Comparative study of the sensitivity of two freshwater gastropods, *Lymnaea stagnalis* and *Planorbarius corneus*, to silver nanoparticles: bioaccumulation and toxicity

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**A R T I C L E   I N F O**

Keywords: Nanotoxicity Freshwater gastropods Species-specificity Metals Physiological traits

**A B S T R A C T**

Metal-based nanoparticles (NPs) are considered detrimental to aquatic organisms due to their potential accumulation. However, little is known about the mechanisms underlying these effects and their species-specificity. Here we used stable silver (Ag) NPs (20 nm, from 10 to 500 μg/L) with a low dissolution rate (≤2.4%) to study the bioaccumulation and biological impacts in two freshwater gastropods: *Lymnaea stagnalis* and *Planorbarius corneus*. No mortality was detected during the experiments. Ag bioaccumulation showed a dose-related increase with an enhanced concentration in both species after 7d exposure. *L. stagnalis* displayed a higher accumulation for AgNPs than *P. corneus* (e.g., up to 18- and 15-fold in hepatopancreas and hemolymph, respectively) which could be due to the more active *L. stagnalis* having greater contact with suspended AgNPs. Furthermore, the hepatopancreas and stomach were preferred organs for bioaccumulation compared to the kidney, mantle and foot. Regarding biological responses, the hemolymph rather than hepatopancreas appeared more susceptible to oxidative stress elicited by AgNPs, as shown by significantly increasing lipid peroxidation (i.e., formation of malondialdehyde). Neurotoxicity was detected in *L. stagnalis* when exposed to high concentrations (500 μg/L). Comparison with impacts elicited by dissolved Ag revealed that the effects observed on AgNPs exposure were mainly attributable to NPs. These results highlighted the relationship between the physiological traits, bioaccumulation, and toxicity responses of these two species to AgNPs and demonstrated the necessity of species-specificity considerations when assessing the toxicity of NPs.

1. Introduction

Invertebrates comprise more than 99% of global species richness with a high diversity (Ruppert et al., 2004). In aquatic ecosystems, invertebrates are crucial components of food webs and fulfil many ecosystem services, such as being food sources and aiding nutrient cycling (Ruppert et al., 2004). Their species distribution, abundance and diversity convey information about environmental health and ecosystem sustainability (Prather et al., 2013). Gastropods, in particular, are increasingly used as excellent biomonitors for environmental sustainability (Prather et al., 2013). Their species distribution, abundance and diversity convey information about environmental health and ecosystem sustainability (Prather et al., 2013).

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https://doi.org/10.1016/j.envpol.2022.119999

Received 29 June 2022; Received in revised form 12 August 2022; Accepted 15 August 2022

Available online 28 August 2022

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environmental pollution indicators given their wide geographic distribution, ease of availability, and sensitivity toward chemicals (Amorim et al., 2019; Chatel et al., 2020; Herbert et al., 2021). The L. stagnalis has been used as an excellent biological model for ecotoxicological studies and is applied in OECD test guidelines (Amorim et al., 2019; Ducrot et al., 2014). Notably, interspecific variation should be considered when comprehensively evaluating environmental risks. For example, the freshwater gastropod Biomphalaria glabrata showed much higher nerve injuries than P. corneus upon pesticide exposure (Garate et al., 2020), and the freshwater amphipod Gammarus pulex accumulated more diclofenac than Hyalella azteca (Fu et al., 2020). Although those researches enriched information for risk assessments, it is little known how the specific species variations exactly contribute to the disparities to pollutants.

Manufactured nanoparticles (NPs) are extensively used in various commercial products. Silver (Ag) NPs, in particular, display great antimicrobial and antiseptic properties, and are the most commonly used NPs in products since 2012 (The Nanodatabase, 2022). The massive utilization of AgNPs raises the probability of their release into the aquatic environment, which could threaten ecological resources (Kaegi et al., 2019; Chatel et al., 2020). The L. stagnalis and the freshwater amphipod Gammarus pulex accumulated more diclofenac than Hyalella azteca (Fu et al., 2020). Although those researches enriched information for risk assessments, it is little known how the specific species variations exactly contribute to the disparities to pollutants.

One recent study demonstrated that AgNPs exhibited lower toxicity (LC50, 96h) of AgNPs was determined to be ~48 μg/L in snails of a related species, Lymnaea lutetiae (Ali, 2014). Referring to toxicity, some reviews have summarized that dissolved ions (Ag+) are exclusively responsible for the toxicity of AgNPs to aquatic organisms (Caixeta et al., 2020; Walters et al., 2014). Conversely, the AgNPs-induced toxicity appeared to originate from the particles, rather than Ag+, for the freshwater gastropod, Bellamya aeruginosa (Bao et al., 2018). Thus, particle-specificity should be considered besides the Ag+’s effect when thoroughly evaluating NPs impacts on organisms. The interspecific variations may also be one of the reasons for the differing outcomes, which have hardly been addressed thus far.

One recent study demonstrated that AgNPs exhibited lower toxicity to Daphnia magna in surface water than in M4 culture medium (Hu et al., 2018). The most substantial difference between those two media is the significant presence of natural organic matter (NOM, mg/L) in the former (Hu et al., 2018; Wang and Liu, 2022), which interacts with NPs and increases the NPs’ stability. Generally, NPs of smaller sizes displayed higher toxicity, thus the aggregates/agglomerates formed by the combination of AgNPs with NOM in surface water may play a key role in the toxicity difference (Hu et al., 2018). Additionally, our recent review also highlights the need for research on consideration of NPs toxicity with respect to natural water to better understand their bioavailability and biological effects (Wang and Liu, 2022). This is also the reason why Volvic® mineral water was used in the present study to simulate natural pond water (Auffan et al., 2018; Auffan et al., 2020).

