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Effects of silver sulfide nanoparticles on the earthworm *Eisenia andrei*

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ABSTRACT

The massive production and use of silver nanoparticles (Ag NPs) have led to their increasing release into the environment. Even though the antimicrobial and cytotoxic effects of native nanoparticles have been well studied, the environmental impacts of transformation products such as silver sulfide nanoparticles (Ag_2S NPs) have not been elucidated. In the present study, we assessed the toxicity of Ag_2S NPs and silver nitrate ($AgNO_3$), as a source of Ag, to the earthworm *Eisenia andrei* using a nominal concentration of 5 mg Ag kg^{-1} soil. We used the OECD guidelines to assess effects on weight loss and mortality for 14 days. After exposure, we also extracted the immune effector cells (coelomocytes) and conducted a battery of biomarker tests. To ensure the quality of the toxicological results, the structural changes of NPs during the experiment and the uptake of silver by the earthworms were monitored. During the experiment, mortality effects were not detected, but a weight loss was observed in the earthworms exposed to Ag_2S NPs. Altough Ag_2S NPs were engulfed by *E. andrei* cells, neither phenoloxidase activity nor lipid peroxidation differed from the untreated control group. Cells from earthworms treated with Ag_2S NPs exerted very broad value range of nitric oxide (NO) generation, suggesting an imbalance in the NO metabolism. Overall, this study suggests minimal risks associated with Ag_2S NPs exposure to earthworms. However, further studies are needed to assure no immunotoxicological or chronic effects on a wider range of terrestrial organisms.

1. Introduction

During the last two decades, the production of engineered nanomaterials (ENMs) has increased globally (Gharailou, 2018). From the different available ENMs, silver nanoparticles (Ag NPs) have been one of the most produced. Due to their very strong antimicrobial/antibacterial effects, Ag NPs are currently used in many industries, and their production increases together with the growth of the global market (Gharailou, 2018). The massive production of Ag NPs has led to the increasing emission of these anthropogenic particles into the environment, where they could affect a wide range of living organisms. For instance, Ag NPs are toxic to aquatic organisms such as crustaceans or fish (Bondarenko et al., 2013; Mohsenpour et al., 2020), and produce cytotoxicity in human cell lines (Tortella et al., 2020).

One of the main environmental pathways of Ag NPs is wastewater treatment plants (WWTPs). Kaegi et al. showed that Ag NPs could be

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transformed into silver sulfide nanoparticles (Ag_2S NPs) in non-aerated WWTP tanks in less than 2 h (Kaegi et al., 2011). This transformation is size-dependent so that large NPs (100 nm) are not strongly affected (approximately 90% of Ag NPs remain in their metallic form), while smaller NPs (10 nm) undergo an almost complete transformation – sulfidation (95%) (Kaegi et al., 2013). A large amount of NPs is retained during wastewater treatment in the sewage sludge (Blaser et al., 2008). Nowadays, about 35% of the sewage sludge produced in the European Union is used as agricultural fertilizer (Eurostat, 2021), so there is a risk that NPs and their transformation products reach soil ecosystems. Only a relatively small part (2–3%) of Ag NPs released into soil ecosystems via sludge applications are known to have their native form, while the vast majority (78–86%) are introduced as Ag_2S NPs (Adam et al., 2018). Moreover, some studies have shown that Ag_2S NPs can be retained in soil ecosystems for longer periods than Ag NPs due to their lower water solubility and the little reactivity with soil (Li et al., 2019; Navarro et al., 2014). Therefore, studies aimed at assessing the ecotoxicological risks of Ag_2S NPs and other transformation products to plants and soil fauna are needed.

Several studies have described the phytotoxic potential of Ag_2S NPs in the plant system. These studies indicated root uptake and translocation to leaves, which ultimately caused a reduction in the shoot length and root biomass (Wang et al., 2015, 2017). A slow-release mechanism has also been described, in which Ag_2S NPs accumulated in plant tissues slowly released Ag^+ ions, causing secondary toxicity (Wang et al., 2015, 2017). According to Baccaro et al. (2018) and Courtois et al. (2020), Ag NPs can be ingested by earthworms and absorbed in their gut, causing potential toxicity. Moreover, these authors suggested that earthworms could biotransform Ag NPs into Ag_2S NPs (Baccaro et al., 2018; Courtois et al., 2020).

