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Toxicokinetics and bioaccumulation of silver sulfide nanoparticles in benthic invertebrates in an indoor stream mesocosm

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Abstract

Mesocosms allow the simulation of environmentally relevant conditions and can be used to establish more realistic scenarios of organism exposure to nanoparticles. An indoor mesocosm experiment simulating an aquatic stream ecosystem was conducted to assess the toxicokinetics and bioaccumulation of silver sulfide nanoparticles (Ag_2S NPs) and $AgNO_3$ in the freshwater invertebrates *Girardia tigrina*, *Physa acuta* and *Chironomus riparius*, and determine if previous single-species tests can predict bioaccumulation in the mesocosm. Water was daily spiked at 10 μgL^{-1}. Ag concentrations in water and sediment reached values of 13.4 μgL^{-1} and 0.30 μgg^{-1} in the Ag_2S NP exposure, and 12.8 μgL^{-1} and 0.20 μgg^{-1} in the $AgNO_3$. Silver was bioaccumulated by the species from both treatments, but with approximately 1.5, 3 and 11 times higher body Ag concentrations in $AgNO_3$ compared to Ag_2S NP exposures in snails, chironomids and planarians, respectively. In the Ag_2S NP exposures, the observed uptake was probably of the particulate form. This demonstrates that this more environmentally relevant Ag nanoform may be bioavailable for uptake by benthic organisms. Interspecies interactions likely occurred, namely predation (planarians fed on chironomids and snails), which somehow influenced Ag uptake/bioaccumulation, possibly by altering organisms´ foraging behaviour. Higher Ag uptake rate constants were determined for $AgNO_3$ (0.64, 80.4 and 1.12 $L_{water}g^{-1}{}_{organism}day^{-1}$) than for Ag_2S NPs (0.05, 2.65 and 0.32 $L_{water}g^{-1}{}_{organism}day^{-1}$) for planarians, snails and chironomids, respectively. Biomagnification under environmentally realistic exposure seemed to be

low, although it was likely to occur in the food chain *P. acuta* to *G. tigrina* exposed to $AgNO_3$. Single-species tests generally could not reliably predict Ag bioaccumulation in the more complex mesocosm scenario. This study provides methodologies/data to better understand exposure, toxicokinetics and bioaccumulation of Ag in complex systems, reinforcing the need to use mesocosm studies to improve the risk assessment of environmental contaminants, specifically NPs, in aquatic environments.

Keywords: nanomaterials, uptake and elimination, exposure routes, sediments, single-species tests, risk assessment.

1. Introduction

The increasing application of engineered nanoparticles in products and processes, and their inevitable release into the environment, have highlighted the need to understand their effects on ecosystems (Lead et al., 2018). Silver nanoparticles (Ag NPs) are one of the most produced NPs, being widely applied in medical and consumer products due to their broad-spectrum antibacterial properties (Jiang et al., 2017; Yan and Wang, 2021). Therefore, an increasing body of research has been conducted on Ag NP fate and toxicity in the different environmental compartments (Ahamed et al., 2021; Lead et al., 2018; Selck et al., 2016). Wastewater effluents are one of the main sources of Ag NPs into aquatic systems (Cervantes-Avilés et al., 2019) and during wastewater treatment they may react with sulfur to form silver sulfide NPs (Ag_2S NPs) (He et al., 2019; Kaegi et al., 2011). Ag NPs tend to aggregate and settle from the water column to the sediment phase in surface waters. Therefore, the sediment compartment can be an important sink for Ag NPs (Furtado et al., 2015; Lowry et al., 2012) with predicted concentrations up to 88.2 µg Ag NP kg^{-1} by 2020 (Sun et al., 2016). Their accumulation in sediments may lead to subsequent Ag NP exposure of biofilms and benthic species, with risk for toxicity and biomagnification across aquatic food webs (Clark et al., 2019). In soft freshwaters in the presence of natural organic matter, Ag NPs may form relatively stable

dispersions in the water column and can migrate over long distances, posing risks to pelagic organisms (Li et al., 2020; Tangaa et al., 2016), while hard waters with higher ionic strength, especially those impacted by effluent discharges, show particle settling. Therefore, understanding exposure, toxicokinetics and bioaccumulation potential of NPs has become crucial for assessing their environmental risk (Petersen et al., 2019). The bioaccumulation potential of NPs has recently been reviewed (Handy et al., 2018; Kuehr et al., 2021a; Petersen et al., 2019; Wang and Liu, 2022), showing that most studies involved single-species tests under standard laboratory conditions (Kuehr et al., 2021b). Such studies can provide important mechanistic information on the NP uptake by individual organisms, but do not reflect the complexity of environmental systems (Colman et al., 2014; Lead et al., 2018). Moreover, most studies used pristine Ag NPs, while Ag_2S NPs can be considered as a more persistent and relevant Ag nanoparticulate form in the environment (Auvinen et al., 2017; Clark et al., 2019; Kaegi et al., 2015; Lead et al., 2018). Recent studies have attempted to replicate more complex scenarios to assess the fate and effects of NPs under realistic environmental conditions (Avellan et al., 2020; Bone et al., 2015; Bour et al., 2016; Mondal et al., 2022). In the environment, exposure can be complex, especially in aquatic systems where organisms can be simultaneously exposed via sediments, water and food (Warren et al., 1998). Furthermore, the bioaccumulation of substances, including NPs, not only depends on their characteristics but also on the exposure pathway, feeding habits and physiology of the exposed organisms (Brooks et al., 2009). Interspecies interactions (e.g., competition, predation, avoidance) can influence bioaccumulation by altering the organism's behaviour (Sloman, 2007). Experimental designs such as mesocosms are used to simulate more complex exposure scenarios to increase environmental relevance, while allowing replication (Nikinmaa, 2014). The need for mesocosm studies to enhance environmental realism and improve NP assessment has been raised (Lead et al., 2018; Selck et al., 2016), as well as the importance of using toxicokinetic approaches to understand their potential bioaccumulation and risk (Petersen et al., 2019).

Considering this, an indoor mesocosm experiment simulating a natural stream environment was conducted to assess the toxicokinetics and bioaccumulation of Ag_2S NPs and $AgNO_3$ in freshwater organisms. The main objectives were to: 1) determine the toxicokinetics in freshwater benthic

invertebrates of Ag_2S NPs, representing an environmentally aged Ag NP form, and compared to $AgNO_3$ as a metal salt control, 2) evaluate potential Ag bioaccumulation and determine potential biomagnification in this more complex scenario, and 3) determine if previously conducted single-species tests can predict bioaccumulation in the mesocosm test scenario (Silva et al., 2020, 2022, 2023). Several species were used in the mesocosm, here the focus is on the planarian, *Girardia tigrina*; the snail, *Physa acuta* and the midge larvae, *Chironomus riparius*. Previous studies using lower-tier experiments have shown Ag bioaccumulation by invertebrates exposed to Ag_2S NPs and $AgNO_3$ (Baccaro et al., 2018; Khodaparast et al., 2021; Silva et al., 2020, 2022, 2023). Other studies have reported different toxic effects in standard laboratory tests and mesocosm experiments (Bone et al., 2015; Bour et al., 2016). Taking this into account, we tested the hypotheses that the three aquatic invertebrate species could bioaccumulate Ag from Ag_2S NPs and $AgNO_3$, and that single-species tests cannot predict bioaccumulation in the complex conditions of a mesocosm experiment. To our knowledge, this is the first study evaluating the toxicokinetics of Ag NPs in invertebrates in a freshwater mesocosm experiment.

