0.05$) were found between k_1 values which can be due to very wide 95% CI. k_2 values differed significantly ($X^2_{(1)} > 3.84$; $p < 0.05$) between Ag₂S-NPs and AgNO₃ (Table 4).

3.5 Ag concentrations in shells

Total Ag concentrations in the shells were much lower than in the soft body samples, at around 25% or less of the concentration measured in the soft body fractions on the last day of the uptake phase (Table 5). In general, shells of snails exposed to Ag-spiked water and clean sediment had lower concentrations that remained relatively constant during the experiment (Fig. S4†). Higher shell Ag concentrations were obtained for snails exposed to Ag-spiked sediment, especially those exposed to 60 nm Ag-NPs, which may be related to the much higher exposure concentrations (Table 5 and Fig. S4†). In general, Ag concentrations in shells did not tend to increase or decrease in any phase of the three experiments and varied throughout the test period for some exposures (Fig. S4†).

The three experiments revealed for some exposures an increase of Ag% in the shell compared to the Ag concentration measured in the soft body at day 14 (Table 5). Snails exposed to Ag₂S-NPs in the Ag-spiked water and Ag-spiked water and clean sediment tests had increased Ag% in shells at day 14, due to the increase of Ag concentrations in the shell and the decrease in the soft body due to elimination. For AgNO₃ in the Ag-spiked sediment test, Ag concentrations in the shell were 2852% at day 14 (Table 5), but this is related to the fact that only one replicate (soft body) was available with a very low Ag concentration (0.37 µg Ag g⁻¹ dw).

4. Discussion

4.1 Nanoparticle stability

Data on NP stability revealed dissolved material of approximately 29% for 60 nm Ag-NPs in UPW for all timepoints (Table S1†), which could suggest the presence of some free ionic Ag in the received solutions as a result of

Table 3 The relative contribution of uptake from water (APW) and sediment to the total uptake of Ag in *Physa acuta* exposed to 3–8 nm, 50 nm and 60 nm Ag-NPs, Ag₂S-NPs and AgNO₃, in the Ag-spiked water and clean sediment test

Ag form	% uptake from water	% uptake from sediment
3–8 nm	64.4	35.6
50 nm	62.6	37.4
60 nm	62.4	37.6
Ag ₂ S-NPs	25.8	74.2
AgNO ₃	61.1	38.9

dissolution during storage.⁶¹ However, in APW a lower dissolution of the 60 nm Ag-NPs was observed when compared to the 3–8 nm and 50 nm Ag-NPs (Fig. S2 and Table S2†). This could mean either higher NP stability or faster precipitation of other larger Ag particles due to Ostwald ripening or formation of other Ag phases (e.g. AgCl, Ag₂S) in APW.^{62,63} This is further supported by the observed decrease in dissolution of the 60 nm Ag-NPs in APW (Table S2†). On the other hand, the 3–8 nm and 50 nm Ag-NPs presented in both cases (UPW and APW) a gradual increase in dissolution over time. While in UPW the behaviour between the two was similar, in APW faster dissolution was observed for the 3–8 nm Ag-NPs than for the 50 nm Ag-NPs. This is probably due to the free ions present in the APW and their interaction with the surface of these NPs. Alkane coated 3–8 nm Ag-NPs are least reactive in APW since alkane (paraffin), as a saturated hydrocarbon, is less susceptible to van der Waals forces⁶⁴ compared to the negatively charged citrate used in 50 nm Ag-NPs. The effects of the different medium were apparent in the Z-potential changes for all Ag-NPs, as the presence of free ions in the APW solutions affected the Stern layer of ions formed around the NPs, regulating the observed Z-potentials.⁶⁵ In fact this was also apparent in the changes in hydrodynamic sizes, which (compared to UPW) increased for 3–8 nm and 50 nm Ag-NPs and Ag₂S-NPs while their Z-potentials decreased thus

reducing the ability of the NPs to repel each other. The opposite was observed for the 60 nm Ag-NPs, i.e. decreased hydrodynamic size and increased Z-potential. The effect of the ionic strength of APW was visible in the Z-potential changes of the Ag₂S-NPs, the stability of which was apparent through the low dissolution rates in both UPW and APW.