Earthworms' body cavity is filled with coelomic fluid containing several types of immune cells, called coelomocytes, which differ in morphology and function. There are two main coelomocyte types – eleocytes having mainly nutritive function and producing antimicrobial factors, and amoebocytes (hyaline and granular) representing effectory immunocytes (Engelmann et al., 2016). Amoebocytes provide information about the immune capacity of the earthworms exposed to different pollutants, including ENMs (Boraschi et al., 2020). Coelomocytes have recently been used to evaluate sublethal effects and immunotoxicity on earthworms exposed to several types of NPs (e.g., TiO_2 NPs, Ag NPs, and CuO NPs; (Boraschi et al., 2020; Gautam et al., 2018; Hayashi et al., 2012; Navarro Pacheco et al., 2021a). In these studies, oxidative stress markers (i.e., reactive oxidative species production, lipid peroxidation), gene expression of molecular biomarkers, and DNA damage have been assessed, showing that immunocompetent cells can be successfully used as markers of exposure and effects. However, to date, a little number of studies have used these immunocompetent cells to assess the toxicity of NP transformation products.

This study aimed to expand our knowledge on the toxicity of Ag NP transformation products on soil organisms. For this, we assessed the effects of Ag_2S NPs, as environmentally relevant transformation products of Ag NPs, on the earthworm *Eisenia andrei (E.andrei)*. We exposed the earthworms to Ag_2S NPs (5 mg Ag kg^{-1} dry soil) for 14 days following the OECD standard protocol (OECD, 1984). Several classical ecotoxicological parameters were determined (e.g., weight loss, mortality) as well as Ag uptake by the earthworms and structural changes of the test NPs. Moreover, cytotoxicity was assessed by staining of extracted coelomocytes with trypan blue. Then, cellular viability, nitric oxide (NO) production, phenoloxidase (PO) activity, and oxidative stress were analyzed. Our study contributes to increase our understanding on the toxicity mechanisms of Ag NP transformation products on terrestrial organisms and to characterize their potential environmental risks.

2. Material and methods

2.1. Chemical and reagents

For soil exposure, LUFA soil 2.1 was purchased from LUFA Speyer (Speyer, Germany). 20.4 ± 11.9 nm size distribution of Ag_2S NPs colloids stabilized in 1 mg mL^{-1} 55 kDa Polyvinylpyrrolidone (PVP) aqueous media were obtained from Applied Nanoparticles S.L. (Barcelona, Spain). Silver nitrate ($AgNO_3$), 0.4% trypan blue solution, nitrate standards ($NaNO_2$), sulphanilamide, phosphoric acid, L-Arginine, *N*-alpha-naphthyl-ethylenediamine, and dopamine hydrochloride were purchased from Sigma Aldrich (Steinheim, Germany). HCl and HNO_3 used for the extraction and quantification of silver were purchased from Penta chemicals (Prague, Czech Republic). The Ag ICP standards were obtained from Merck (Darmstadt, Germany). For coelomic fluid collection, 5 μL capillary Brand™ micropipettes (Sigma Aldrich; Wertheim, Germany) were used. Sodium dodecyl sulfate (SDS) was purchased from Serva (Heidelberg, Germany).

2.2. Experimental design

E. andrei earthworms were used as model organisms in this study. Adult earthworms with clitellum were placed into the prepared high-density polyethylene (HDPE) containers with a volume of 1.5 L. Ten earthworms (300 mg body weight) were used per 750 g soil according to OECD guideline 207 (OECD, 1984). A total number of 60 earthworms per treatment were collected for the study.

The artificial soil was prepared following the OECD guideline 207 (OECD, 1984) with slight modifications. LUFA 2.1 soil was used as the standard soil for the preparation of the experimental assay. Dried LUFA 2.1 soil was placed in the containers and moistened to 50% of its water holding capacity with suspensions of Ag_2S NPs and a solution of $AgNO_3$ in distilled water. $AgNO_3$ was used as a control of Ag^+ ions effects. Thus, this ion control ($AgNO_3$) aided to determine whether the observed effects were derived from the NPs themselves or by the ions released. Control groups were moistened with distilled water. The nominal concentration of 5 mg Ag kg^{-1} dry soil was chosen for Ag_2S NPs and $AgNO_3$ to reach sublethal effects in the earthworms. According to Peixoto et al. (2020) and Starnes et al. (2015), similar concentrations of Ag_2S NPs have shown significant effects in the soil bacterial communities and increased mortality in *Caenorhabditis elegans*, respectively. After spiking the respective Ag forms used in the experiment, the soils were thoroughly mixed to achieve a homogenous distribution of the different suspensions of Ag_2S NPs and $AgNO_3$. The earthworms were kept in a container of LUFA 2.1 soil for 24 h before their introduction into the experimental soils for acclimatization. Afterward, the different experimental soils (control group, Ag_2S NPs, and $AgNO_3$) were kept in darkness at 20 °C for 14 days.