2. Materials and Methods

2.1 Test species and culturing

The species *Girardia tigrina* (planarians), *Physa acuta* (snails) and *Chironomus riparius* (chironomid larvae) were bred and maintained under controlled laboratory conditions (20 ± 1 ºC and 16:8h light: dark photoperiod) at the University of Aveiro, Portugal. Due to their photonegative nature (Saraiva et al., 2020), *G. tigrina* were maintained in plastic containers covered with aluminium foil. These containers were filled with American Society for Testing Materials (ASTM) hard water (ASTM, 1980), having a pH between 7.5 and 7.8. The planarians were fed *ad libitum* once a week with bovine liver or *C. riparius* larvae. ASTM medium was fully renewed immediately after feeding and 2 days

after. Adult planarians (size 1.5 to 2 cm) used in the present experiment were not fed 1 week prior to the experiment to ensure a uniform metabolic state (Oviedo et al., 2008). Groups of approx. 50 snails were kept in glass aquariums, filled with 3 L of artificial pond water (APW) (Naylor et al., 1989), with continuous aeration and a basic pH between 7.9 and 8.2 to avoid shell fracturing. The medium was partially renewed every other day and fully renewed once a week. The animals were fed *ad libitum* every other day with ground fish food, TetraMin® (Tetrawerke, Melle, Germany). Snails of approximately two months old were used in the experiment. *C. riparius* larvae were cultured in plastic containers with fine inorganic sediment (<1 mm grain size, previously burned at 500 °C for 4 h) and ASTM hard water (pH between 7.3 and 7.9), at a depth ratio of 1:4, respectively. Continuous aeration was provided to the cultures. Sediment was fully renewed monthly, and ASTM hard water was fully renewed every two weeks. Larvae were fed *ad libitum* three times a week with a suspension of ground TetraMin® (Tetrawerke, Melle, Germany). For the experiment, egg ropes were isolated from the main *C. riparius* culture. After hatching, larvae were fed every other day and 2nd instar larvae (6 to 7 days post-hatching) were used in the mesocosm test.

2.2 Nanoparticles

Silver sulfide nanoparticle were obtained as a colloid suspension [Ag_2S NPs in polyvinylpyrrolidone (PVP), 1.32 g Ag_2S L^{-1}; reported size by the manufacturer 20.4 ± 11.9 nm; crystal structure: 72% of Acanthite and 28% of Argentite] and were supplied by Applied Nanoparticles (Barcelona, Spain), a partner of the EU H2020 NanoFASE project (http://www.nanofase.eu/). These NPs were synthetised *ab initio* to simulate an environmentally aged Ag NP form to increase the exposure's realism. Detailed characterization of the Ag_2S NP colloids is described in the supplementary information (SI). Silver nitrate ($AgNO_3$, Sigma Aldrich, CAS number 7761-88-8, 99% purity, as crystalline powder) was used as the metal salt control. The stability of the Ag_2S NPs in the test medium was evaluated by monitoring their hydrodynamic size, zeta potential and dissolution at

different time points (0, 0.5, 1, 2, 4, 8, 24 and 48 hours), and experimental details and results can be found in the SI.

2.3 Experimental design

2.3.1 Mesocosm experiment

The experiment used an indoor modular mesocosm system at the Applied Ecology and Ecotoxicology group, at the University of Aveiro, Portugal, maintained at 15 ± 1 ºC (air temperature) and 16:8h light: dark photoperiod. The system was composed of 36 artificial streams made of glass (each of 2 m length, 0.200 m width and 0.225 m depth) arranged in triplicates. Each triplicate was supplied with water from a shared sump (Figure S1A and B). The system was divided into 12 triplicates, with 4 sets randomly assigned to each treatment: control, Ag_2S NPs and $AgNO_3$. The final water volume of each triplicate (3 streams and the sump) was approx. 207 L, and the water was operated in recirculation mode. The bottom of each stream was covered with a layer of sediment (99% sand, < 2 mm, previously burned at 500 ºC for 4h) mixed with ground alder leaves (*Alnus glutinosa*; 1% w/w), giving a total of 7 kg of sediment per stream. Then, each stream was filled with 35 L of APW (Naylor et al., 1989), freshly prepared and enriched with 0.028 g L^{-1} $NaSiO_3.9H_2O$, 0.008 g L^{-1} K_2HPO_4 and 0.085 g L^{-1} $NaNO_3$, to simulate the mineral concentrations of the Mau river, located in an unpolluted area in Sever do Vouga, Portugal (Vidal et al., 2014). At day zero, APW medium pH was 7.81-7.90. Alder leaves collected from the riparian vegetation at São Pedro de Alva, Portugal, were used to provide organic matter to the sediment and food for the test organisms. In all streams, water was maintained at a constant flow rate of approx. 4 L/min, as measured in the Mau river (Campos et al., 2020). Unglazed ceramic tiles (20 cm^2) were incubated in the Mau river two weeks prior to the start of the experiment, to allow natural colonization of biofilm, which served as food source for snails. Ceramic tiles also served as a shelter for planarians.

The systems were acclimated for 2 days to allow equilibration of the water chemistry, after which organisms were introduced into each stream: the invertebrates *G. tigrina* (planaria, 50/stream), *P. acuta* (snail, 60/stream), *C. riparius* (midge larvae, 150/stream), and *Lumbriculus variegatus* (blackworm, approx. 900 mg/stream). *Daphnia magna* (water flea; 0.25% ration for the fish equating to approximately 13 *Daphnia*/stream/day) and the rainbow trout *Oncorhynchus mykiss* (3/stream) were kept separately in submerged plastic chambers in the streams. The number of organisms of each species was based on the biomass necessary for chemical analysis, while accounting for possible predation and mortality in the system (Campos et al., 2020; Clark et al., 2023; OECD, 2007; Silva et al., 2020, 2022, 2023). Ceramic tiles with biofilm were placed in each stream's inflow, middle, and outflow (Figure S1C). One day after all organisms were introduced, each triplicate Ag_2S NP and $AgNO_3$ treatment was spiked daily at the shared sump, to maintain a nominal concentration of 10 µg Ag L^{-1}. This concentration (sub-lethal) was selected based on previous bioaccumulation experiments with the test species (see below) and to ensure reliable Ag measurements in the different compartments. The mesocosm experiment lasted for 14 days and organisms were exposed for the entire test duration. In this paper, the sampling procedure and toxicokinetic data for *G. tigrina*, *P. acuta* and *C. riparius* are presented. Details for the other species are reported by Clark et al. (2022), which also reports the fate and concentrations of Ag in water, sediments and biota of this mesocosm experiment. Therefore, some data derived from Clark et al. (2022) is also presented in this paper as it is crucial for understanding Ag exposure, uptake and toxicokinetics in the test species.

2.3.2 Single-species experiments

Single-species tests were conducted prior to the mesocosm experiment with *G. tigrina* (Silva et al., 2022), *P. acuta* (Silva et al., 2020) and *C. riparius* (Silva et al., 2023) to determine the toxicokinetics of Ag_2S NPs and $AgNO_3$ through different exposure routes. In these previous studies, the three species were exposed in independent single-species tests to waterborne Ag_2S NPs and

$AgNO_3$ at a nominal concentration of 10 µg Ag L^{-1} and clean sediment. A detailed description of the experimental design of these single-species can be found in Silva et al. (2020, 2022, 2023). To compare with the mesocosm results, the toxicokinetic data of these single-species tests were remodelled only considering the uptake phase, and therefore the data on the single-species tests presented here differ from those published by Silva et al. (2020, 2022, 2023).