4.2 Dissolved Ag concentrations

No dissolved Ag was detected in the water at day 0 in any of the Ag-spiked water and clean sediment test, although detection was possible for Ag-spiked water test samples (Tables S3 and S5†). Some explanations could be: 1) an analytical error at day 0; 2) perhaps the different water spiking methods applied may have influenced the results; 3) the presence of sediment may have accelerated complexation; 4) formation of AgCl precipitates during sample digestion. Nevertheless, dissolved Ag concentrations measured in both Ag-spiked water and Ag-spiked water and clean sediment tests may have been underestimated. Some Ag loss may have occurred, by adsorption to the container walls, especially in the case of glass containers such as those used in the Ag-spiked water and clean sediment test.⁶⁶ Additionally, complexation of Ag⁺ with Cl[−] (also other ions such as Ca²⁺) may have promoted faster sedimentation and decreasing Ag concentrations in the water column.⁴¹ In addition, chloride can form a AgCl(s)⁰ shell on NP surfaces reducing or even inhibiting dissolution.⁶⁷

Ag-NPs with similar nominal sizes were tested however, differences in their behaviour and consequent bioavailability were observed. The PVP-coated 60 nm Ag-NPs presented higher concentrations of dissolved Ag in both experiments than the citrate-coated 50 nm Ag-NPs, hence, these differences may be attributed to the coating type. However, sterically stabilized PVP-coated Ag-NPs are considered to be more stable than charge stabilized citrate-coated Ag-NPs. The PVP polymer is strongly bound to the NP surface while citrate stabilizes by electrostatic repulsion and is weakly bound to

Fig. 3 Uptake and elimination kinetics of 3–8 nm, 50 nm and 60 nm Ag-NPs, Ag₂S-NPs and AgNO₃ in the freshwater snail *Physa acuta* exposed for 7 days to sediment spiked at a nominal concentration of 10 mg Ag kg^{−1} and then transferred to clean sediment for 7 days, in the Ag-spiked sediment test. Lines represent the fit of a one-compartment model to the data, which represent Ag concentrations measured in individual snail soft bodies.

Table 4 Measured concentrations (mg Ag kg⁻¹) given as mean and standard deviation (mean $\pm$ SD; $n = 3$) and toxicokinetic parameters for 3–8 nm, 50 nm and 60 nm Ag-NPs, Ag₂S-NPs and AgNO₃ in *Physa acuta* exposed to sediment spiked at a nominal concentration of 10 mg Ag kg⁻¹, in the Ag-spiked sediment test. k_1 is the uptake rate constant, k_2 the elimination rate constant, k_g the growth rate constant and SF the stored fraction. 95% confidence intervals (CI) are given in brackets. Different letters within a column indicate statistically significant differences ($\chi^2_{(u)} > 3.84$; $p < 0.05$)

Exposure route	Ag form	Measured concentration ^a (mg Ag kg ⁻¹)	k_1 (g _{sediment} g ⁻¹ organism per day)	k_2 (day ⁻¹)	SF	k_g (day ⁻¹)
Sediment	3–8 nm	9.83 $\pm$ 1.89	7.24(—)	2.02(–8.62–12.7)	0.22(–0.55–0.99)	–0.01
	50 nm	7.99 $\pm$ 0.94	2.56(n.d.)	0.01(n.d.)	1(n.d.)	–0.01
	60 nm	10.2 $\pm$ 1.63	2.03(1.38–2.68)	0.001(n.d.)	1(—)	0
	Ag ₂ S-NPs	10.2 $\pm$ 0.82	16.5(—)	1.71(–4.89–8.31)	0.02(–0.05–0.08)	–0.01
	AgNO ₃	8.58 $\pm$ 0.71	2.98(–0.18–6.13)	0.14(–0.82–1.09)	0(–4.56–4.56)	–0.02

(—) very wide 95% confidence intervals. (n.d.) not possible to determine 95% confidence intervals. ^a Mean concentrations measured at days 0, 3 and 7 for each treatment, used as exposure concentrations for the kinetic model.

the NP surface. Studies have reported lower dissolution of citrate-coated Ag-NPs than PVP-coated Ag-NPs in UPW⁶⁸ and lake/river waters,²³ probably because citrate may reduce the oxide layer at the surface to zero-valent Ag, decreasing ion release. However, other studies have also determined higher dissolution of citrate-coated Ag-NPs than PVP-coated Ag-NPs in *Daphnia* test medium⁶⁹ and in freshwater and seawater.⁷⁰

As mentioned above, results on NP stability showed a gradual increase in dissolution of the 50 nm Ag-NPs in APW (Table S2†) but very low dissolved Ag concentrations were detected in water samples from Ag-spiked water and Ag-spiked water and clean sediment tests (Tables S3 and S5†). It can be hypothesized that fast complexation of the available Ag ions may have occurred in APW leading to a faster precipitation, explaining the lower dissolved Ag concentrations measured. On the other hand, 60 nm Ag-NPs revealed very low % dissolution in APW samples from the NP dissolution tests (Table S2†), which is the opposite of what was observed in water samples from the Ag-spiked water and Ag-spiked water and clean sediment tests (Tables S3 and S5†). Nonetheless, it should be kept in mind that the exposure concentration was much lower (10 μ g Ag L⁻¹) than the concentration used in the NP dissolution tests (1 mg Ag L⁻¹) and thus the behaviour could have been different.⁵³ It is possible that at the snail's exposure Ag concentration, the 60 nm Ag-NPs may have had the highest dispersion stability in APW, thus being more susceptible to dissolution.³⁶

Comparable low dissolved Ag concentrations were also measured in water samples spiked with similar concentrations of the same 3–8 nm Ag-NPs as used in this study.⁴¹ As expected, very low dissolution was measured for Ag₂S-NPs in both spiking and dissolution tests (Tables S3 and S5†).