2.3. Weight loss and mortality

Fourteen days after the start of the exposure period, earthworm mortality and weight loss were assessed according to the OECD 207 guideline (OECD, 1984). Immobilization of the animal, and a dead body found in the container were considered as mortality.

2.4. Characterization of NPs before and after exposure

Ag_2S NPs were synthesized and characterized by Applied

Nanoparticles (Barcelona, Spain). According to the manufacturer, images of freshly prepared NPs were acquired by JEOL1010 transmission electron microscope (TEM; JEOL, Tokyo, Japan) at 80 keV. Then, formvar coated and carbon-stabilized 200-mesh copper grids (Ted-pella Inc., Redding, CA, USA) were immersed in Ag_2S stock solution aliquots diluted 1:10 with Milli-Q water. Then, the samples were left to air-dry for a minimum of 12 h. The ImageJ software (NIH, Bethesda, Maryland, USA) was used for calculating the mean size and size distribution from the acquired TEM images. Dynamic Light Scattering (DLS) and Zeta Potential (ζ-Potential) on a Malvern Zetasizer Nano ZS90 analyzer (Malvern Instruments Ltd., Worcestershire, UK) was used to characterize the colloidal properties of the Ag_2S NPs colloid as supplied, including hydrodynamic diameter, and surface charge. To comply with the technical experimental limits, samples were diluted at 1:5 with Milli-Q water. Agilent Cary 60 UV–Vis Spectrophotometer (with spectra limits in the range of 300 to 800 nm; Agilent Technologies Inc., Santa Clara, CA, USA) was used to measure the UV–Vis absorption spectra of three NP colloids. The spectra analysis was performed after the dilution of the Ag_2S NP colloid at 1:10 with Milli-Q water. The identification of Ag_2S nanoparticles/aggregates in soil samples was performed using a field-emission scanning electron microscope (FE-SEM) Hitachi SU6600 (Hitachi High-Tech Corporation, Tokyo, Japan) with ultrahigh point-to-point resolution (1–2 nm) in combination with elemental mapping using integrated energy-dispersive X-ray (EDX) spectroscopy.

2.5. Extraction and quantification of silver in soil and worm tissue

Prior to further analyses, the samples of soil and earthworms were lyophilized and thoroughly homogenized. The extraction of total silver was performed using microwave-assisted acid digestion according to a recently published protocol (Baccaro et al., 2018). Briefly, 0.3 g of the soil or worm sample was transferred into a polytetrafluoroethylene vessel and was supplemented by 8 mL of an acid mixture (3:1, 37% HCl: 69% HNO_3, v:v). The samples prepared in the vessels were subsequently digested in a microwave system (MARS 5, CEM Corporation, USA) using a temperature ramp from 160 °C (20 min) to 200 °C (40 min). An inductively coupled plasma optical emission spectrophotometer (ICP-OES, 5110 Series, Agilent Technologies, Santa Clara, CA, USA) was used to determine the concentration of dissolved silver in the cultivation medium. Samples were centrifuged four times (18,400 *g*; 4 °C, 10 min) to sediment undissolved impurities. The supernatants were analyzed by ICP-OES to determine the Ag concentration. Soluble Ag concentration was determined in parallel also for a solution of $AgNO_3$ (1, 10, and 100 μg mL^{-1} of Ag) in 3% HNO_3. Metal detection and quantification in the samples were carried out in triplicate. Instrumental errors were less than 3%.

2.6. Extrusion of coelomic fluid

The extrusion of coelomocytes was performed by mild stimulation with electricity (9 V battery) after 14 days of exposure with Ag_2S NPs and $AgNO_3$. Firstly, before the stimulation, the earthworms were kept in sparkling water, and then they were carefully dried with tissue paper. Afterward, the earthworms were placed in a Petri dish and they were electrically stimulated to obtain coelomic fluid and coelomocytes. The coelomic fluid expelled from earthworms was collected using 5 μL capillary Brand™ Blaubrand® micropipettes, transferred into Eppendorf tubes, and kept on ice prior to subsequent analyses (cell viability, nitric oxide generation, and phenoloxidase activity).