2.4 Sampling procedure in the mesocosm test

One stream of each set of 4 triplicates assigned to each treatment was destructively sampled at days 2, 7 and 14. On each sampling day, water (10 mL; *n* = 4 per treatment) and sediment (20-30 g; *n* = 4 per treatment) were collected near each stream's inflow, middle, and outflow for total Ag analysis. Sampled organisms were left to depurate for 24 h in containers with clean APW medium. Some snails were stored without depuration or washing to evaluate potential biomagnification considering a more realistic scenario, which includes any adsorbed Ag on the organism and Ag in the digestive tract. Organisms were rinsed in ultra-pure water, pooled and frozen at -80 ºC until total Ag analysis. Snail shells and soft bodies were separated and analysed separately. Prior to digestion, samples were freeze-dried and weighed. Water samples were daily taken from each stream for total Ag analysis, and for routine measurements of water chemistry parameters, such as temperature, pH, dissolved oxygen (DO), electrolytes and total ammonia. On days 1, 6 and 13, water was sampled from each stream at 10 min, 1 h, 2 h, 4 h and 24 h after dosing to outline the exposure profile between spiking periods. On days 2 and 14, water, sediment and sediment pore water samples were taken for Transmission Electron Microscopy (TEM) analysis (see SI for the methodology).

2.5 Sample digestion and Ag analysis of biota, water and sediment samples of the mesocosm test

Total metal analysis in biota followed the methodology of Clark et al. (2019). Samples were digested in Eppendorf tubes with 0.5 mL of neat nitric acid (Fisher, Primar Plus Trace Metals Analysis Grade), for 2h in a water bath at 65 ^{o}C. After cooling, samples were diluted to 1.5 mL using ultrapure deionised water and stored in the dark until analysis. Samples were analysed for total Ag by ICP-MS. To assess accuracy and recovery of the procedure, the certified reference material DORM-4 (National Research Council Canada) was analysed as described above, with a recovery of 80.9% ± 15.2% (± SD; n = 20). Water samples were immediately acidified with 0.5 mL of neat nitric acid (Fisher, Primar Plus Trace Metals Analysis Grade) and analysed by ICP-MS for total Ag concentrations. To determine particulate Ag content, water was sampled and immediately stored at 4 ^{o}C until analysis by iCAP RQ ICP-MS in time resolved analysis. Total Ag concentrations in sediment were determined at days 0, 2, 7 and 14. About 30 g moist sediment (n = 12 *per* treatment) was dried at 85 ^{o}C. Sub-samples of around 250 mg dry sediment were digested in covered 50 mL glass beakers using 10 mL neat nitric acid, following Chen and Ma (2001). A more detailed description of the sampling procedure and analysis of biota, water and sediment samples can be found in Clark et al. (2022).

2.6 Toxicokinetic modelling

To describe Ag toxicokinetics in the test organisms, one-compartment models were fitted to the measured body Ag concentrations in time of *G. tigrina*, *P. acuta* and *C. riparius*. A model commonly used to determine toxicokinetics of metals was applied (model 1 – single exposure route), using the total Ag concentration in the water or sediment as separate exposure routes (Ardestani et al., 2014). Model 2 – double exposure route - was used to understand toxicokinetics accounting for sediment and water as simultaneous exposure routes (van den Brink et al., 2019). Organisms increased in

weight during the test. Therefore, exponential growth rate constants were calculated from the organism dry weight (dw) changes during the experiment and included in both models to account for the effect of biomass changes on the estimated body Ag concentrations. The equations for the models are shown below.

Model 1 – single exposure route (Ardestani et al., 2014):

$$Q(t) = C_0 + \left(\frac{k1}{(k2+k_g)}\right) * C_{exp} * \left(1 - e^{\left(-(k2+k_g)*t\right)}\right)$$

Model 2 – double exposure route (van den Brink et al., 2019):

$$Q(t) = C_0 + \left(\frac{(C_{\exp water}*kw + C_{\exp sed}*ks)}{(k2+k_g)}\right) * \left(1 - e^{\left(-(k2+k_g)*t\right)}\right)$$

Where Q(t) = Ag internal concentration in the organisms at time t days (µg Ag $g^{-1}_{organism}$ dw); *k1* = uptake rate constant from water or sediment (L_{water} $g^{-1}_{organism}$ day^{-1} or $g_{sediment}$ $g^{-1}_{organism}$ day^{-1}); *k2* = elimination rate constant (day^{-1}); C_0 = background internal concentration measured at day 0 (µg Ag $g^{-1}_{organism}$ dw); $C_{exp\ water}$ = Ag exposure concentration in water (µg Ag L^{-1}); $C_{exp\ sed}$ = Ag exposure concentration in sediment (µg Ag g^{-1}); k_w = uptake rate constant from water (L_{water} $g^{-1}_{organism}$ day^{-1}); k_s = uptake rate constant from sediment ($g_{sediment}$ $g^{-1}_{organism}$ day^{-1}); k_g = growth rate constant (day^{-1}).

For model 2, the relative contribution (in %) of each uptake route to the total Ag uptake was determined as follows:

Water uptake route:

$$\left(\frac{(C_{\exp water} * kw)}{(C_{\exp water} * kw + C_{\exp sed} * ks)}\right) * 100$$

Sediment uptake route:

$$\left(\frac{(C_{\exp sed} * ks)}{(C_{\exp water} * kw + C_{\exp sed} * ks)}\right) * 100$$

Ag uptake patterns and toxicokinetic parameters derived from the mesocosm test were compared with results from previous single-species tests (Silva et al., 2020, 2022, 2023). To enable reliable comparison, data of the single-species tests were modelled considering only the uptake phase and applying the same models as described above.

2.7 Calculation of Bioconcentration, Bioaccumulation and Biomagnification Factors

Kinetic bioconcentration (BCF_k; L g^{-1}), kinetic biota-to-sediment accumulation ($BSAF_k$; g g^{-1}) and biomagnification factors (BMF) were calculated to relate body concentrations to Ag exposure levels in water, sediment and food, respectively, following the equations proposed by Arnot and Gobas (2006) and Petersen et al. (2019):

$$BCF_k/BSAF_k = \frac{k1}{k2} \qquad BMF = \frac{C_o}{C_d}$$

Where *k1* and *k2* are the uptake and elimination rate constants described above; C_o is the concentration in the organism (predator) (µg Ag $g^{-1}_{organism}$ dw) and C_d is the concentration in the diet (prey) (µg Ag $g^{-1}_{organism}$ dw) after 7 or 14 days of exposure. Values of BMF > 1 indicate biomagnification potential.

2.8 Statistical analysis

Equations were fitted to the raw data and toxicokinetic parameters and corresponding 95% confidence intervals (CI) were estimated by non-linear regression in SPSS (version 25). Akaike Information Criteria tests (AIC and AICc) were applied to select the best fitting models (data not shown). Generalised Likelihood Ratio Tests (GLR) were applied to determine the significance of differences of *k1* and *k2* parameters between Ag forms, species, or between mesocosm and single-species tests. One-way analysis of variance (ANOVA) (SigmaPlot 12.5 software) was used to compare organism recovery between treatments. Two-way ANOVA followed by the Holm-Sidak method ($p < 0.05$) (SigmaPlot 12.5 software) was also applied for analysis of treatment and time as factors in Ag concentrations of water or sediment samples, and of organisms. Data transformations were conducted whenever ANOVA assumptions were not fulfilled.