4.3 Ag-spiked water test

Similarities between internal Ag concentrations in the snails, uptake curves and k_1 values of 60 nm Ag-NPs and AgNO₃ suggest that uptake was mainly from dissolved Ag (Fig. 1, Table 1). The higher dissolved Ag concentrations determined for these exposures confirmed that snails were more exposed to dissolved Ag with the 60 nm Ag-NPs than with other nanoforms (Table S3†). However, differences in elimination kinetics suggest that uptake of NPs also occurred upon exposure to 60 nm Ag-NPs and thus the snails dealt in a different way with particulate and dissolved Ag. Probably, dissolved Ag was not eliminated from the body at least during the 7 day depuration period (explaining the k_2 close to zero and SF of 1 for AgNO₃ exposure) and the gradual elimination observed in snails exposed to 60 nm Ag-NPs was presumably of the nanoparticulate form (Fig. 1, Table 1). Other studies have also reported uptake of the nanoparticulate form, which in some cases was dominant when compared to dissolved Ag uptake. *Lymnaea stagnalis* exposed to waterborne Ag also revealed comparable uptake rate constants for AgNO₃ and humic acid-coated Ag-NPs,

Table 5 Ag concentrations ($\mu\text{g Ag g}^{-1} \text{ dw}$) (mean $\pm$ SD) and Ag% in shells at the end of the uptake phase (day 7) and at the end of the elimination phase (day 14), in *Physa acuta* exposed to 3–8 nm, 50 nm and 60 nm Ag-NPs, Ag₂S-NPs and AgNO₃ in all tests. Ag% in shells are given in relation to the Ag concentration measured in soft bodies at days 7 and 14

Ag form	Ag-spiked water test				Ag-spiked water and clean sediment test				Ag-spiked sediment test			
	Day 7	Day 14	Ag% at day 7	Ag% at day 14	Day 7	Day 14	Ag% at day 7	Ag% at day 14	Day 7	Day 14	Ag% at day 7	Ag% at day 14
3–8 nm	1.4 $\pm$ 0.7	1.6 $\pm$ 0.1	12	5.4	4 $\pm$ 3.6	5.2 $\pm$ 2.9	25.5	28.5	14.5 $\pm$ 5.8	7.1 $\pm$ 3	8.2	5.7
50 nm	2.5 $\pm$ 1.1	6.3 $\pm$ 2.2	12.9	51.2	1 $\pm$ 1.6	1 $\pm$ 0.5	10.8	9.4	7.3 $\pm$ 4.3	2.7 $\pm$ 0.7	6.6	1.6
60 nm	3.3 $\pm$ 1.6	1.8 $\pm$ 2	4.7	7.7	3.9 $\pm$ 3.9	1.8 $\pm$ 1.6	12.5	4	17.6 $\pm$ 3.7	18.5 $\pm$ 4.2	13.2	10.8
Ag ₂ S-NPs	1.3 $\pm$ 0.3	7.8 $\pm$ 2.8	17.2	153	0.1 $\pm$ 0.1	0.4 $\pm$ 0.7	2.5	23.3	9.5 $\pm$ 11.7	3.9 $\pm$ 3	6.2	47.9
AgNO ₃	6.1 $\pm$ 4.4	3.5 $\pm$ 0.8	7.5	3.7	1.7 $\pm$ 2	1.7 $\pm$ 0.9	3.2	3.8	18.5 $\pm$ 3.7	10.4 $\pm$ 0.3	13.7	2852

which were clearly higher than for citrate-coated Ag-NPs.³² Elimination of Ag from AgNO₃ exposure was very slow while from NP exposures it was relatively fast, further suggesting that uptake of nanoparticulate Ag may also have occurred.³² Uptake of Ag from PVP Ag-NPs in *L. stagnalis* was slower than for AgNO₃ when considering total Ag exposure concentrations, but higher when accounting only for ionic Ag concentrations.³⁶ This “excess” uptake in Ag-NP exposures was attributed to particulate Ag as the main uptake driver, likely because NP concentrations exceeded dissolved Ag concentrations,³⁶ which was also the case in the present work. When comparing the relative contribution of each Ag form, Croteau *et al.* predicted that around 80% of the bioaccumulation of citrate-coated Ag-NPs by *L. stagnalis* was driven by uptake of particulate Ag, at concentrations close to those used in this work.⁵³

The lowest uptake rate and internal Ag concentrations were obtained for Ag₂S-NPs, although it can also be related to the lower Ag concentration measured in the test medium (Fig. 1, Table 1). Nevertheless, this Ag form presented one of the highest k_1 , very close to the k_1 values of AgNO₃ and 60 nm Ag-NPs and not differing significantly ($X^2_{(1)} < 3.84$; $p > 0.05$) (Table 1). Therefore, considering their very low dissolution (Table S3†), Ag₂S-NPs seemed available for uptake by the snails as particles.