2.6.1. Cell viability

For cell viability determination after the exposure, the coelomocytes were extracted from 15 animals of each treatment (control group, Ag_2S NPs, and $AgNO_3$) and stained 1:1 (v:v) by 0.4% trypan blue solution. A hemocytometer (Bürker chamber, The Paul Marienfeld GmbH & Co, Lauda-Königshofen, Germany) was used to count the cells and each sample was analyzed in two technical replicates. Based on the number of stained (dead) cells and the total cell count, the viability (%) was determined using the following equation:

$$Viability\ (\%) = [1 - (Dead\ cells/Total\ number\ of\ cells)\,]\ x\ 100$$

2.6.2. Nitric oxide (NO) generation

Nitric oxide generation was assessed using the colorimetric reaction between NO generated by coelomocytes with Griess reagent. After 14 days of exposure, the coelomic fluid of the exposed and control earthworms was collected as described above, and a calibration curve was prepared using nitrate standards (0–200 μM $NaNO_2$). A mixed solution containing 1% sulphanilamide, 5% phosphoric acid, and 1 mM L-Arginine was prepared, and an aliquot of 28 μL was added to 60 μL of the sample (coelomic fluid) or nitrate standard on ice. The samples were centrifuged (14,000 *g*; 10 min, 4 °C), and 20 μL of the supernatant was transferred to a 384-well plate. Afterward, 20 μL of 1% *N*-alpha-naphthyl-ethylenediamine was added to the samples. The plate was shaken for 5 min before absorbance measurement at 540 nm. A blank (distilled water) was included in each analysis. Ten samples per each treatment and duplicates of the standards were analyzed.

2.6.3. Phenoloxidase activity

After the exposure of cells to Ag_2S NPs and $AgNO_3$, phenoloxidase activity in extracted coelomocytes was assessed. Firstly, an aliquot of 200 μL of 2 mM SDS and 4 mM dopamine hydrochloride in phosphate-buffered saline (PBS) was mixed with 10 μL of coelomic fluid. Then, 40 μL of the mixed suspension was transferred into a 384-well plate, shaken for 10 s, and the absorbance was measured every 2 min for 8 h (490 nm, TECAN Infinite M200 Pro; Männedorf, Switzerland). The samples (15 for each treatment – control group, Ag_2S NPs, and $AgNO_3$) were analyzed in quadruplicate.

2.7. Malondialdehyde production

The concentration of malondialdehyde (MDA) in earthworm tissue was determined by high-performance liquid chromatography with fluorescence detection (HPLC-FLD) using a derivatized complex with thiobarbituric acid according to a previously optimized protocol for toxicity evaluation of iron NPs towards bacteria species and earthworms (Semerad et al., 2020; Semerád et al., 2018). Sample preparation was modified slightly for the earthworm tissue according to the following steps. Briefly, earthworms washed previously with distilled water and stored at −20 °C were thawed, transferred into 1 mL of PBS buffer with 0.5 g of glass beads, and vigorously shaken by a high-throughput cell homogenizer MP FastPrep-24 (MP Biomedicals, Santa Ana, CA, USA) set at 5.5 m s^{-1} for one minute. Then, 0.5 mL of the tissue homogenate was used for MDA extraction, derivatization, and analysis according to the aforementioned publication.

2.8. Data presentation and statistical analyses

One-way ANOVA with Bonferroni's post-test was carried out to assess significant effects of the sampling time, and the treatments on coelomocytes viability, nitric oxide generation, phenoloxidase activity, and

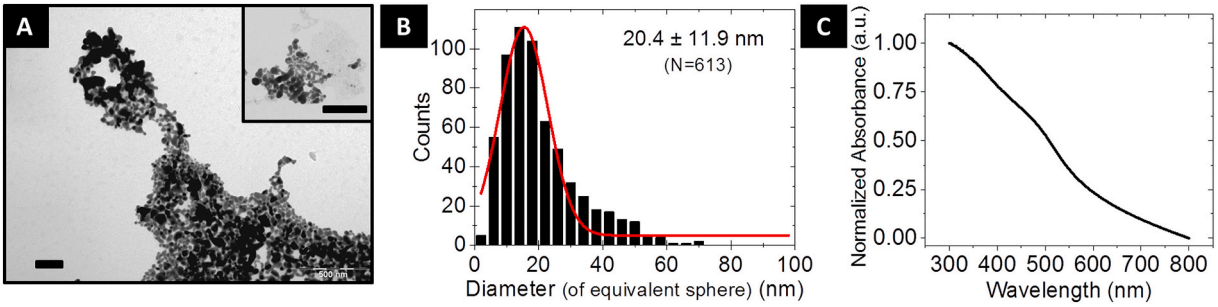

Fig. 1. Characterization data of the Ag_2S NP colloids used in this study. A) shows a low-magnification TEM image with a higher-magnification top-right inset with the scale bar equaling 200 nm; B) shows their corresponding size distribution by analysis of the TEM images (20.4 ± 11.9 nm; 613 NPs analyzed); C) shows their normalized UV/Vis spectra in colloidal dispersion in Milli-Q water.

weight loss. Lipid peroxidation did not follow the parametric assumptions of ANOVA, and was analyzed with the non-parametric Kruskal-Wallis test followed by the Dunn's multiple comparison test. All statistical analyses were performed using the GraphPad Prism software (8.4.3 version, San Diego, CA,USA) or OriginLab software 2019b (Northampton, MA, USA). Statistically significant differences were assumed when the calculated *p*-value was <0.05.