3. Results

3.1 Nanoparticle characterization

The characteristics of the Ag_2S NP colloids in the stock suspension are summarized in Tables S1 and S2, and corresponding TEM images are shown in Figures S2 and S3. The mean measured particle diameter was 20.4 ± 11.9 nm and zeta-potential -23.8 ± 4.5 mV in milli-Q water (Figure S2, Table S1). The particle elemental composition by TEM-EDX showed high Ag content, from 70% to 85% (Figure S3, Table S2), which indicates that the particles were not 100% pure Ag_2S NPs. Nevertheless, these Ag_2S NP particles were synthesized (Ag_2S) to be studied as a model of sulfidised Ag_2S NPs, instead of Ag NPs undergoing sulfidation. In APW medium, Ag_2S NPs revealed negligible dissolution at a test concentration of 1 mg Ag L^{-1} over 48 h (Table S3). DLS values indicated strong agglomeration at time 0, with a hydrodynamic size of 336 ± 26.6 nm (by intensity; mean ± SD; n=3),

which remained relatively constant over 48h. Zeta-potential values also were stable during the 48h (Table S3).

3.2 Exposure medium: water and sediment

Total Ag concentrations in water from the $AgNO_3$ treated mesocosms gradually increased until day 7 after which they stayed close to the nominal concentration of 10 µg Ag L^{-1}, at around 13 µg Ag L^{-1} until the end of the experiment (Table 1). Clark et al. (2022) reported that in the Ag_2S NP treatment of this mesocosm, Ag concentrations increased until day 11, reaching a peak of around 28 µg Ag L^{-1}, followed by a decrease until the end of the experiment with concentrations of about 12-13 µg Ag L^{-1} (Table 1). Total Ag concentrations in water from control streams were below the detection limit. Concentrations at days 7 and 14 differed significantly from day 2 (two-way ANOVA, $p < 0.05$) in both treatments and no significant differences (two-way ANOVA, $p > 0.05$) were detected between concentrations of Ag_2S NPs and $AgNO_3$ (Table 1). Clark et al. (2022) conducted sp-ICP-MS measurements in water samples from the Ag_2S NP and $AgNO_3$ treatments, and showed Ag particles in both exposures, whose number increased over time. Figure S4 and Table S4 present the TEM image and Ag and S composition (%) of some particles detected in the Ag_2S NP treatment at day 2, showing that particulate Ag was detected by TEM in APW in this treatment, but not in the other treatments or sampling days. No particulate Ag was detected by TEM in sediment pore water of the Ag_2S NP and $AgNO_3$ treatments. Table S5 presents the pH, temperature (ºC) and dissolved oxygen concentration (mg L^{-1}) measured at different time points, which stayed maintained relatively stable during the experiment and within the acceptable ranges for water quality (for electrolyte composition of the APW during the experiment see Clark et al. (2022)).

Total Ag concentrations in the sediment before being introduced in the system revealed a background concentration of 0.00487 ug g^{-1} (4.87 µg Ag kg^{-1}) at day 0, which increased throughout the test period. For Ag_2S NP and $AgNO_3$ treatments, total Ag concentrations in sediment significantly

increased (two-way ANOVA, Holm-Sidak method, $p < 0.05$) from day 0 to day 14 (Table 1). No significant interactions were found between treatment and time (two-way ANOVA, $p > 0.05$) in water or sediment samples. Clark et al. (2022) show that a small fraction of the total Ag was extracted from sediment by water in all treatments, and around 12% and 28% of the total Ag measured were extracted by diluted acid from sediment sampled at day 14 in Ag_2S NP and $AgNO_3$ treatments, respectively.

3.3 Toxicokinetics of Ag in benthic invertebrate species in mesocosm tests

Upon destructive sampling of mesocosms, of the *G. tigrina* introduced at day 0, 74-76% were recovered at day 7 and 75-87% after 14 days, without much difference between treatments. Recovery of live snails in control, Ag_2S NP and $AgNO_3$ exposures was 51%, 71% and 70% at day 7 and 35%, 47% and 61% after 14 days, respectively, from the initial number of snails introduced at day 0. At day 7, only 6.5%, 7.5% and 3.2% of chironomid larvae were recovered in the control, Ag_2S NP and $AgNO_3$ treatments, respectively, and no larvae were found at day 14. No significant differences in organism recovery between treatments were found for the three species (one-way ANOVA, $p > 0.05$).

Figure 1 shows the uptake kinetics of Ag in *G. tigrina* and *P. acuta* (soft body) when exposed for 14 days to Ag_2S NP and $AgNO_3$ in the mesocosm test. Chironomid larvae were not found in the streams on the last sampling day, therefore Ag uptake concentrations are only shown till day 7. Identical uptake curves were obtained through modelling with water (model 1; Figure 1) or sediment as single exposure route (model 1) or double exposure (model 2). For planarians, toxicokinetics were only determined accounting for water as (single) exposure route, as no indication was found that they ingest sediment.

The highest Ag uptake rates and body Ag concentrations were observed for all invertebrates in the $AgNO_3$ treated mesocosms. In general, higher body Ag concentrations were found in snails, followed by planarians, but in the Ag_2S NP exposures chironomid larvae revealed higher body concentrations than planarians (Figure 1). Body Ag concentrations were significantly higher (two-way

ANOVA, Holm-Sidak method, $p < 0.001$) in planarians exposed to $AgNO_3$ than to Ag_2S NPs at days 7 and 14, being around 11 times higher at day 14. For chironomids, body concentrations were significantly higher (two-way ANOVA, Holm-Sidak method, $p < 0.05$) for $AgNO_3$ compared to Ag_2S NP treatments at days 2 and 7. Silver concentrations in snails peaked at day 7 upon Ag_2S NP (body burden, 53 µg Ag g^{-1} dw) and $AgNO_3$ (body burden, 119 µg Ag g^{-1} dw) exposures, and were significantly higher (two-way ANOVA, Holm-Sidak method, $p < 0.001$) in the $AgNO_3$ treatment. At day 14, internal Ag concentrations in snails exposed to $AgNO_3$ were not significantly different from those exposed to Ag_2S NPs, being of 59.2 and 41.3 µg Ag g^{-1} dw, respectively (Figure 1).

Table 2 displays the toxicokinetic parameters calculated when considering water or sediment as exposure routes. Both water and sediment Ag concentrations were similar between Ag_2S NP and $AgNO_3$ treatments (Table 2). All organisms revealed higher *k1* values in the $AgNO_3$ exposures. Snails revealed the highest *k1* water and *k1* sediment values (Table 2). Significant differences were only found between *k1* water values of planarians and chironomids in the Ag_2S NP treatment ($X^2_{(1)} > 3.84$; $p < 0.05$). Snails had higher *k2* values, especially for $AgNO_3$, while planarians and chironomid larvae presented *k2* values of zero or almost zero. Considering the double exposure (water and sediment), the same *k1* water and *k2* values as for single water exposure were determined, except for snails exposed to $AgNO_3$. In turn, much lower *k1* sediment values were obtained from double exposure modelling than in single-exposure, however, differences were not significant as no 95% CI could be estimated (Table 2). Water seems to be responsible for almost 100% of the Ag uptake by snails and chironomid larvae (Table 2).