In this study, we used AgNPs as model NPs to compare the sensitivity of L. stagnalis and P. corneus to environmental chemical compounds. Bioaccumulation is critically important to link the environmental chemistry of NPs to biological responses (Croteau et al., 2014; Lekamge et al., 2018). First, we compared the bioaccumulation of AgNPs (exposure concentration 0 to 500 μg/L) in the two species, i.e. L. stagnalis and P. corneus. For invertebrates, the hepatopancreas (i.e., midintestinal gland) comprehensively mediates metabolism and immunity function (Rösser, 2014), and hemolymph contributes importantly to the innate immunity and is a well recognized model for NPs’ toxicity studies (Canesi and Procházková, 2014; Perez and Fontanetti, 2011). Thus, we then elucidated potential biological responses (immune responses and oxidative stress) caused by different Ag forms (i.e., NPs and Ag+) in these two tissues of the species. Given that the two selected gastropods exhibit different species traits, we hypothesized that they will show different AgNP bioaccumulation patterns and thus dissimilar accumulation in tissues and translocation to the circulatory fluid, followed by consequences elicited by alterations in the metabolic state of hepatopancreas and hemocyte functions.

2. Experimental

2.1. Nanoparticle chemical and colloidal stability

Citrate (Cit)-coated spherical AgNPs (Biopure) with a primary size of 18.4 ± 2.4 nm at 1.01 mg Ag per mL in 2 mM citrate was purchased from Nanocomposix (San Diego, USA). To better understand the physical-chemical transformation and determine the colloidal stability of AgNPs and their kinetics of dissolution over time (2h, 1d, 2d, and 7d) without organisms, a preliminary exposure to Cit-AgNPs in 100 mL Volvic® water in 400 mL glass beakers was performed. AgNPs were suspended in Volvic® water at predicted environmental and supra-environmental concentrations (100 and 500 μg/L, respectively) (Zhao et al., 2021) and beakers were covered with aluminum foil to prevent evaporation and exclude dust. This set-up was conducted in a climate-controlled room at 20 °C under a 16:8 h light/dark artificial photoperiod regime to ensure the conditions were consistent with subsequent gastropod exposure.

At each sampling time, the hydrodynamic size and ζ-potential of the AgNP suspensions were measured using a NanoZetaSizer (Malvern Instruments Inc., UK). Moreover, silver released from the particles was measured at different time points using inductively coupled plasma-mass spectrometry (ICP-MS, Agilent 7700x, Agilent Technologies, USA). To this end, 4 mL of the particle suspensions were sampled and placed in an Ultra Centrifugal Filter Unit (Amicon®) and centrifuged at 7500 g for 30 min at 4 °C in a microcentrifuge (Sigma 3-18 KI; Sigma). The 2 mL collected supernatant was diluted two-fold in ultrapure water with 1% v/v nitric acid before ICP-MS analysis.

2.2. Gastropod collection and acclimation

L. stagnalis and P. corneus were collected from a protected aquatic garden (47° 22’ 18.413” N, 5° 48’ 24.439” E, Autoreille, France). Gastropod individuals were allowed to acclimatize in the laboratory in a climate-controlled room at 20 °C under a 16:8 h light/dark artificial photoperiod regime for two weeks. A quarantine period was applied for several days before acclimatization. Fresh lettuce was fed to gastropods once daily during the acclimation period. Before exposure, L. stagnalis and P. corneus of comparable sizes (length: 2.95 ± 0.20 cm and 2.88 ± 0.29 cm, respectively) were collected from among free-floating gastropods and transferred to aquariums filled with Volvic® water for one day; food was withheld during this period to depurate their guts.

2.3. AgNPs exposure and sample collection

In this study, five concentrations of AgNPs (10, 50, 100, 250 and 500 μg/L) and one concentration of Ag+ (11.96 μg/L, Ag+ released from AgNPs of 500 μg/L at 7d, determined as described in section 2.1) were selected. The exposure medium was prepared in 1 L glass beakers with Volvic® water. Six gastropods were added per exposure condition (42 gastropods for each species in total). Two types of endpoints were assessed in the gastropods following exposure to AgNPs: 1) bioaccumulation and 2) biological responses. The exposure time (7d) was chosen because this time had previously been shown to allow for Ag accumulation (Croteau et al., 2011b, Silva et al., 2022) and could trigger biological responses of freshwater gastropods to metal NPs (Ma et al., 2017). Gastropods were not fed during the whole exposure to minimize faecal scavenging. No mortality in both species of gastropods was observed for all treatments during the exposure.

After exposure, the six gastropods of the same species from each
exposure condition were rinsed with deionized water and wiped with facial tissue. Firstly, hemolymph (approximately 500 μL per gastropod) was collected with the micropipette tip by tickling the gastropod’s foot sole (Boisseaux et al., 2016). The hemolymph was pooled and collected in conical tubes and kept on ice to minimize cellular adhesion. The pooled hemolymph preparations were then divided into two portions for bioaccumulation and biochemical assay analysis. For the biochemical assay, once sampled, hemolymph was immediately processed for measurement of total hemocyte counts, hemocyte mortality, and reactive oxygen species (ROS) generation, while the remaining hemolymph was stored at −80 °C for the enzymatic activity measurement.

After hemolymph sampling, three gastropods were randomly selected for tissue Ag determination. To prevent potential metal contamination, all the glassware was soaked overnight in acid (15% HNO₃ and 5% HCl), and rinsed with ultrapure water three times before experimentation (Croteau et al., 2011a). The gastropods were plunged into hot water (70 °C) for 90 s to prevent contraction before dissection (Schols et al., 2021). Hepatopancreas, stomach, kidney, mantle and foot were dissected and frozen at −20 °C before Ag bioaccumulation analysis. The remaining three gastropods per treatment were placed individually and snap frozen at −80 °C for the biochemical assays.