The 3–8 nm and 50 nm Ag-NPs presented similar k_1 values, uptake rate curves and also very low dissolution. However, 3–8 nm Ag-NPs and AgNO₃ showed the same elimination patterns, k_2 and SF values, which may suggest the uptake was from dissolved Ag in spite of the very low dissolution of these Ag-NPs (Fig. 1, Tables 1 and S3†). Gonçalves *et al.* attributed the toxicity of 3–8 nm Ag-NPs to *P. acuta* mainly to NP properties rather than to Ag⁺.⁴¹ Uptake rate constants of Ag in the oligochaeta *Tubifex tubifex* exposed to waterborne AgNO₃ were nearly 25-times higher than for Ag-NPs (the same type as the 3–8 nm Ag-NPs as used in the present work) and no significant loss was detected after 20 days depuration of either Ag form,³⁸ as also observed in this work. Although cellular uptake and internalization pathways may differ between Ag⁺ and Ag-NPs, once within the cells these forms might experience the same processes, which can explain the similar elimination patterns observed for AgNO₃ and 3–8 nm Ag-NPs in the present work.^{71,72} For

50 nm and 60 nm Ag-NPs and Ag₂S-NPs, SF was 0, which can indicate that snails handled Ag differently than in the other exposures (Table 1).

The uptake mechanism of Ag⁺ is mainly *via* ion transport channels, such as the proton-coupled Na⁺ channels, while for Ag-NPs uptake might be *via* endocytosis.⁵⁹ Khan *et al.* demonstrated that uptake pathway is determinant of the intracellular fate and toxicity of the Ag-NPs in the estuarine mudsnail *Peringia ulvae*.³⁴ They combined these results with those of Khan *et al.*,³³ suggesting a “Trojan horse” elimination mechanism for Ag-NPs, whereby a fast Ag efflux followed by a slow elimination were observed: Ag-NPs internalized through a caveolae-mediated endocytosis pathway and micropinocytosis were not directed to lysosomes and could have been responsible for the fast efflux. Ag-NPs taken up *via* clathrin-mediated endocytosis were directed towards lysosomal degradation, promoting ion release. The intracellular released Ag⁺, jointly with Ag⁺ uptake from Ag-NP dissolution in the medium, could have been eliminated slowly from the snails.³⁴ These processes are dependent on the NP properties, such as size (~120 nm seemed optimal for NP uptake *via* clathrin-mediated processes and 50–100 nm for caveolae-mediated pathways³⁴) and charge, since positively charged NPs are preferentially internalized by clathrin-mediated endocytosis,⁷³ and their interactions with the media.³⁴ Considering this, it is fair to suggest that multiple possible uptake pathways for Ag-NPs along with the intrinsic and extrinsic NP properties and interactions with the test medium may have contributed to the differences in uptake and elimination kinetics observed in the present study.

Interestingly, in this test, Ag₂S-NPs presented higher k_1 values, similar to 60 nm Ag-NPs and AgNO₃, (Table 1) but similar uptake rates with 3–8 nm and 50 nm Ag-NPs (Fig. 1) which could be related with the lower total Ag concentration of Ag₂S-NPs in the medium, as mentioned above. Additionally, different sizes of NP agglomerates could have contributed to differences in their uptake by the snails. However, Ag₂S-NPs also showed higher k_2 values than the other pristine Ag-NPs (Table 1). One hypothesis is dissolution. Even though the low dissolution observed for 3–8 nm and 50 nm Ag-NPs, some ionic Ag seemed to be taken up, which could have led to higher internalization, explaining

the lower elimination compared to Ag₂S-NPs. Furthermore, the NPs/agglomerates taken up by the snails could have determined their size- and charge-dependent internalization pathways (e.g. clathrin- or caveolae-mediated endocytosis) and consequently their elimination. It can be further hypothesized that intracellular dissolution of NPs occurred leading to additional Ag⁺ that was slowly eliminated, and that dissolution only occurred or was higher for pristine NPs than for Ag₂S-NPs. Nevertheless, the lack of information on these factors (e.g. complete characterization of the NPs in the test medium at the concentration used, internal distribution of Ag) limited deeper analyses.