3.4 Bioaccumulation and evaluation of potential trophic transfer

Table 3 shows the BMF for planarians, calculated considering: 1) chironomid larvae (at day 7) as diet, 2) depurated snails (at days 7 and 14) as diet, 3) non-depurated/non-washed snails (at days 7 and 14) as diet, and 4) accounting with the mean body Ag concentrations of non-depurated snails and larvae, as planarians probably fed on both organisms. For planarians, no BCF_k values could be

determined for both treatments as no elimination was seen, so *k2* = 0. Chironomid larvae exposed to Ag_2S NPs also had *k2* = 0 making it impossible to determine BCF_k or $BSAF_k$ values, but they showed to bioaccumulate Ag following $AgNO_3$ exposure, with a BCF_k of 56 L g^{-1} and a $BSAF_k$ of 6150. Snails seemed to bioaccumulate Ag from both Ag forms, but BCF_k and $BSAF_k$ were lower for Ag_2S NPs (5.64 L g^{-1} and 389, respectively) than for $AgNO_3$ (11.4 L g^{-1} and 856, respectively). For *G. tigrina*, BMFs considering chironomid larvae or snails (depurated and non-depurated) as diet were higher in the $AgNO_3$ exposure. Planarians revealed a BMF of 1.05 when considering depurated snails as their food in the $AgNO_3$ treatment (Table 3). The BMF values (non-depurated snails and larvae) at day 7 were similar to those calculated accounting only for snails, in both Ag_2S NP and $AgNO_3$ exposures (Table 3).

3.5 Comparison of toxicokinetics between mesocosm and single-species tests

Figure 2 and Table 4 present data of the three species exposed in independent single-species tests, which contained non-spiked sediment and water spiked to a nominal concentration of 10 µg Ag L^{-1}. In these single-species tests, Ag concentrations in sediments increased during the uptake phase due to settlement from the water column, so sediment exposure was also accounted for in the toxicokinetics calculations for snails and chironomid larvae. Planarians showed no significant Ag uptake from Ag_2S NPs and very low uptake from $AgNO_3$ in the single-species tests, showing body concentrations ~40 times higher upon exposure in $AgNO_3$-treated mesocosms than in the respective treatment of the single-species test. Body Ag concentrations in snails exposed to $AgNO_3$ were around 18 times higher than in the Ag_2S NP exposure of single-species tests at the end of the uptake phase, while chironomid larvae revealed 3.2 times higher Ag accumulation upon exposure to Ag_2S NPs than to $AgNO_3$. At equivalent exposure time (48 h) in both treatments of the single-species tests, similar body Ag concentrations were measured in snails (38.5 µg Ag g^{-1} dw in Ag_2S NP and 15.9 µg Ag g^{-1} dw in $AgNO_3$ exposures) and chironomids (35.7 µg Ag g^{-1} dw in Ag_2S NP and 10.9 µg Ag g^{-1} dw in $AgNO_3$ exposures). Higher *k1* through water and sediment were determined for snails in both

treatments. Remarkably high *k1*s sediment were also generally obtained, particularly for snails (Table 4).

Table 4 also presents data from the mesocosm tests modelled with water or sediment as exposure routes to enable direct comparison. Mesocosm data was modelled considering equivalent exposure times as for the single-species data, i.e. for planarians and snails the data was modelled until day 7 and for chironomids until 48 h. Since chironomid larvae were sampled every 12 h in the single-species tests, graphs and kinetic parameters of both single-species and mesocosm tests are presented in hours (Figure 2, Table 4). In general, planarians and snails reached higher body Ag concentrations at respective times in the mesocosm test compared to the single-species tests, while the chironomid larvae reached similar or higher concentrations in the single-species test (Figure 2). For *G. tigrina* exposure to $AgNO_3$, mesocosm and single-species tests showed an increasing Ag uptake pattern, although uptake was much lower in the single-species test (Figure 2), being significantly lower at day 7 (two-way ANOVA, Holm-Sidak method, $p < 0.05$), and *k1* and *k2* values did not differ ($X^2_{(1)} < 3.84$; $p > 0.05$) between tests (Table 4).

Snails exposed to Ag_2S NPs in both mesocosm and single-species tests showed a fast Ag uptake during the first day, which continued until day 2 in the mesocosm test. In snails from single-species tests the uptake curve reached a steady state from day 1 until the end of the uptake phase. For snails in the mesocosm test, a steady state was reached later, from days 7 to 14 (Figures 1 and 2). Snails from the mesocosm test revealed significantly higher body Ag concentrations (two-way ANOVA, Holm-Sidak method, $p < 0.05$) at day 7 in the Ag_2S NP exposure, and at days 2 and 7 (two-way ANOVA, Holm-Sidak method, $p < 0.001$) in the $AgNO_3$ exposure than snails from the single-species test. Upon $AgNO_3$ exposures, snails in the single-species test exhibited a very different uptake pattern, showing a gradual increase with time (Figure 2), with *k1* and *k2* values being significantly lower ($X^2_{(1)} < 3.84$; $p > 0.05$) than for the mesocosm exposure (Table 4). In turn, for snails exposed to Ag_2S NPs in the single-species test, values of *k1* sediment and *k2* were significantly higher ($X^2_{(1)} < 3.84$; $p > 0.05$) than for the mesocosm test (Table 4).

Chironomid larvae from the single-species test exposed to Ag_2S NPs reached body Ag concentrations around 10 times higher (two-way ANOVA, Holm-Sidak method, $p < 0.001$) at day 2

(35.7 µg Ag g^{-1} dw) than those from the mesocosm (3.42 µg Ag g^{-1} dw) (Figure 2). Chironomid larvae showed similar gradual Ag uptake patterns for $AgNO_3$ exposures in both mesocosm and single-species tests during the first 24h, after which uptake in the single-species test slowed down. Still, body Ag concentrations were not different between mesocosm and single-species tests in larvae from this exposure (two-way ANOVA, $p > 0.05$). For chironomid larvae, no significant differences were found in kinetic parameters between mesocosm and single-species tests ($X^2_{(1)} < 3.84; p > 0.05$) (Table 4).

The *k2* of zero for the planarians prevented the calculation of BCF_k values, so no comparison can be made between tests. For snails, BCF_k obtained upon exposure to Ag_2S NP-treated mesocosms was around 4.5 times higher than in the single-species test, while BCF_ks were very similar between tests in exposures to $AgNO_3$. For chironomids, BCF_ks were around 4.5 times higher in the Ag_2S NP and almost 35 times higher in the $AgNO_3$ mesocosm exposures, respectively, than in single-species tests (Table 4).

4. Discussion

This is the first study to assess the toxicokinetics of Ag_2S NPs in benthic invertebrates in a complex system such as a mesocosm experiment. The main findings include the demonstration of the uptake and accumulation of Ag_2S NPs by benthic invertebrates, but the absence of biomagnification of this Ag form. Overall, the metal salt showed to be more bioavailable than the Ag_2S NPs, and water was the most important uptake route for both nano and dissolved Ag forms. When comparing the mesocosm experiment and single-species tests, bioaccumulation seems to be underestimated by the latter.