2.4. Ag bioaccumulation

Ag concentrations in the different tissues and hemolymph were measured using ICP-MS (Liu et al., 2017). Gastropod tissues were freeze-dried in a vacuum freezing machine at −80 °C for 72 h (Christ® Gamma 1–16/2–16 LSC, Germany). Dried samples were ground to a homogenous powder, then weighed and digested with 1 mL 65% HNO₃ and 1 mL 30% H₂O₂ in Teflon vessels at 120 °C overnight. For hemolymph, 100 μL sample were digested with 1 mL HNO₃ and 1 mL H₂O₂ in Teflon vessels at 120 °C overnight. After cooling down to room temperature, the remaining solution was collected and diluted with MilliQ water to 3 mL and stored at 4 °C before ICP-MS determination. The reference aliquots were spiked with the solution of AgNPs to obtain 0, 1, 5 and 50 μg/L for tissues and 0, 1, 5 and 50 μg/L for hemolymph. Their supernatants were collected for the assay. The final values were calculated from standard curves, where the resuspended standard was being provided with a U/mL unit.

Intracellular ROS generation of hemolymph was detected using the CellROX® Green Reagent (C10444, Life Technologies Europe B.V., Zug, Switzerland) via Microplate Reader (Synergy H1, Bio Tek) with fluorescence excitation at 485 nm and emission at 520 nm. The biomarker determination (i.e., SOD, CAT, GST and MDA) of hemolymph followed the same commercial kit instructions as mentioned above. Briefly, the hemolymph was centrifuged, and cell pellets were resuspended in ice-cold PBS and centrifuged again. The supernatants were collected for the assay. The final values were calculated with the same method mentioned above.

2.5.2. Immune response

Total hemocytes were counted on the hemocytometer under the microscope (Olympus BX-UCB). Hemocyte mortality was assessed using the propidium iodide (PI, P3566, 1 mg/mL, Invitrogen, USA) exclusion assay. The final values were calculated with the same method. Hemocyte mortality was calculated as the mean fluorescence intensity (MFI) using the Microplate Reader (Synergy H1, Bio Tek) with fluorescence excitation at 535 nm and emission at 617 nm. The total protein content was measured using the Protein Quantification Kit-Rapid (51254, Sigma-Aldrich, USA).

2.6. Statistical analysis

Statistical analysis was performed using the software GraphPad Prism 9.3.1. One-way analysis of variance (ANOVA) with AgNP concentration as the fixed factor, followed by Dunnett’s tests, was applied to test its effects on the bioaccumulation and physiological parameters. Regression analysis in bioaccumulation (concentration-response calculation) was performed using a four-parameter non-linear regression equation. For comparison between AgNPs and Ag⁺ effects, an unpaired t-test was applied. Principal Component Analysis (PCA) and correlation matrices were applied using the packages Factoshiny and Corrplot within the R statistical environment (version 4.1.2) to identify and visualize the pattern of endpoints among treatments.

3. Results and discussion

3.1. Physicochemical properties and ion release of AgNPs

The characteristics of the AgNPs in the stock suspension as provided by the manufacturer can be found in Fig. S1. The Z-averaged hydrodynamic diameters of the AgNPs after 2h, 1d, 2d and 7d of suspension in
Volvic® water are shown in Fig. 1a. Generally, the hydrodynamic sizes ranged from ~25 nm to ~50 nm, where smaller sizes occurred at 500 μg/L. The ζ-potential of AgNPs in all the suspensions was around −10 mV (Fig. 1a), which is higher than that in stock solution (~39 mV), probably because of the divalent cations (e.g., Ca²⁺ and Mg²⁺) present in Volvic® water (Fig. S2), which may neutralize the surface charge of the AgNPs.

In the present study, the increased dissolution (from 0.69% to 2.4%, 11.97 μg/L at 7d) was observed in 500 μg/L AgNP suspensions over time (Fig. 1b). However, the dissolution of 100 μg/L remained largely unchanged (from 0.49% to 0.65%). At each time point, the % of released Ag⁺ in 500 μg/L AgNPs suspension was higher than that in 100 μg/L (Fig. 1b). In contrast, previous studies reported that the dissolution rate was much higher for 10 μg/L AgNP (5.5%) than for 100 μg/L (2.47%) in deionized water after 96 h exposure, respectively (Ali, 2014). Notably, studies have suggested that Ag⁺ could complex with Cl⁻ in aqueous solutions which decreases the measured Ag⁺ (Chen et al., 2013). Hence, the lower dissolution at 100 μg/L in the present study was possibly due to the low ratio of Ag⁺ to Cl⁻ (from Volvic water medium) compared with that at 500 μg/L. Furthermore, in our case, the smaller aggregate/agglomerate size at 500 μg/L likely contributed to the maintenance of a higher specific surface area of AgNPs and greater dissolution.

3.2. Bioaccumulation in tissues and hemolymph

Generally, NPs bioaccumulation studies so far focused on the whole soft tissue of gastropods, i.e., CuONPs in L. stagnalis and Potamopyrgus antipodarum, and AgNPs in Peringia ulvae (Croteau et al., 2014; Khan et al., 2015; Ramskov et al., 2014). In our present study, the Ag content was measured in various tissues (hepatopancreas, stomach, kidney, mantle and foot) and biofluid (i.e., hemolymph) after 7d exposure, allowing to examine Ag’s distribution profile (range from 0 to 500 μg/L) in different tissues. The tissues were chosen on the basis that they have previously been shown to be target organs for NP accumulation (Caixeta et al., 2020; Kuehr et al., 2021) and because of the important role of hemolymph served in the immune system (Iwanaga and Lee, 2005).