3. Results

3.1. Characterization of NPs

According to the TEM image (Fig. 1A), freshly prepared Ag_2S NPs have a rounded crystal shape, and the formed colloid shows some degree of agglomeration; however, this is common in metal oxides and metal sulfide NPs. The size of Ag_2S NPs was determined immediately after the synthesis by the ImageJ software through the acquired images JEOL1010 transmission electron microscope (JEOL, Japan) working at 80 keV and reached values of 20.4 ± 11.9 nm (Fig. 1B). Ag_2S NPs were supplied in 1 mg mL^{-1} 55 kDa PVP aqueous media to minimize NPs agglomeration and to infer high colloidal stability. To ensure the exposure of earthworms to unchanged NPs, the characterization was performed again, just before the *in vivo* experiment. Using UV/Vis spectra analyses by Nanodrop 2000c Spectrophotometer (Thermo Scientific™, V1.0, Willmington, DE, USA), the patterns obtained (data not shown) were similar to the manufacturer ones (Fig. 1C), indicating the

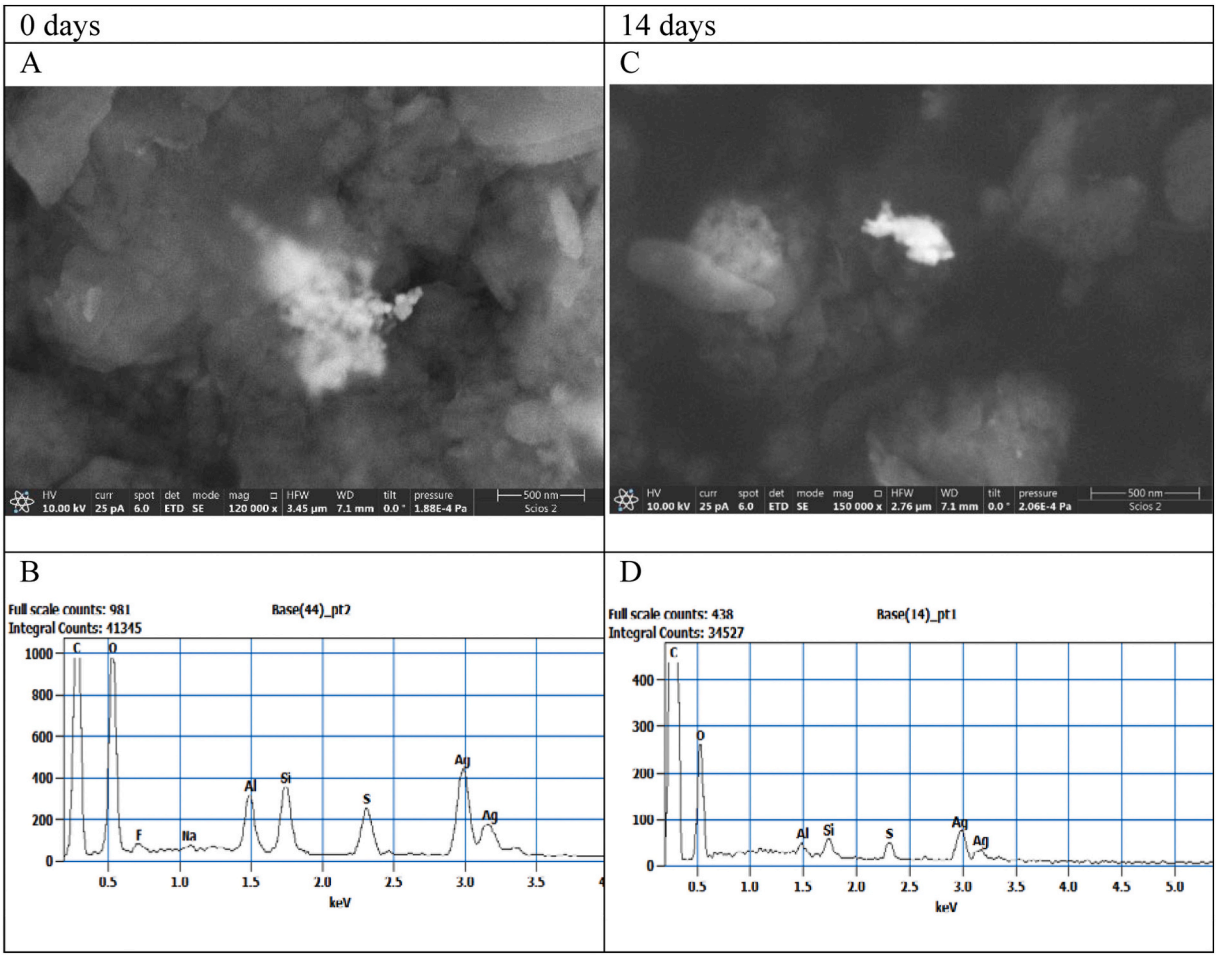

Fig. 2. SEM images (A, C) and EDS spectra (B, D) of Ag_2S NPs before, and after 14-days of exposure, respectively.