4.1 Exposure medium: water and sediment

Total Ag concentrations in the water showed a gradual increase over time, then stabilizing in the latter part of the mesocosm experiment close to the nominal 10 µg Ag L^{-1}. This observation suggests that during the initial days of spiking the Ag was not yet in a dynamic-steady state with all compartments in the mesocosm, with some of the daily dose being removed from the water column and partitioned to sediments and/or glass walls of the mesocosm, and the organisms. Results of sp-ICP-MS indicated that Ag was mainly in the particulate form in the water column for Ag_2S NP and $AgNO_3$ treatments (see Clark et al. (2022)). For Ag_2S NPs, negligible Ag dissolution is expected due to its high stability, even though these particles were not pure Ag_2S NPs. $AgNO_3$ is expected to form dissolved Ag species in the aqueous media that will likely bind to –SH groups or other ligands (e.g., humic acids, on the alder leaves in the sediment) and/or adsorb to the surface of particulate minerals and/or bind to Cl^- and form insoluble silver chloride complexes, with posterior settling from the water column to the sediment (see Bradford et al. (2009)). Settling of Ag_2S NPs is also expected given the ionic strength of the APW. The increasing Ag concentrations in the sediment (Table 1) confirmed this tendency of Ag to partition from the water to the sediment in both treatments, with sediments becoming a sink for Ag. This is in accordance with other studies where sediment was the main sink for Ag in Ag NP and $AgNO_3$ treatments (Bradford et al., 2009; Jiang et al., 2017). The Ag fate in water and sediment of this mesocosm experiment is discussed in more detail by Clark et al. (2022).

4.2 Toxicokinetics of Ag in invertebrate species in the mesocosm test

In both treatments, generally higher body Ag concentrations were found in snails than in planarians (Figure 1). Despite planarians occupying the same ecological niche as the snails, this likely reflects that bioavailability also depends on the organisms' feeding strategies and physiology (Brooks et al., 2009; De Jonge et al., 2010). Different Ag accumulation was observed between two pulmonate

freshwater snails exposed to Ag NPs, potentially due to the different physiological species traits. *L. stagnalis* showed higher Ag accumulation likely due to climbing higher in the beaker or floating, while *P. corneus* stayed preferably at the bottom of the beaker (Wang et al., 2022). Freshwater snails are good indicators of metal contamination in freshwater due to their ability to accumulate high levels of metals (Spyra et al., 2019). Freshwater snails have been shown to efficiently accumulate Ag from Ag NPs or $AgNO_3$ in water, and from sediment (Ramskov et al., 2015; Stoiber et al., 2015), with some studies reporting either greater Ag uptake from $AgNO_3$ (Bao et al., 2018; Dai et al., 2013) or from particulate Ag (Croteau et al., 2014). The chironomid larvae accumulated similar amounts of Ag as the planarians (Figure 1), also generally less than the snails. The hepatopancreas of freshwater gastropods is the preferred organ for xenobiotics accumulation, and was the major organ for Ag accumulation in the freshwater snail *Bellamya aeruginosa* (Bao et al., 2018). Therefore, it is unsurprising that higher internal Ag concentrations were observed in *P. acuta* compared to planarians and chironomids in this mesocosms.

Based on the single-species tests and on toxicity experiments performed by our research team, the Ag concentrations reached in water and sediment of the mesocosm were unlikely to cause toxicity to chironomids (Lopes, 2015), therefore the low recovery of larvae in this mesocosm is more likely due to predation rather than to the bioaccumulation of Ag to toxic levels. Similar observations with chironomids have been made in other mesocosm experiments, where less than 7% of the *C. riparius* larvae were recovered at the end of the test, which was also attributed to predation rather than to toxicity of CeO_2 NPs (Bour et al., 2016). Bioaccumulation can be affected by external biotic factors such as species interactions (Diepens et al., 2015), and thus predation may have had a considerable impact in the mesocosm experiment. For example, the presence of planarians (predator) could have hampered feeding behaviour of chironomid larvae (prey) that must leave their tube to feed on surface sediment particles. Although fish were kept in chambers, their chemical cues could also have affected invertebrate behaviour, such as decreasing foraging behaviour (Paterson et al., 2013), as observed for *C. riparius* and *P. acuta* (Hölker and Stief, 2005; Justice and Bernot, 2014). Since most of the species used in our mesocosm test were benthic, some competition for space was theoretically possible and could have affected their foraging behaviour and consequently food uptake (Diepens et

al., 2015). Competition and avoidance behaviour between *Physa* and *Chironomus* species have been reported (Devereaux and Mokany, 2006; Gresens, 1995).

Internal Ag concentrations were significantly higher in the three species exposed to $AgNO_3$, although the measured Ag concentrations in water indicate that the total Ag exposure was similar for both Ag forms (Figure 1, Table 1). In previous single-species tests on *P. acuta* and *G. tigrina*, Ag was also more bioavailable in $AgNO_3$ than in Ag_2S NP treatments (Silva et al., 2022). For *C. riparius*, internal Ag concentrations were significantly higher upon exposure to Ag_2S NPs than to $AgNO_3$ in single-species tests (Figure 2). However, a study reported that water spiked with Ag NPs and sulfide showed slower Ag uptake into larvae compared to water spiked with Ag NP only (Lee et al., 2016). In our earlier bioaccumulation tests (Silva et al., 2022, 2022, 2023) and in the mesocosm (Table S3), Ag_2S NPs were probably taken up by the three species as particulate Ag as almost no dissolution was observed. Huang et al. (2020) reported that copper(I) was a driver for Ag_2S dissolution and consequently increased Ag availability to wheat. Pradas del Real et al. (2017) reported dissolution of Ag_2S NPs at the root surface of wheat, which could have been favoured by root exudates. This suggests that natural abiotic and biotic factors may trigger transformations at the sediment/bio-interface, that may increase Ag bioavailability for benthic organisms. This however, needs further investigation under environmentally realistic conditions, *e.g.,* in field studies.

According to Visual Minteq speciation calculations for $AgNO_3$, only 7.2% was present as free Ag^+, the remaining Ag being complexed with Cl, probably as particles (further details in Clark et al. (2022)). The spontaneous formation of AgCl particles from $AgNO_3$ in media containing NaCl is now well-established (Besinis et al., 2014; Clark et al., 2019). These results suggest that in both treatments exposure was most to particulate Ag in water. In the $AgNO_3$ exposure, AgCl complexes formed could be more bioavailable to the organisms and their re-solubilisation may explain the higher bioavailability of Ag in the $AgNO_3$ than in the Ag_2S NP treatment. Such secondary particles are taken up (Clark et al., 2021) and their re-solubilisation in acidic intracellular compartments such as lysosomes could, in theory, also lead to the release of Ag ions (Buffet et al., 2014). Nevertheless, higher exposure to dissolved Ag in water of the $AgNO_3$ may be expected due to the continuous water spiking.