In the present study, bioaccumulation differences between the two species was observed: all tissues of L. stagnalis showed a higher Ag content than in P. corneus (Fig. 2). For example, at 50 μg/L AgNPs exposure concentration, L. stagnalis accumulated around 14-fold and 9-fold more Ag compared to P. corneus in hepatopancreas and stomach, respectively (Fig. 2a and b). This difference in Ag bioaccumulation could be attributed to differences of their species’ traits. Indeed, we observed that the P. corneus prefers to stay at the bottom of the beaker during the experiment, while the L. stagnalis spends most of the time climbing higher or floating to the surface (Video S1 in the online SI). When L. stagnalis moves to the water surface, they open the pneumostome fully for aerial respiration and contract mantle muscles to expel gas from the lung (Nargeot and Puygrenier, 2019). Notably, AgNPs could remain suspended in natural freshwaters, stabilizing particles against agglomeration (Chinnaponse et al., 2011), which is also the case in our experimental condition (Fig. 1a). Therefore, the more active L. stagnalis would be expected to have greater contact with NPs, which might explain the differential bioaccumulation results. Indeed, different physiological traits might contribute to the bioconcentration outcomes between and even for the same species. For example, different residence times on the surface resulted in the considerable variance of the insecticide chlorthion to accumulate within L. stagnalis (Legierse et al., 1998), confirming the substantial role of physiological species traits in bioaccumulation processes.

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2022.119999.

In addition to the difference of bioaccumulation between the two species, a varied pattern among organs was also observed. The dose-response curve of bioaccumulation in different tissues as a function of AgNP exposure concentration, as shown in Fig. 2, demonstrates that all tissues showed increasing bioaccumulation with increasing AgNP concentration. Notably, for the hepatopancreas and stomach, significant increases were observed from 50 μg/L on (Fig. 2a and b). However, for the remaining organs (kidney, mantle and foot), higher concentrations (starting from 100 μg/L) of the AgNPs were required to obtain significant bioaccumulation compared to that in the control (Fig. 2c, d and e). Ingestion has been commonly recognized as a pathway for NP uptake into aquatic organisms (Croteau et al., 2014; Kuehr et al., 2021). The AgNP uptake sequence might contribute to the disparity of bioaccumulation among organs. For gastropods, after ingestion, salivaries
and enzymes from the intestine aid in the extracellular digestion of ingested feed particles in the stomach (Carriker, 1946). Small particles (<4 μm) can penetrate the pyloric filter and enter the hepatopancreas for subsequent intracellular digestion (Carriker, 1946; Dillon, 2000). As the major metabolic tissue, the hepatopancreas is responsible for xenobiotics biotransformation, redistribution to other tissues and elimination (Bao et al., 2018; Livingstone, 1998). Thus, AgNPs might migrate from the hepatopancreas epithelia to adjacent connective tissue (e.g., mantle or kidney) (Robinson and Ryan, 1988). Hence, compared with other tissues, the hepatopancreas and stomach might be the first organs in contact with the AgNPs. The bioaccumulation and tissue distribution of metal might also be related to the amount of metal-binding proteins and chelates in different tissues (Kuehr et al., 2021). Previous studies reported that the hepatopancreas was the major target tissue for NP accumulation in freshwater gastropods, exemplified by AgNP and CuONP exposure to Bellamya aeruginosa, presumably due to a high content of metal-binding proteins (Bao et al., 2018; Ma et al., 2017). Studies on bioaccumulation are particularly important to link the environmental chemistry of NPs to biological responses (Garner et al., 2018). Although numerous studies showed that NPs generate toxicity to hemolymph of gastropods (Caixeta et al., 2020), few of them addressed the NP accumulation in this biofluid, with the exception of a recent study where TiO$_2$NPs were visualized in the hemolymph of the land snail Cornu aspersum (Bobori et al., 2020). It is generally accepted that NPs and their aggregates or agglomerates are able to cross the gut barrier and translocate to hemolymph; endocytic pathways were identified as the primary mechanism of NP internalization at the hemocyte level (Moore et al., 2009; Sendra et al., 2020; Shao et al., 2020). In the present study, along with the increase of AgNP concentration, the Ag content in hemolymph increased, with this increase being significant at 500 μg/L in both species (Fig. 2f). The Ag content in L. stagnalis was higher than in P. corneus, e.g. 453.34 ± 104.99 μg/L at the highest AgNP exposure, which is nearly 15-fold greater than in P. corneus (30.52 ± 1.47 μg/L). The differences in Ag accumulation in the hemolymph of the two gastropods is consistent with that of the tissue profile, which confirms the migration of AgNPs from the tissues to the hemolymph.

Although ICP-MS analysis does not allow to distinguish how much of this accumulation originated from AgNPs or Ag$^+$, the low dissolution of AgNPs in the exposure medium (≤2.4%) suggests that most of the accumulated Ag originated from the AgNPs. A previous study showed that polyvinylpyrrolidone-coated platinum nanoparticles (PtNPs) and CuONPs displayed greater bioavailability than their ionic form in L. stagnalis (Croteau et al., 2014; Sikder et al., 2018), which indicates that dissolution of NPs had a negligible influence on metal uptake by the freshwater gastropods.

3.3. Oxidative stress in hepatopancreas and hemolymph

Chemical bioaccumulation in organisms is generally considered to be a prerequisite for adverse effects (Luoma and Rainbow, 2008). Our findings demonstrate oxidative stress induction by AgNPs in the hepatopancreas and hemolymph of both gastropods (Fig. 3). The hepatopancreas and hemolymph have been used for assessing NPs’ effects in gastropods as a detoxifying organ and because of its important role in the detoxification process. The induction of oxidative stress in the hepatopancreas and hemolymph was confirmed by the increased concentrations of the marker proteins such as glutathione S-transferase (GST) and superoxide dismutase (SOD) (Fig. 3). The concentrations of these proteins were significantly higher in the AgNP-exposed gastropods compared to the control, indicating the induction of oxidative stress.