4.4 Ag-spiked water test and sediment test

Higher Ag body concentrations and higher uptake rates were observed in snails exposed to 60 nm Ag-NPs and AgNO₃, which can be related once again with the higher dissolution of the 60 nm Ag-NPs (Fig. 2, Tables 2 and S5†). The high uptake rate obtained for Ag₂S-NPs may be explained by the rapid uptake in the first day, indicating that these NPs were easily available. After day 2, the uptake curve declined following the body concentration pattern. This can be due to the higher k_2 values observed for this Ag form, indicating that probably elimination overcame uptake after day 2, and therefore this Ag form was easily eliminated from the body (Fig. 2, Table 2). Body Ag concentrations from exposures to 60 nm Ag-NPs and AgNO₃ were in general lower in this experiment when compared with the Ag-spiked water test, which can be related with the lower dissolution of these Ag forms in this test, possibly as a result of heteroaggregation with sediment particles (Table S5†). Furthermore, the medium was not renewed in this experiment, unlike in the Ag-spiked water test. In the Ag-spiked water and clean sediment test, Ag body concentrations in general increased over time during the uptake phase suggesting continuing Ag influx in the snails exposed to the different Ag treatments. Ag-NPs are likely to agglomerate and deposit into sediments, which combined with the low dissolution indicates that Ag was likely associated with sediment but still available to the snails, either by ingestion of sediment particles or by direct contact with the skin and possibly adsorption to the foot. Citrate-coated 50 nm Ag-NPs are expected to rapidly agglomerate and to sedimentate.^{17,74} Again, the very low dissolution may have led to lower body concentrations in the snails at least during the first 2 days for the 50 nm and 3–8 nm Ag-NPs.

The possible ingestion of sediment by snails may also have influenced Ag effluxes. In contrast to what was observed in the Ag-spiked water test, snails exposed to 60 nm Ag-NPs were not able to eliminate Ag, at least during the 7 day depuration period, while snails exposed to 50 nm Ag-NP and AgNO₃ revealed higher Ag elimination (Fig. 2, Table 2). Using the double exposure model (model 2) could bring a more realistic approach to the exposure scenario, considering that in the environment NPs may deposit from the water column

to the sediment and benthic organisms can simultaneously be exposed to both exposure routes.²⁰ However, the lack of 95% CI for all k_w and k_s values may raise uncertainties about this model (Table 2). Looking at the results from single exposures, some differences are observed between the kinetic curves. There is a clear difference in the uptake curves obtained for Ag₂S-NPs when modelling sediment as single exposure route (model 4) (Fig. S3†). There is a relatively fast increase of Ag body concentrations during day 1 and then a gradual increase until day 7, followed by a sharp decrease on the first day of the elimination phase. This sharp decrease was also observed in snails exposed to AgNO₃, showing a fast decline of their body concentrations during the first day and stabilising from day 9 onwards (Fig. S3†). *Lumbriculus variegatus* exposed to AgNO₃ also demonstrated a biphasic elimination, with rapid loss of the majority of Ag during the first 6 h of depuration, followed by very slow elimination over 6 days.⁷¹ Elimination may have been more efficient with ingestion of clean sediment by *L. variegatus* during the depuration phase, since clean sediment passing through the gut may assist in the removal of Ag.⁷¹ Therefore, in the present work, the fast drop can be a result of an efflux of Ag associated with sediment particles and the “stable” phase may be Ag⁺ that is not eliminated, probably mostly taken up as dissolved Ag not associated with sediment particles. Background concentrations in sediments were probably maintained during the depuration phase, so it should not be disregarded as having some influence on elimination. The estuarine polychaete *Nereis diversicolor* revealed Ag uptake by the gut epithelium after ingestion of sediment spiked with citrate-capped Ag-NPs and AgNO₃.⁷⁵ In this organism, the Ag accumulation process from Ag-NPs was mainly associated with organelles, inorganic granules and heat denatured proteins, and accumulation of dissolved Ag was predominantly in the metallothionein fraction.⁷⁵ Metallothioneins (MTs) are metal binding proteins playing an important role in the detoxification and storage of metals present in many species,⁷⁶ including freshwater snails.⁷⁷ Moreover, the sequestration of Ag⁺ by MTs has been previously demonstrated.^{76,78,79} The much higher BCF in the freshwater amphipod *Hyalella azteca* exposed to AgNO₃, and the delayed/incomplete Ag elimination observed in AgNO₃ and Ag-NP exposures was explained by Ag⁺ association with metal-binding proteins, such as MTs.⁷⁹ It is possible that Ag⁺ accumulation in *P. acuta* could be associated with MTs, leading to a slower elimination of this form. Therefore, it can also be hypothesised that the slow elimination observed for some Ag-NPs could be of ionic Ag (from NP dissolution in exposure medium or intracellular dissolution) sequestered by MTs, while particulate Ag forms were eliminated faster. This highlights the role of ions, possibly contributing to higher Ag accumulation in nanoparticulate exposures. Moreover, the fast elimination observed in snails from Ag₂S-NP exposures suggests that the nanoparticulate form is probably not internalized in tissues and rapidly eliminated. In this test, uptake could have been mainly of Ag₂S-NPs associated with

sediment particles and were probably rapidly eliminated from the body with the excreted sediment. The low gut residence time could have limited internalization into the gut epithelium. However, comparing elimination rates between Ag-NP and AgNO₃ exposures of the present study showed that, in some cases, the elimination of Ag from the snails in nanoparticulate exposures was slower than for AgNO₃ (e.g. 3–8 nm and 60 nm Ag-NPs). This could be an indication of potential slow elimination of Ag-NPs. For instance, NPs could be taken up *via* clathrin-mediated endocytosis and directed towards lysosomal degradation, slowing down their elimination, as seen for the mudsnail *P. ulvae*.³⁴ In turn, lysosomal degradation can lead to ion release and possibly to further Ag⁺ association with MTs.