Values of *k1* for the uptake of Ag from water were lower in the planarians than in the chironomid larvae and snails (Table 2). A study reported the *k1* values for Cd, Cu and Zn uptake in the planarians *Dugesia japonica* and *G. tigrina* being up to 10 times lower compared with molluscs, crustaceans and oligochaetes. It was suggested that the absence of gills and water current-creating structures may lead to lower metal uptake from water in organisms like planarians (Wu and Li, 2017). Still, a study reported Ag uptake by the planarian *Schmidtea mediterranea* (belonging also to the family of Dugesiidae) exposed to $AgNO_3$ and Ag NPs, but Ag seemed more available and more toxic to this species in the $AgNO_3$ treatment (Leynen et al., 2019). Planarians primarily take up chemicals from the water via the integument (Kapu and Schaeffer, 1991), which may involve facilitated diffusion of metal ions into the epithelial cells (Handy and Eddy, 2004). Invertebrates secrete mucus, and the negatively charged matrix of the highly conserved mucoproteins is very good at chelating and effectively trapping cations in the mucus layer (e.g., snail mucus in APW, Schlichter 1982), including metals (Handy and Eddy, 2004). However, mucus offers only a transient protection and eventually the underlying epithelium will be exposed. Ag was detected at concentrations up to 0.083 µg L^{-1} in the epidermal mucus of the planarian *S. mediterranea* after 2 days of exposure to 15 mg L^{-1} of uncoated and PVP-coated Ag NPs (Leynen et al., 2019). Planarians can ingest food particles by phagocytosis and the internalisation of other nanoparticles has also been demonstrated by mechanisms including endocytosis (Ermakov et al., 2019; Salvetti et al., 2020, 2015). Thus, in the present study, the planarians could have ingested and taken up intact Ag_2S NPs or any AgCl particles formed from $AgNO_3$ in the APW. The higher body Ag concentrations of planarians exposed to $AgNO_3$ may also result from feeding on their prey (snails, chironomid larvae and blackworms (Ilic et al., 2018)), which also showed higher accumulation in $AgNO_3$ exposures. This is in keeping with previous observations where metal accumulation in the predator is strongly related to metal concentrations in their prey items (De Jonge et al., 2010). It also suggests that dietary rather than waterborne exposure is more important to total Ag accumulation by planarians. In the present mesocosm study, the data modelling for planarians and chironomid larvae showed no elimination of Ag in either treatment (low/negligible elimination rate constants, Table 2). However, the mesocosm study design did not include an elimination phase, therefore the modelling of the Ag elimination needs to be confirmed by observation.

Nonetheless, the bioaccumulation and retention of Ag is well-known for aquatic organisms (Ratte, 1999).

Comparing the contribution from water and sediment to total Ag uptake revealed that water contributed with nearly 100% in both treatments (Table 2), which agrees with our previous findings for *P. acuta* (Silva et al., 2020). Still, caution should be taken when considering the contribution of water or sediment to total uptake because no 95% CI could be determined for *kw* and *ks*. The very high *k1* values estimated for snails and chironomid larvae when accounting for Ag concentrations in the sediment may suggest that exposure to sediment may not have contributed much to Ag uptake in both treatments, confirming that water is the main Ag uptake route. Chironomid larvae can be vulnerable to water exposure when foraging or when pumping water for tube irrigation (De Haas et al., 2005), and accumulate metals from the overlying water and pore water (Bervoets et al., 1997; Gimbert et al., 2018). Still, exposure to sediments may have happened, as chironomids ingest sediment and snails may have accidentally ingested sediment particles when feeding on sediment organic matter. Nevertheless, a higher contribution from sediments would be expected for chironomid larvae, considering that they probably fed on sediment organic matter and particles. Aggregates of CeO_2 NPs (Bour et al., 2014) and Al_2O_3 NPs (Lorenz et al., 2017) were observed in the digestive tract of CeO_2 exposed *C. riparius* larvae, indicating uptake of NPs alone and/or associated with sediment particles. After settling into the sediment phase, Ag was probably mostly adsorbed onto sediment organic matter, but some labile pool of Ag was expected to be available for both Ag forms (see above). This suggests that at some point some Ag may have been exchanged to the pore water, likely enhanced by the intense reworking activity of the chironomid larvae, for instance, to become available for transdermal uptake, which seemed to be easier for ionic than for nanoparticulate Ag due to the ability of ionic Ag to cross cells via ion transport channels, such as proton-coupled Na^+ channels (Khan et al., 2015). This may explain the higher accumulation and uptake in the $AgNO_3$ treatment.

4.3 Bioaccumulation and evaluation of potential trophic transfer

The model predictions (Table 2) suggest low elimination rates and therefore the potential for bioaccumulation in all test organisms of total Ag from either $AgNO_3$ or Ag_2S NP exposures. At day 7, the uptake and elimination rate constants were used to calculate BMF values for the planarians, either assuming the planarians were eating chironomid larvae alone, snails alone, or both larvae and snails (Table 3). Overall, regardless of the type of prey item, the BMF values for $AgNO_3$ are 5-7 fold greater than those for the equivalent exposure to Ag_2S NPs. However, the BMF values were also dependent on the type of prey item, with depurated snails giving rise to higher BMF values than eating chironomid larvae alone, or predating on a combination of snails and larvae (Table 3). Indeed, all the BMF values were < 1, indicating no biomagnification, except when the food consisted of depurated snails, which was of 1.05 in the $AgNO_3$ exposure (Table 3). In a natural aquatic food web from a Chinese lake, Ag NPs were biomagnified in a fish food web and revealed higher bioaccumulation potential than total Ag (Xiao et al., 2019). Trophic transfer of Ag from Ag NP, Ag_2S NP and Ag^+ exposures was observed from *Daphnia magna* to *Danio rerio*, but no biomagnification occurred (Xiao et al., 2022).

For the snails and chironomids, it was also possible to calculate bioaccumulation factors for accumulation from the water and sediments respectively. Higher BCF_k and $BSAF_k$ were found for *P. acuta* exposed to $AgNO_3$ than to Ag_2S NPs, again indicating the metal salt was more bioavailable than the nano form. The BCF_ks for snails in the present study for $AgNO_3$ (11.4 L g^{-1}) and Ag_2S NPs (5.64 L g^{-1}) are slightly higher but of similar magnitude to other reports. BCFs were also higher for the freshwater snail *Cipangopaludina chinensis* after 7 days of exposure to $AgNO_3$ (0.92) than to citrate (0.1) and PVP-capped (0.23) Ag NPs in a paddy microcosm (Park et al., 2018). The freshwater snail *Potamopyrgus antipodarum* clone B revealed a much higher BSAF (~2) upon exposure to Ag NPs than to $AgNO_3$ (~0.3), while *P. antipodarum* clone A showed similar and low BSAFs in both treatments (Ramskov et al., 2015). The $BSAF_k$ values here for *P. acuta* are 100 fold greater, perhaps because the sediment was very sandy with little organic matter to chelate the Ag. Clearly, the precise $BASF_k$s for snails depends on the physical properties of the sediment, and the biology/strain of the animals.

Upon exposure to $AgNO_3$ chironomids revealed $BSAF_k$ values of 6150, around 7 times higher than snails, even though the snails overall accumulated more total Ag than the chironomids (Figure 1), but this could be because *k2* were negligible for chironomids, while snails showed some elimination of Ag in the $AgNO_3$ treatment. It should be noted that the *k2* values used to estimate the BCF_k/$BSAF_k$ were determined by calculation only in the absence of an elimination phase in the study design, making them less certain. Moreover, since steady state was generally not achieved in the different mesocosm compartments (water, sediment, and biota), the biomagnification factors should be interpreted with caution. Nevertheless, it was the aim of this study to evaluate potential biomagnification under this more complex scenario.