Fig. 3. The levels of biomarkers in the hepatopancreas (a–j) and hemolymph (k–t) of L. stagnalis and P. corneus (mean ± SE, n = 3). Different numbers of asterisks represent statistical differences compared with the control and the differences between 500 μg/L and Ag’ treatments: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
the immune system of the invertebrates, respectively (Baroudi et al., 2020; Bhagat et al., 2016; Caixeta et al., 2020; Lekamge et al., 2018). It is known that NPs can induce oxidative stress in aquatic organisms by producing ROS, such as superoxide radicals (O$_2^-$) (Caixeta et al., 2020; Lekamge et al., 2018). The antioxidant defense systems have been shown to be important in eliminating ROS (Bhagat et al., 2016). SOD catalyzes the dismutation of O$_2^-$ to oxygen (O$_2$) and hydrogen peroxide (H$_2$O$_2$), and then CAT converts H$_2$O$_2$ to O$_2$ and water. GST not only participates in the transition process from H$_2$O$_2$ to water, but also in the defense against lipid peroxides. MDA (a marker of lipid peroxidation) has been suggested as a sensor against NPs in gastropods (Kaloyianni et al., 2020). AChE activity is widely used as a biomarker of neurotoxicity for marine invertebrates after metal exposure (Deidda et al., 2021). Notably, no published data on the effect of AgNPs exposure on AChE activity in freshwater gastropods exists.

With regard to the hepatopancreas, our results showed that AgNP exposure induced no lipid damage in either gastropod after 7d exposure, demonstrated by insignificant changes in MDA contents compared to control (Fig. 3d and i). Similar results were also observed in the hepatopancreas of B. aeruginosa after CuONP exposure (Ma et al., 2017). SOD, in particular, played an essential role for L. stagnalis and P. corneus to react to AgNP stress. Results showed that SOD activity was lower in all treatments than those in the control, with a significant decrease at 250 and 500 μg/L AgNP exposure levels for both species of snails (Fig. 3a and f). For P. corneus, GST activity was inhibited at all the exposure concentrations (Fig. 5h), indicating that this enzyme was also critical to help respond to oxidative stress. A significant increase in AChE activity was only observed in L. stagnalis upon exposure to 250 and 500 μg/L AgNP (Fig. 5e). Similar AChE activation after AgNPs exposure was reported in D. magna (Ulm et al., 2015). Considering the high accumulation of Ag in the hepatopancreas of L. stagnalis, the binding of Ag to AChE might stimulate the catalytic function of AChE (Najimi et al., 1997).

In the hemolymph, significant lipid damage (increased MDA contents) was observed in all AgNP-exposed (except 10 μg/L) L. stagnalis and P. corneus (Fig. 3o and t). In agreement with our study, previous studies reported that MDA levels of the hemolymph of freshwater gastropods Biophalaria alexandrina (Fahmy et al., 2014) and L. luteola (Ali, 2014) increased after ZnONP and AgNP exposure, respectively. AgNPs exposure caused a significant increase in ROS levels at 500 μg/L (Fig. 3f and p), which is in agreement with previous studies where ROS levels were estimated to increase in gastropod cells after iron oxide NPs treatment (Sidiropoulou et al., 2018). SOD activity significantly increased in the hemolymph of L. stagnalis and P. corneus exposed from 0 to 250 μg/L and 0–50 μg/L, followed by a decrease for the remaining concentrations to near the control level (Fig. 3i and q). The subsequent decrease of SOD in the present study might be due to the involvement of other antioxidant defense system constituents (e.g., GST increased significantly at the highest concentration, which will be discussed below), which helps to cope with oxidative stress. GST activity was generally increased in the hemolymph of both species at 500 μg/L (Fig. 3n and s). Compared with L. stagnalis, more antioxidative enzymes of P. corneus participated in the AgNP stress responses. CAT decreased at the higher concentrations (250 and 500 μg/L) in P. corneus (Fig. 3r), while no significant change occurred in L. stagnalis. Previous studies also found a decrease in CAT activities in the freshwater gastropod L. luteola exposed to CuONPs (21 μg/L) for 5d (Ali et al., 2015).

The above results indicated that SOD and GST are more involved in the oxidative stress response of L. stagnalis and P. corneus to AgNPs exposure than other enzymes. Although no lipid damage was observed in the hepatopancreas of both gastropods, AgNP exposure generated neurotoxicity (i.e., enhanced AChE) to L. stagnalis. Furthermore, the hemolymph appears more susceptible to oxidative stress originating...
from AgNPs compared to the hepatopancreas in L. stagnalis and P. corneus.

3.4. Immune responses in hemolymph

The hemocyte count is considered an indicator of innate immunological status of invertebrates (Kacsoh and Schlenke, 2012). Experimental exposure to AgNPs for 7d yielded a significant decrease in total hemocyte count (Fig. 4a), which indicates immune suppression in both species of gastropods. Our results are in line with previous studies which reported that AgNP and TiO$_2$NP exposure decreased the hemocyte counts of the freshwater gastropod, Lymnea luteola (Ali, 2014; Ali et al., 2015). The hemocyte counts reflect the success of a defence response to some extent, and hemocytes reduction appears to result from cell lysis or cell movement from circulation to tissues (Perez and Fontanetti, 2011).

The increase of ROS production generated by AgNPs at 500 $\mu$g/L was of biological significance, which resulted in a significant increase in the hemocyte mortality in both species (Fig. 4b). The increased hemocyte mortality as observed in the present study is in accordance with the findings for L. luteola after AgNPs exposure (Ali, 2014).

Proteins in hemolymph serve for maintaining the osmotic pressure and regulating intravascular water distribution, which directly affect the hemolymph flow dynamics (Rawi et al., 1995). In the present study, significant decreases in protein content were observed at higher AgNP concentrations (250 and 500 $\mu$g/L) (Fig. 4c), which is in accordance with the finding in the freshwater gastropod B. alexandrina after three weeks of ZnONP exposure (Fahmy et al., 2014). The reduction in protein content may reflect damage in the hepatic parenchyma, which is the origin of protein generation (Rawi et al., 1995).

As summarized in Fig. 5, our findings show that exposure to AgNPs led to the accumulation of total Ag in different tissues and hemolymph and subsequent changes in the biochemical parameters of the hepatopancreas (the detoxification organ) and the hemolymph (the immune response). The AgNP exposure impairs the immune biomarkers and generates oxidative stress in the hemolymph. The highest exposure concentration induced AChE enhancement indicating that neurotoxicity occurred.