Tangaa *et al.*, determining the relative importance of Ag uptake *via* water and sediment to *T. tubifex*, showed that sediment was the most relevant exposure route for both Ag-NPs and AgNO₃, based on the $k_d = 10^5$ (sediment:water distribution coefficient) for Ag in freshwater ecosystems.³⁸ The present study revealed that water contributed the most to Ag uptake in *P. acuta* for all Ag forms, except Ag₂S-NPs (Table 3). Looking at the similarities of the uptake patterns between Ag-spiked water and Ag-spiked water and clean sediment tests, it may be assumed that the water determined uptake, although at a certain point Ag is expected to be predominantly in the sediment. However, for Ag₂S-NPs this may not be the case, as uptake from sediment contributed 74.2% of the total Ag uptake (Table 3). Furthermore, uptake and elimination kinetics obtained for this Ag form with sediment as the single exposure route (model 4) are similar to those observed in the Ag-spiked sediment test (Fig. 3 and S3†).

4.5 Ag-spiked sediment test

Sediment Ag concentrations were relatively constant throughout the experiment (Table S6†). Although the overlying water was not analysed, the Ag concentration available for uptake in water was assumed to be negligible when compared to the sediment. Ramskov *et al.* detected >98% of Ag in the sediment phase from citrate-coated Ag-NP and AgNO₃ treatments, and levels were mostly below the detection limit in the water.³⁵ Bao *et al.* detected <0.6% of Ag (by mass) lost from the sediment to the overlying water.³⁷ Although not analysed in this work, Ag-NP behaviour and speciation may undergo alterations after sediment spiking. Interestingly, Ag₂S-NPs showed similar kinetics as in the Ag-spiked water and clean sediment test when modelled with sediment as the single exposure route (Fig. 3 and S3†). On the other hand, the 3–8 nm Ag-NPs showed an opposite trend to the water-exposure tests, presenting higher k_1 and k_2 values. In the Ag-spiked sediment test, AgNO₃ and 50 nm and 60 nm Ag-NPs had similar uptake curves and k_1 values. The 50 nm and 60 nm Ag-NPs also presented similar elimination rates, k_2 and SF (Fig. 3, Table 4). This suggests that dissolution of AgNO₃ or Ag-NPs in the sediment-spiked

test may have had less influence than in the water-spiked tests, thus Ag uptake could have been mainly associated with ingestion of sediment particles. Thus, it seems that the behaviour (e.g. dissolution, agglomeration) of the pristine Ag-NPs and AgNO₃ changed in spiked sediment when compared with the water exposures, indicating that sediment had a large influence. In addition, the very different concentration range should also be considered. On the last day of the uptake phase, internal Ag concentrations in snails exposed to Ag₂S-NPs in sediment were 50 times higher when compared with those in snails exposed to the Ag-spiked water and clean sediment test, while for 3–8 nm and 50 nm Ag-NPs internal concentrations were around 11 to 12 times higher, respectively, and for 60 nm Ag-NPs and AgNO₃ 2.5 to 4 times higher, respectively (data not shown). The large difference determined for Ag₂S-NPs in relation to the other Ag forms confirmed that sediment played a more relevant role in the uptake of the simulated aged Ag form. Therefore, despite the higher body Ag concentrations determined in this experiment, much higher body Ag concentrations would be expected considering the 1000 times higher sediment concentrations than those measured in the tests with Ag-spiked water and clean sediment (Tables S4 and S6†). Bioavailability of Ag⁺ is expected to decrease in sediment due to complexation with inorganic ligands such as chlorides and sulfides, which can also modify Ag-NP speciation and dissolution.^{24,37} Instead, it is likely that Ag-NP dissolution products may persist as complexes and soluble species.⁷¹ Since the sediment was burnt in the present experiment prior to the exposure, sorption of Ag to organic matter was not possible, leading to higher bioavailability than would be typical in the environment. In the present test, the analogous uptake rates observed for pristine Ag-NPs and AgNO₃ may imply similar Ag bioavailability. Similar bioavailability for both aqueous and particulate Cu to *Potamopyrgus antipodarum* in sediments has also been reported.⁸⁰ Despite the comparable bioavailabilities, the dissimilarities observed in elimination rate constants between treatments suggest that different Ag forms were taken up and processed differently by snails. Notably, pristine Ag-NPs in this study showed steady elimination, whilst the AgNO₃ elimination rate decreased considerably (Fig. 3). The differences in uptake and elimination of AgNO₃ in snails exposed to the spiked sediment compared with the water exposures further supports the hypothesis that Ag from the AgNO₃ exposure was mostly taken up associated with sediment particles. Elimination of 3–8 nm Ag-NPs had a fast decline on the first day of elimination and remained constant thereafter (Fig. 3). In Khan *et al.*, when accounting for a two-component efflux rate in *P. ulvae* exposed to Ag-NPs, fast loss was higher and probably shorter for Ag-NPs than for AgNO₃, but the slow efflux was almost identical for both Ag forms. The authors suggested that for snails exposed to Ag-NPs, fast elimination corresponds to the particulate form and slower loss to dissolved Ag from Ag-NP dissolution *in vivo*, supporting the “Trojan Horse” concept.³³