4.4 Predicting bioaccumulation in complex exposure: comparison of toxicokinetics between mesocosm and single-species tests

For regulatory purposes, chemical substances' toxicity and bioaccumulation potential are usually assessed by conducting single-species laboratory tests. However, in order to protect biodiversity, it is required to perform more complex and realistic exposure scenarios (Kefford et al., 2021). Considering this, one of the aims of the present study was to assess whether bioaccumulation tests with single exposure routes and single organisms can predict bioaccumulation in a complex but more realistic scenario such as a mesocosm. Overall, the single-species tests seem not to be able to reliably predict bioaccumulation of both dissolved and nano Ag forms in the more complex scenario of this mesocosm as body Ag concentrations were generally significantly higher (and so were BCF_k; Table 4) in both Ag_2S NP and $AgNO_3$ treatments in the mesocosms compared to single-species tests, pointing to underestimation of the bioaccumulation in the latter. Toxicokinetic parameters did not differ between the two experiments for planarians and chironomids, however, it should be noted that in some cases the large scatter in the data leading to very wide 95% CI hampered reliable comparisons. For snails exposed to $AgNO_3$, BCF_k values in mesocosm and single-species tests were similar, although the kinetic *k*1 and *k*2 values were significantly different between these experiments. Furthermore, the

differences between single-species and mesocosm tests generally seem to be equivalent for Ag_2S NP and $AgNO_3$. Importantly, as no uptake seemed to have occurred in the single-species Ag_2S NP exposures for planarians, the observed Ag uptake in the Ag_2S NP treated mesocosms indicates a higher bioavailability of this Ag form to the planarians in the mesocosms. This is most likely related to the simultaneous exposure to different routes (water, food and sediment pore water) and to the relatively constant Ag concentrations in water due to continuous spiking. The continuous water flow may have also helped maintaining some Ag in suspension, likely becoming more available for uptake. Furthermore, the absence of Ag uptake in Ag_2S NP exposures in single-species tests may indicate that water is a less important uptake route for planarians than food for this Ag form. While *k2* was zero in $AgNO_3$ treated mesocosms, planarians seemed to be able to eliminate Ag following $AgNO_3$ exposure in single-species tests, even though *k2* values were not significantly different (Table 4).

For snails, the single-species test seemed to correctly predict the steady state obtained later in the mesocosms (Figures 1 and 2), but this is probably related to their ability to eliminate these NPs, and the steady state level was much higher than expected based on the single-species test. The overall significantly higher *k1* and *k2* values found in the mesocosm relative to the single-species tests in both treatments suggests that uptake and elimination by snails was different between the types of experiments (Table 4). Even though exposure concentrations (mean values) in water were relatively similar between the experiments, the increase in body Ag concentrations in snails exposed to Ag_2S NPs from the mesocosms may be, in part, the result of the increasing Ag concentrations in water (Tables 1 and 4), while in the single-species test concentrations in water decreased over time likely due to sedimentation. In the $AgNO_3$ treatment, uptake patterns were distinct and body Ag concentrations of snails from the mesocosm were significantly higher than from the single-species test at day 2 (Figure 2), but Ag concentrations in water at day 2 were still low (Table 1) and similar to those in the single-species test at the equivalent time (Silva et al., 2020). Although Ag concentrations were not measured in biofilms due to their very low biomass, snails could have used them as food source, being another uptake route for snails in mesocosms. Biofilms have shown a high bioaccumulation of Ag from Ag NP and $AgNO_3$ exposures (Park et al., 2018) and constitute relevant exposure routes to snails (Cleveland et al., 2012). Diet (diatoms) was the main Ag uptake route for *L.*

stagnalis in Ag NP and $AgNO_3$ exposures (Croteau et al., 2011). Another aspect that was never observed in the single-species tests with *P. acuta* exposed to $AgNO_3$ is that the considerable decrease in internal Ag concentrations already starting during the uptake phase, which was also seen in snails exposed to $AgNO_3$ in the mesocosms at day 14 (Figure 1).

For chironomids, Ag from the Ag_2S NP treatment was probably more bioavailable in the single-species test than in the mesocosms, as shown by the significantly higher body Ag concentrations despite the 4 times lower total Ag concentrations measured in sediments from the single-species tests (Silva et al., 2023). The similar body Ag concentrations and uptake curves between $AgNO_3$ treatments of single-species and mesocosm tests suggest that single-species tests may predict bioaccumulation until 48h, pointing to similar bioavailability of Ag in the $AgNO_3$ treatment from both experiments. In turn, it may also support the fact that Ag uptake by the chironomid larvae was more important through water than via sediment. However, the very short exposure time (48h) may not be realistic or robust enough to build conclusions, and the low number of replicates and time points available for the chironomids in the mesocosm experiment may have affected the reliability of these conclusions. The different larval instar and depuration period used in mesocosm (2nd instar larvae and 24 h-depuration) and single-species tests (4th instar larvae and 4 h-depuration (Silva et al., 2023) may not be disregarded as possibly having some influence on the differences in Ag bioaccumulation observed. Other studies on nanomaterials have also shown differences in results between mesocosms and lab studies. For instance, Bour et al. (2016) determined toxicity of CeO_2 NPs to *Pleurodeles* larvae that was not observed in parallel standardized single-species tests. Bone et al. (2015) also obtained different toxicity patterns of Ag NPs and $AgNO_3$ between laboratory experiments and mesocosms, with higher toxicity of the Ag NPs than of $AgNO_3$ observed in the latter. In line with several studies (Auffan et al., 2019; Ayadi et al., 2021; Carboni et al., 2021), the obtained results stress the need to include mesocosm studies to assess fate, toxicity and bioaccumulation of Ag NPs in freshwater benthic invertebrates. However, studies comparing bioaccumulation of other nanomaterials in standardized tests with mesocosms experiments are still scarce, highlighting the need to conduct more studies to reach a consensus for the different nanomaterials.

5. Conclusions

Due to its negligible dissolution, the sulfidated Ag nanoform is considered less bioavailable and consequently less harmful to biota than non-sulfidated Ag NPs. However, some studies have reported higher bioavailability of this form than of Ag NPs, and nano-specific effects caused by Ag_2S NPs in organisms. This study demonstrated that this relevant Ag nanoform is likely to be bioavailable for uptake by benthic organisms, therefore, its environmental safety has to be considered carefully to avoid underestimation of its potential effects. Silver from $AgNO_3$ exposure, either in particulate or dissolved form, appeared to be more bioavailable for uptake, with higher uptake and body Ag concentrations than when exposed to Ag_2S NPs, and water seemed to be the most important uptake route for the three species. Biomagnification under this environmentally realistic exposure scenario seemed to be low, even though it is likely to occur in the food chain *P. acuta* to *G. tigrina* in the $AgNO_3$ treatment. This mesocosm study allowed determination of toxicokinetics and bioaccumulation in complex scenarios, including simultaneous exposure routes and species interactions (*e.g.,* predation), that cannot be observed in single-species assays, providing a crucial bridge between laboratory and field conditions. The single-species tests generally seemed not able to reliably predict Ag bioaccumulation in the more complex environment of the mesocosms. To conclude, mesocosm studies should be considered complementary to existing regulatory frameworks procedures to allow a more realistic environmental risk assessment of nanomaterials in aquatic ecosystems. To our knowledge, this is the first study evaluating the toxicokinetics of Ag_2S NPs in benthic invertebrates in a freshwater mesocosm experiment.

Conflicts of interest

There are no conflicts of interest to declare.

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