3.5. Toxic mechanism of AgNPs and Ag$^+$

There is controversy over the toxic mechanism of AgNPs. As some studies indicated (Gao et al., 2021; Shen et al., 2015; Yan et al., 2021) that the Ag$^+$ was a major contributor to AgNP toxicity; however, other studies suggested that the toxicity of AgNPs was attributed to the particles (Bao et al., 2018; Carrasco-Quevedo et al., 2019). Some metal and metal oxide NPs, such as AuNPs and TiO$_2$NPs, even if not capable of releasing ions, could also trigger toxic effects by inducing oxidative stress (Arini et al., 2020; Girardello et al., 2016), suggesting a particle-specific effect. In our study, there were significant differences in biological responses between 500 $\mu$g/L AgNP and Ag$^+$ treatments. Except for the hemocyte mortality for P. corneus, all the immune and oxidative stress responses in hemolymph are different (Figs. 3 and 4).

These findings suggested different toxic mechanisms between AgNPs and Ag$^+$. Ag$^+$ may be complexed by various organic/inorganic ligands abundant in natural water, substantially reducing Ag$^+$ bioaccumulation (Azimzada et al., 2017). Particularly in our case with chloride (Cl$^-$) containing medium, the Ag$^+$ might combine with Cl$^-$ and form insoluble AgCl, which is low in toxicity. However, it is undeniable that Ag$^+$ in natural water could exert toxicity in organisms (Gao et al., 2021; Shen et al., 2015; Yan et al., 2021). AgNPs’ toxicity may originate from the Ag$^+$ effect and the particle-specific effect (Xu et al., 2019). Considering the higher toxicity (e.g., enhanced ROS production and MDA content) induced by AgNPs compared with Ag$^+$, we supposed that the particle-specific effects might play a major role for AgNPs in exerting toxicity on freshwater gastropods.

Fig. 5. Schematic diagram illustrates the AgNPs influencing the fitness of gastropods by bioaccumulation and altered immunity and antioxidant defence. Red shapes indicate the biomarkers determined in the present study. White and black arrows indicate biomarker changes of L. stagnalis and P. corneus, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
between Ag accumulation in the hemolymph and tissues (NPs accumulation and oxidative stress. Positive correlations were found between Ag accumulation in the hepatopancreas, a negative correlation of the Ag content with SOD and MDA change of hemolymph and CAT and AChE of hepatopancreas were the variables contributing most to the separation observed between the two species (Fig. 6b, Table S2). The correlation analysis between various measured attributes of L. stagnalis and P. corneus is depicted in Fig. S3 and Table S3. In both species, a significant positive relationship between Ag content in hemolymph was observed with ROS and MDA, whereas a negative correlation was noted with total hemocyte counts and protein content. In the hepatopancreas, a negative correlation of the Ag content with SOD and CAT was recorded. Those findings indicate an important link between the two species (Fig. 6b, Table S2).

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3.6. Principal component and correlation data analysis

PCA was performed on all accumulation and biological variables and on all the treatments for both gastropod species (Fig. 6, Table S2). The biplot of the PCA (Fig. 6a) demonstrated that L. stagnalis and P. corneus form distinct clusters along the first principal component (PC1), which explained 52.07% of the total variance. The variables contributing the most to this separation are shown in the PCA plot of variables (Fig. 6b) where the direction and length of the vectors display the magnitude and correlation among vectors. The high values in the color scale indicate a high contribution to the PCA. More variations were explained for L. stagnalis than P. corneus, illustrated by the area covered by the ellipses, which verified that L. stagnalis are more sensitive to exposure than the other gastropod. It was concluded that Ag accumulation, ROS and AChE are grouped, which indicates a positive correlation with each other. ROS and MDA change of hemolymph and CAT and AChE of hepatopancreas were the variables contributing most to the separation observed between the two species (Fig. 6b, Table S2).

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4. Conclusions

The present study provided deeper insight into the differential AgNPs bioaccumulation and immunological and molecular responses of two freshwater gastropods, L. stagnalis and P. corneus, using AgNPs as model NPs. The fitness of these two freshwater gastropods was investigated concerning bioaccumulation, antioxidant defence and immunity. As a general trend in both species, Ag bioaccumulation was augmented with an increase of exposure concentration. However, L. stagnalis accumulated higher Ag levels than P. corneus, which was thought to be due to differential physiological species traits. The hepatopancreas and stomach showed a significant increase of accumulation from 50 μg/L AgNPs on, which was not the case in the other studied tissues (kidney, mantle and foot). As for the biological response, the hemolymph of L. stagnalis and P. corneus are more susceptible to AgNP exposure-induced oxidative stress compared to the hepatopancreas. Although no lipid damage was found in the hepatopancreas, neurotoxicity was observed in this tissue. Furthermore, although the effects produced by Ag+ should not be underestimated, the Ag particulate form better explained the biological responses, indicating that the NPs themselves play a significant role in exerting toxicity. The results provide valuable information for the toxicity of NP toxicity on freshwater gastropods, demonstrating that bioaccumulation and the underlying mechanisms of NPs vary depending on the species’ sensitivity related to physiological traits. This work also highlights that further studies are needed to better understand the species-specific stress responses.

Credit author statement

Ting Wang: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Visualization. Pierre Marle: Conceptualization, Validation, Formal analysis, Investigation, Writing - review & editing. Vera I. Slaveykova: Review & Editing. Kristin Schirmer: Investigation, Review & Editing. Wei Liu: Conceptualization, Validation, Formal analysis, Investigation, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

Wei Liu acknowledges the financial support from the Swiss National...
Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2022.119999.

References

All, D., 2014. Oxidative stress-mediated apoptosis and genotoxicity induced by silver nanoparticles in freshwater snail Lymnaea luteola L. Biol. Trace Elem. Res. 162, 333–341.

Ali, D., Ali, H., Alarifi, S., Kumar, S., Serajuddin, M., Manthi, A.P., Ahmed, M., Khan, M., Aedl, S.F., Shaik, M., 2015. Impairment of DNA in a freshwater gastropod (Lymnaea stagnalis) after exposure to titanium dioxide nanoparticles. Arch. Environ. Contam. Toxicol. 68 (3), 543–552.