Citrate- and PEG-coated Ag-NPs and AgNO₃ experienced similar dissolution in sediments spiked at a nominal concentration of 2.5 mg kg⁻¹.⁷¹ Transdermal uptake of dissolution products in sediment pore water was found to be predominantly responsible for Ag accumulation in the oligochaete *L. variegatus* exposed to citrate-coated Ag-NPs and AgNO₃. However, in another experiment, PVP-coated Ag-NPs and Ag₂S-NPs at a nominal concentration of 2.5 mg kg⁻¹ did not dissolve within sediments and no Ag was accumulated by *L. variegatus*, while the opposite was found upon AgNO₃ exposure.⁷¹ Other studies reported higher Ag accumulation in benthic organisms exposed to the ionic counterpart than to the particulate form in sediments.^{37,73} Ramskov *et al.* however, reported higher Ag accumulation in *P. antipodarum* clone A only from AgNO₃ exposures and significantly higher particulate Ag accumulation in *P. antipodarum* clone B from citrate-coated Ag-NP exposures.³⁵ Others reported higher accumulation of NPs than the ionic counterpart.⁸¹ Pang *et al.* further indicated that particle selection by deposit feeders may determine NP bioavailability.⁸¹ Freshwater pulmonated snails exhibit structures and particle-related processes in the gut that can act as barriers to NPs, such as sorting mechanisms in their digestive tract.⁸² Briefly, ingested particles are ground and sorted in the gizzard. Particle-size selection occurs in the ciliated passages system of the pylorus, where only particles ≤4 μm pass into the digestive gland for extracellular/intracellular digestion, and larger particles pass into the intestine for excretion.^{55,81–83} In the present experiment, there was no indication of size-dependent uptake, however, looking at the elimination kinetics it is possible that some particle/form selection may have occurred in the snails. In addition, cuticle (gastric shield) and mucus secreting cells in the stomach (and associated glycoproteins) also act as barriers to NP translocation across the epithelium in snails.⁸³ The high internal Ag concentrations determined for *P. acuta* and the very low mortality observed reflect the high tolerance to metals of these organisms. Detoxification strategies such as metallothioneins, phytochelators, or storing metals in insoluble granules to prevent biochemical reactions are also part of the barrier that freshwater snails, among other invertebrates, developed to deal with high metal accumulation.^{83,84} The digestive gland of gastropod molluscs is the main location of accumulation and biotransformation of xenobiotics.⁸⁵ The digestive gland was the major target tissue for Ag accumulation in the freshwater snail *Bellamya aeruginosa* exposed to sediment-associated Ag nanoparticles.³⁷ *P. acuta* is considered an invasive species that can survive in sewage treatment plants and drains, therefore, this species may likely have developed strategies to cope with metal contaminated environments.⁴⁴ This high ability further suggests that although Ag⁺ and Ag-NPs were probably bioavailable for uptake and internalization, they were sequestered and inactive thus no toxicity was observed in the Ag-spiked sediment test.