stability of the studied NPs. In other words, the nanoparticles did not undergo any changes prior to the study. A more detailed characterization of the tested NPs was provided by the study by Baccaro et al. (2018), where the same material and the same batch were used.

For understanding the fate and the possible mechanisms behind the potential adverse effects of Ag_2S NPs, soil samples collected at 0 and 14 days were analyzed by scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS). Samples from the beginning of the exposure (Fig. 2A) were obtained, and an EDS spectra analysis was performed to detect elemental S and Ag from the Ag_2S NPs (Fig. 2B). After 14 days of exposure with the earthworms, Ag_2S NPs were still visible in the SEM image and detected by the EDS analysis (Fig. 2C, and D).

After the confirmation of the nano-size and stability of NPs in soil, the following step to ensure the quality of the toxicological results was to determine the real exposure concentrations of Ag_2S NPs/$AgNO_3$. Therefore, the quantification of total Ag^+ ions by ICP-OES was performed. As shown in Table 1, the measured concentrations of Ag^+ ions in the experimental soil were similar to the nominal concentration.

3.2. Uptake of Ag_2S NPs by earthworms

To evaluate the uptake of silver in the form of Ag_2S NPs and $AgNO_3$ by the earthworms, 3 animals from the different treatments were homogenized, and their Ag content was analyzed. Both treatments resulted in an uptake of Ag in the earthworm bodies, 6.4 ± 0.3 and 10.8 ± 3.0 mg Ag kg^{-1} dry bodyweight for Ag_2S NPs, and $AgNO_3$, respectively (Table 2).

3.3. Weight loss and mortality

No dead body or immobilized animal was found after 14 days of exposure. The weight of earthworms in the control group increased significantly over the 14 days of the experiment (336.6 mg at $t = 0$ and 357.5 mg at $t = 14$ days). The weight of earthworms exposed to Ag_2S NPs decreased over the 14 days (327.5 mg at t = 0, and 311.6 at t = 14 days), and remained unchanged after exposure to $AgNO_3$ (341.8 mg at t = 0, and 341.4 at t = 14 days). The comparison of the treatments on day 14 showed a significant effect of the Ag_2S NPs on the weight of the earthworms as compared to the controls, and the $AgNO_3$ treatment (Fig. 3).

Table 1
Concentrations of Ag^+ ions detected in the soil at the start (0 day), and at the end (14 days) of the experiment. Values show the mean ± SD; n = 3.

Time exposure Treatment	Soil (mg Ag kg^{-1})	
	0 day	14 days
Control	0.0 ± 0.0	0.0 ± 0.0
Ag_2S NPs	6.6 ± 0.8	4.4 ± 0.5
$AgNO_3$	4.9 ± 0.4	6.4 ± 0.7

Table 2
Concentrations of Ag^+ ions measured in earthworms after 14 days of exposure to Ag_2S NPs, and $AgNO_3$. Values show the mean ± SD; n = 3.

Time exposure Treatment	Earthworm (mg Ag kg^{-1} dry weight)
	14 days
Control	0.1 ± 0.0
Ag_2S NPs	6.4 ± 0.3
$AgNO_3$	10.8 ± 3.2