Amortín, J., Abreu, I., Rodrigues, L., Pinheiro, C., Saraiva, A., Carvalho, A.P., Pinheiro, C., Saraiva, A., Carvalho, A.P., 2018. Tissue distribution of Ag and Au nanoparticles in freshwater snail Biomphalaria glabrata. Arch. Environ. Contam. Toxicol. 67, 192–203.

Fu, Q., Pedrizzi, D., Konfeld, V., Schlechtriem, C., Ganz, V., Derrc, S., Rentch, D., Hollender, J., 2020. Biotransformation changes bioaccumulation and toxicity of diclofenac in aquatic organisms. Environ. Sci. Technol. 54, 4400–4408.

Cao, Y., Wu, W., Qiao, K., Feng, H., Zhu, Z., X. 2021. Bioavailability and toxicity of silver nanoparticles: determination based on toxicokinetic–toxicodynamic processes. Water Res. 204, 117603.

Garate, O.F., Gazaniga, S., Cochon, A.C., 2020. A comparative study of enzymatic and immunological parameters in Planorbis corneus and Biomphalaria glabrata exposed to the organophosphate chlorpyrifos. Aquat. Toxicol. 225, 105544.

Garner, K.L., Qin, Y., Cucurachi, S., Suh, S., Keller, A.A., 2018. Linking exposure and kinetic bioaccumulation models for metallic engineered nanomaterials in freshwater ecosystems. ACS Sustain. Chem. Eng. 6, 12684–12694.

Girardello, F., Leite, C.C., Branco, C.S., Roche, E., Mendes, F., Salvador, M., Henriques, J.P., 2016. Antioxidant defences and haemocyte internalization in Lymnaea stagnalis exposed to TiO2 nanoparticles. Aquat. Toxicol. 176, 190–196.

Herbert, L.T., Gosni, F.P., Paini, C.M., Luquet, C.M., Kristoff, G., 2021. Acute neurotoxicity evaluation of two anticholinesterase insecticides, independently and in mixtures, and a neonicotinoid on a freshwater gastropod. Chemosphere 265, 121071.

Hu, Y., Chen, X., Yang, X., K., H., 2018. Distinct toxicity of silver nanoparticles and silver nitrate to Daphnia magna in M4 medium and surface water. Sci. Total Environ. 618, 838–846.

Iwanga, S., Lee, B.-L., 2005. Recent advances in the innate immunity of invertebrate animals. MBM Reports 3, 109–135.

Kascbo, B.Z., Schlenke, T.A., 2012. High moisture load is associated with increased resistance against paraoxon in Drosophila suzukii, a relative of D. melanogaster. Philos. Trans. B. 367, 3471–3482.

Kaes, M., Pinzet, B., Zuleeg, S., Hagendorfer, H., Mueller, E., Vonbank, R., Roll, M., Burkhardt, M., 2010. Release of silver nanoparticles from outdoor facades. Envir. Pollut. 158, 2900–2905.

Kalogianni, M., Dimitriadis, A., Oxevik, M., Stamouloupolos, D., Feidantis, K., Kastrinakis, G., Gallyon, G., Tsiatoxiou, I., Koumoudouros, G., Bobori, D., 2020. Magnetic nanoparticles effects on adverse responses of aquatic and terrestrial animal models. J. Hazard Mater. 383, 121204.

Khan, F.R., Misra, S.K., Bury, N.R., Smith, B.D., Rainbow, P.S., Luoma, S.N., Valamis-Jones, E., 2015. Inhibition of potential uptake pathways for silver nanoparticles in the estuarine snail Peringia ulvae. Nano Letters 9, 493–501.

Kuehr, S., K., Solberg, A., Schlechtriem, C., 2021. Bioaccumulation assessment of nanomaterials using freshwater invertebrates. Sci. Total Environ. 780, 1–16.

Legiere, C.K., Sijm, D.T., van Leeuwen, C.J., Seinen, W., Hermens, J.L., 1998. Biocinoculation kinetics of chlorobenzene and the organophosphorus pesticide chlordrin in the pond snail Lymnaea stagnalis—a comparison with the guppy Poecilia reticulata. Aquat. Toxicol. 46, 27–32.

Lekangke, S., Ball, A.S., Shukla, R., Nugegoda, D., 2018. The toxicity of nanoparticles to aquatic invertebrates. Rev. Environ. Contam. Toxicol. 248, 1–80.

Liu, X., Chaix, A., Gary-Bobo, M., Angelletti, B., Manion, D., Da Silva, A., Daurat, M., Liobon, L., Garcia, M., Morire, A., 2017. Stealth biocompatible Si-based nanoparticles for biomedical applications. Nanomaterials 7, 288.

Livingstone, D., 1998. The fate of organic xenobiotics in aquatic ecosystems: quantitative and qualitative differences in biotransformation by invertebrates and fish. Comp. Biochem. Physiol. Mol. Integr. Physiol. 120, 499–511.

Luoma, S.N., Rainbow, P.S., 2008. In: Metal Contamination in Aquatic Environments: Science and Lateral Management. Cambridge university press, New York.

Ma, T., Gong, S., Tian, B., 2017. Effects of sediment-associated CuO nanoparticles on Cu bioaccumulation and oxidative stress responses in freshwater snail Bellamya aeruginosa. Sci. Total Environ. 580, 790–804.

Meshcheryakov, V., 1990. The Common Pond Snail Lymnaea stagnalis, Animal Species for Developmental Studies. Springer, pp. 69–80.

Moon, M.N., Readman, J.W., Rice, J.E., Low, D.M., Frickes, P.E., Bensley, A., 2009. Lysosomal cytotoxicity of carbon nanoparticles in cells of the molluscan immune system: an in vitro study. Nano Letters 9, 40–45.