4.6 Ag concentrations in shells

At the last day of the uptake phase, Ag concentrations in shells were up to 25% of the amount measured in the soft bodies (Table 5). Spyra *et al.* detected less than 20% of the metals Cd, Cu and Pb concentrations in shells when compared to the soft body in *P. acuta*.⁴⁴ In the present study, no clear tendency of Ag concentrations to increase or decrease was observed in shells during uptake or elimination phases (Table 5, Fig. S4†). Although the proportion of Ag in shells was higher in comparison to soft bodies at day 14 in some exposures, this can be due to higher Ag concentrations detected in shell samples of the elimination phase and/or to the decrease of concentrations in the respective soft bodies. As an example, Ag concentration in shells exposed to 60 nm Ag-NPs in the Ag-spiked water test decreased from 3.3 to 1.8 μg Ag g⁻¹ dw, while the proportion of Ag increased from 4.7 to 7.7% (Table 5). Soft body Ag concentrations decreased from 70.3 to 23.9 μg Ag g⁻¹ dw at days 7 and 14, respectively (data not shown). It should not be disregarded, however, that dissecting the soft body and shell is prone to human error. Concentrations increased in shells of *Lymnaea peregra* exposed to Pb contaminated sediment but reduced loss occurred in clean medium for 28 days, suggesting that shells may act as a sink for Pb.⁸⁶ Ramskov *et al.* detected higher amounts of accumulated Cu in shells instead of soft body, in CuO-NPs and CuCl₂ treatments.⁴⁵ In another study, *P. antipodarum* snails revealed higher Cu concentrations in the shell after 2 weeks exposure to CuCl₂ and CuO-NPs (6 nm) but, in contrast, during the elimination phase Cu concentrations were higher in soft body and reduced rapidly in the shells.⁸² It has been suggested that CuO-NPs were loosely sorbed onto the shell and were subsequently lost due to snail movement in clean sediment.⁸⁰

Everard and Denny suggested that since empty shells exposed to Pb did not absorb the metal, it can be assumed that metals are incorporated into the shells during their formation and cannot be eliminated. This means that Pb could be incorporated into the shell during growth and not be removed.⁸⁶ Since in most cases snails decreased in weight (probably due to not being fed during the entire exposure period), it is unlikely that Ag incorporation into the shells of *P. acuta* occurred in our experiments. Differences in metal assimilation between soft body and shell were suggested to be related to different uptake modes, as the soft body presents strategies such as formation of granules in different organs that can be excreted, while shells lack specific binding sites and may accumulate metals over time.⁸⁷

Although the empty shells exposed by Everard and Denny did not absorb Pb,⁸⁶ the high adsorption tendency of metals to mollusc shells is extensively studied and so is its use for biomonitoring and remediation of metal contaminated waters.^{88,89} Pb concentrations in shells of the terrestrial snail *Helix aspersa* fed on Pb-dosed diets decreased when concentrations in the soft body also fell, suggesting that shells could be storage sites for Pb when ingested levels are

high, being part of the detoxification strategies of the snails.⁸⁷ Ag-NPs (PAAm coated) and ionic Ag were transported at similar levels from soft body to the extrapallial fluid of the mussel *Mytilus edulis*. It is known that metals transported to the extrapallial fluid can be incorporated into the interior shell surface of the bivalves.⁹⁰

In the present work, no direct and clear correlation between Ag concentrations in these two fractions was observed, and there is no evidence to support the theory of Ag redistribution between soft body and shell (Table 5, Fig. S4†). Considering also the decrease in snail weight, it is more plausible that the Ag detected was, at least in its majority, adsorbed to shells from the contaminated medium.

5. Conclusions

In the present work, uptake and elimination kinetics of pristine and simulated aged Ag-NPs and its ionic form were evaluated in the snail *P. acuta*, exposed through different routes. The results suggest that Ag-NP characteristics largely influenced their bioavailability, leading to different uptake and elimination patterns in snails exposed to different Ag forms within the same exposure route. Snails from tests with Ag-spiked water with and without clean sediment had, in general, more similar uptake kinetics that differed from those of snails exposed to Ag-spiked sediment. This suggests that exposure route had a determining role in the bioavailability of the tested Ag forms. The different elimination kinetics of the same Ag forms in Ag-spiked water with and without clean sediment suggest possible interactions with sediment that changed the Ag form taken up by the snails. This emphasizes the need to properly evaluate NP form in the exposure medium as well as internally. The similar uptake curves observed for all Ag forms (except for Ag₂S-NPs) in the Ag-spiked sediment indicate that sediment greatly influenced Ag behaviour and the form taken up by snails. When accounting for double exposure *via* both water and sediment, water was likely to be the main route. Interestingly, the simulated aged Ag-NP form (Ag₂S-NPs) revealed analogous kinetics in experiments that considered sediment as exposure route, which points to a higher influence of the sediment for Ag₂S-NPs. This was also supported by the higher contribution to Ag uptake from sediment determined for Ag₂S-NP. Moreover, Ag₂S-NPs were not only highly available to snails but were also easily depurated.

The remarkably low elimination and high stored fraction observed in some exposures highlights the bioaccumulation ability of *P. acuta* and may raise concerns about possible adverse consequences in terms of chronic exposure and trophic transfer, especially in scenarios of continued Ag-NP exposure. The shell fraction accumulated lower Ag amounts, which can contribute to transfer to predators that ingest the whole snail. This work highlights the need for additional examination of the overall exposure scenario, such as the factors controlling bioavailability, internalization, and

elimination in different scenarios with full speciation analysis and mass-balance accounting. The results also contribute important hazard data for the environmental risk assessment of Ag-NPs to benthic biota.

Conflicts of interest

There are no conflicts of interest to declare.

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