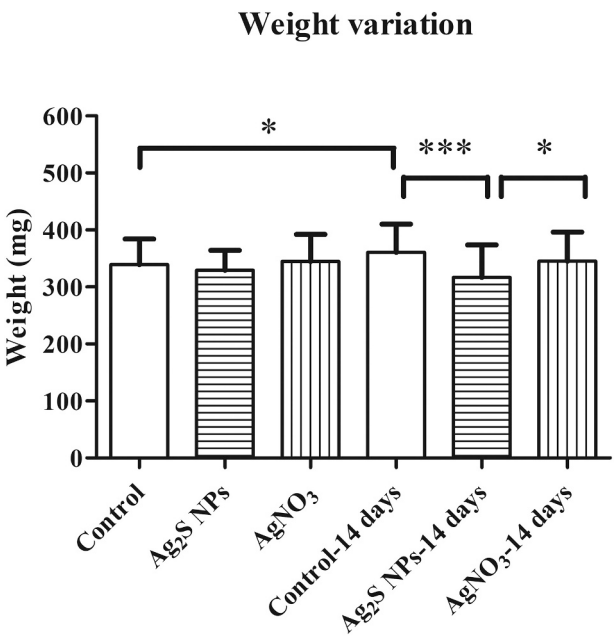

Fig. 3. Bodyweight loss. The body weight (mg) of control earthworms and earthworms exposed to 5 mg Ag kg^{-1} of Ag_2S NPs or $AgNO_3$ at day 0, and day 14. The results are expressed as the mean (mg) ± SD. *, *** indicates significant differences ($p < 0.05$, and $p < 0.001$ respectively, One-way ANOVA, and Bonferroni's multiple comparison test).

3.4. Coelomocytes viability, oxidative stress biomarkers, and phenoloxidase activity

After the exposure, the earthworms were collected, and the cell viability of the extracted coelomocytes was determined. From the provided results, it was clear that the coelomocytes viability was not affected by Ag_2S NPs, and $AgNO_3$, and a statistical difference was observed in $AgNO_3$ in comparison with the control ($p < 0.05$ according to one-way ANOVA, and Bonfferoni's multiple comparison test; Fig. 4A). The cell viability was 71.3 ± 13.9%, 76.7 ± 11.3%, and 78.5 ± 7.2% for earthworm coelomocytes in the control group, Ag_2S NPs, and $AgNO_3$, respectively.

No statistically significant differences ($p > 0.05$ according to one-way ANOVA, and Bonferroni's multiple comparison test) were observed in NO production between the coelomocytes from the earthworms in the control soil (32.9 ± 2.6 μM NO), and the earthworms exposed to Ag_2S NPs (28.4 ± 6.5 μM NO), and $AgNO_3$ (29.9 ± 3.1 μM NO; Fig. 4B). Although there was no increase in NO production, greater variances were observed in samples exposed to Ag_2S NPs (Fig. 4B), which could suggest an imbalance in NO metabolism. Phenoloxidase activity (%) of extruded coelomocytes reached 111.7 ± 42.4% in control earthworms,130.4 ± 38.1% in Ag_2S NPs, and 145.8 ± 65.4% in $AgNO_3$ treated earthworms (Fig. 4C). Similar to NO production and cell viability, no statistically significant differences ($p > 0.05$) between the different exposures were observed according to one-way ANOVA, and Bonferroni's multiple comparison test .

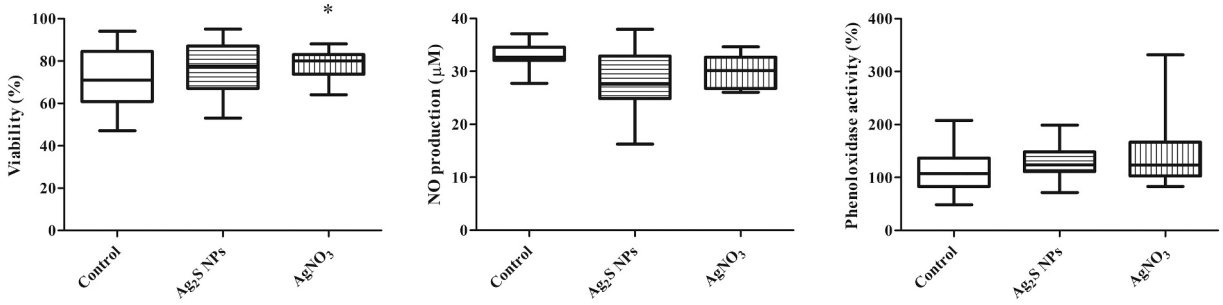

Fig. 4. Viability and functional assays of the control group and groups exposed to 5 mg Ag kg^{-1} soil of Ag_2S NPs or $AgNO_3$ after 14 days. A) Cell viability. B) Nitric oxide (μM; NO) generated by coelomocytes. C) Phenoloxidase activity (%) of coelomocytes. * indicates significant differences ($p < 0.05$, One-way ANOVA, and Bonferroni's multiple comparison test).