Najimi, S., Bouhaimi, A., Daubeze, M., Zekhnini, A., Pellerin, J., Narbonne, J., 2011a. In: Anticholinesterase insecticides in Safflower and Mytilus galloprovincialis as a biomonitor of pollution in agadir marine bay (south of Morocco). Bull. Environ. Contam. Toxicol. 85, 901–908.

Nagoret, P., Pouygre, L., 2019. Operant Learning in Invertebrates. Reference Module in Life Sciences.

Perez, D.G., Fontanetti, C.S., 2011. Hemisomatic responses to invertebrates: a review. Environ. Monit. Assess. 177, 433–479.

Prather, C.M., Pelini, S.L., Dewhurst, W.M., Blech, C.P., Del Toro, I., Ho, C.K., Kominoski, J., Newbold, T.S., 2013. Invertebrates, ecosystem services and climate change. Biol. Rev. 88, 327–348.
Ramskov, T., Selck, H., Banta, G., Misra, S.K., Berhamu, D., Valsami-Jones, E., Forbes, V. E., 2014. Bioaccumulation and effects of different-shaped copper oxide nanoparticles in the deposit-feeding snail Potamopyrgus antipodarum. Environ. Toxicol. Chem. 33, 1976–1987.

Rawi, S., El-Gindy, H., Haggag, A., Abou El Hassan, A., Abdel Kader, A., 1995. Few possible molluscicides from calendula Micrantha officinalis and Ammi majus plants. 1. Physiological effect on B. alexandrina and B. truncatus. J. Egypt.-German Soc. Zool. 16, 69–75.

Robinson, W., Ryan, D., 1988. Transport of cadmium and other metals in the blood of the bivalve mollusc Mercenaria mercenaria. Mar. Biol. 97, 101–109.

Rösszer, T., 2014. The invertebrate midintestinal gland ("hepatopancreas") is an evolutionary forerunner in the integration of immunity and metabolism. Cell Tissue Res. 358, 685–695.

Ruppert, E.E., Fox, R.S., Barnes, R.D., 2004. Invertebrate Zoology: a Functional Evolutionary Approach. Brooks/Cole Publishing Company.

Schols, R., Carolus, H., Hammoud, C., Muzarabani, K.C., Barson, M., Huyse, T., 2021. Invasive snails, parasite spillback, and potential parasite spillover drive parasitic diseases of Hippopotamus amphibius in artificial lakes of Zimbabwe. BMC Biol. 19, 1–21.

Sendra, M., Saco, A., Yeste, M.P., Romero, A., Novoa, B., Figueras, A., 2020. Nanoplastics: from tissue accumulation to cell translocation into Mytilus galloprovincialis hemocytes. resilience of immune cells exposed to nanoplastics and nanoplastics plus Vibrio splendidus combination. J. Hazard Mater. 388, 121788.

Shao, Z., Guagliardo, P., Jiang, H., Wang, W.-X., 2020. Intra-and intercellular silver nanoparticle translocation and transformation in oyster gill filaments: coupling nanoscale secondary ion mass spectrometry and dual stable isotope tracing study. Environ. Sci. Technol. 55, 433–446.

Shen, M.-H., Zhou, X.-X., Yang, X.-Y., Chao, J.-B., Liu, R., Liu, J.-F., 2015. Exposure medium: key in identifying free Ag⁺ as the exclusive species of silver nanoparticles with acute toxicity to Daphnia magna. Sci. Rep. 5, 1–9.

Sidropoulou, E., Feidantitis, K., Kalogiannis, S., Gallios, G.P., Kastrinaki, G., Papaoanou, E., Václavíková, M., Kaloyianni, M., 2018. Insights into the toxicity of iron oxides nanoparticles in land snails. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 206, 1–10.

Sikder, M., Eudy, E., Chandler, G.T., Baloucha, M., 2018. Comparative study of dissolved and nanoparticulate Ag effects on the life cycle of an estuarine mesiobenthic copepod, Amphiascus tenureum. Nanotoxicology 12, 375–389.

Silva, P.V., Pinheiro, C., Morgado, R.G., Verweij, R.A., van Gestel, C.A., Loureiro, S., 2022. Bioaccumulation but no biomagnification of silver sulphide nanoparticles in freshwater snails and planarians. Sci. Total Environ. 808, 151966.

The Nanodatabase, 2022. https://nanodb.dk/en/.

Ulm, L., Krivohlavek, A., Jurasin, D., Ljubojevic, M., Sinko, G., Crnkovic, T., Zuntar, I., Sikic, S., Vinkovic Vrecelj, I., 2015. Response of biochemical biomarkers in the aquatic crustacean Daphnia magna exposed to silver nanoparticles. Environ. Sci. Pollut. Control Ser. 22, 19990–19999.

Walters, C.R., Pool, E.J., Somerset, V.S., 2014. Ecotoxicity of silver nanomaterials in the aquatic environment: a review of literature and gaps in nano-toxicological research. J. Environ. Sci. Health, Part A 49, 1588–1601.

Wang, T., Liu, W., 2022. Emerging investigator series: metal nanoparticles in freshwater: transformation, bioavailability and effects on invertebrates. Environ. Sci. J. Integr. Environ. Res.: Nano 9, 2257–2265.

Xu, M., Yang, Q., Xu, L., Rao, Z., Cao, D., Gao, M., Liu, S., 2019. Protein target identification and toxicological mechanism investigation of silver nanoparticles-induced hepatotoxicity by integrating proteomic and metabolic strategies. Part. Fibre Toxicol. 16, 1–14.

Yan, N., Tang, B.Z., Wang, W.-X., 2021. Intracellular trafficking of silver nanoparticles and silver ions determined their specific mitotoxicity to the zebrafish cell line. Environ. Sci. J. Integr. Environ. Res.: Nano 8, 1364–1375.

Zhao, J., Lin, M., Wang, Z., Cao, X., Xing, B., 2021. Engineered nanomaterials in the environment: are they safe? Crit. Rev. Environ. Sci. Technol. 51, 1443–1478.