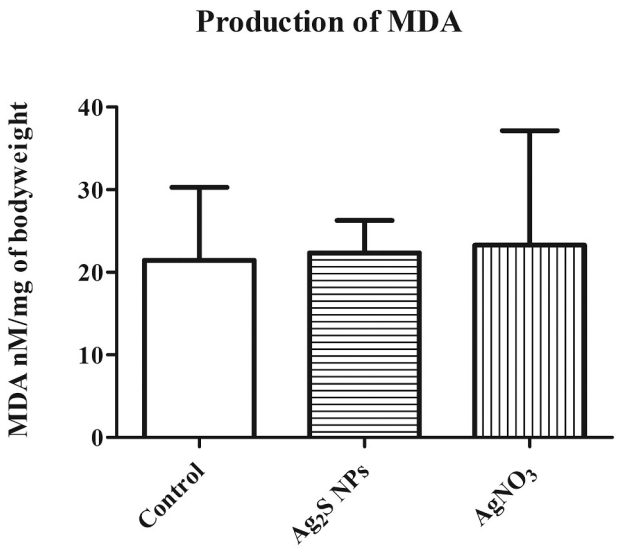

Fig. 5. Malondialdehyde (MDA) production. Malondialdehyde production (nM/mg body weight) of earthworms exposed to the control soil and 5 mg Ag kg^{-1} soil as Ag_2S NPs or $AgNO_3$ at the end of the experiment. Results are expressed as the mean ± SD. No significant differences were observed between the different treatments ($p > 0.05$, Kruskal-Wallis, and Dunn's post-test).

3.5. Lipid peroxidation

The control earthworms produced 21.4 ± 8.8 nM MDA, 22.4 ± 3.9 nM MDA when exposed to Ag_2S NPs, and 23.3 ± 13.9 nM MDA when exposed to $AgNO_3$. MDA determination showed no significant differences ($p > 0.05$) in the formation of MDA between the control and the treatments (Ag_2S NPs, and $AgNO_3$) according to the Kruskal-Wallis, and the Dunn's multiple comparison test (Fig. 5). The concentration of MDA was dependent on the bodyweight of the earthworms; therefore, the results were corrected accordingly.

4. Discussion

The studied Ag_2S NPs are formed in a relatively high quantity during wastewater treatment processes from its precursor, Ag NPs, and could pose a threat to soil organisms via sludge application in agriculture. Many factors are affecting the physicochemical properties, and structure of NPs, such as time, light intensity, temperature, matrix complexity, or the presence of oxygen or organic matter. These changes can affect the toxicity of the NPs. For that reason, the proper characterization of NPs before, and during the exposure is highly needed to accurately describe the state of the NPs, which will assure the robustness of the data and comparability between studies. First, the stability of Ag_2S NPs in the stock solution was assessed by a comparison of the UV/Vis spectra obtained immediately after the fabrication, and at the beginning of the experiment. The characterization showed the stability of the tested NPs, and confirmed the unchanged physicochemical properties as stated by the manufacturer.

The analysis of TEM images of the Ag_2S NPs revealed a mean size of 20.4 nm and a slight state of aggregation (Fig. 1A and B). It has already been shown that the increasing size of metal NPs decreases toxicity (Sukhanova et al., 2018). Moreover, the size of the NPs plays a crucial role in the engulfment of NPs by the cells. It was demonstrated that NPs having a size between 20, and 50 nm can be engulfed by the cells (epithelial cells of the gut, immune cells, etc.; Cornelis et al., 2014) and; therefore, we can hypothesize that the tested NPs would behave similarly. However, there is still a lack of knowledge of how the gut cells ingest the NPs in soil invertebrate organisms.

The bioavailability of NPs also depends on the various factors affecting their mobility. For example, in soil, elevated pH, ionic strength, or ubiquitous microbes could change the physicochemical properties and increase the mobility and solubility of NPs (Cornelis et al., 2014; Schultz et al., 2018). Moreover, earthworms can transform the NPs in their gut by ingesting the Ag NPs, and $AgNO_3$, and excreting Ag particulate forms of reduced size (Baccaro et al., 2018). Even dissolved organic matter (DOM) in the soil could accelerate the dissolution process of the NPs and increase the toxicity (Cornelis et al., 2014). The dissolution of metal NPs (e.g., CuO NPs, Ag NPs, ZnO NPs) is one of the most discussed effects in terms of possible adverse effects (Gupta et al., 2014; Mincarelli et al., 2016; Navarro Pacheco et al., 2021a). The dissolved ions leaching from metal nanoparticles exhibit strong toxicity, sometimes even higher than the parent NPs. Despite this fact, only a very limited part of studies have focused on the kinetics of NP dissolution. Concerning Ag_2S NPs, it has already been suggested that these transformation products are more stable and less soluble than their highly used precursor Ag NPs (Baccaro et al., 2018; Peixoto et al., 2020; Schultz et al., 2018), and most likely these NPs require more time to release the Ag^+ ions. In this way, Peixoto et al. (2020) suggested that long-term exposure to Ag_2S NPs could increase the potentially harmful effects due to Ag^+ ions release (Peixoto et al., 2020).

SEM analysis of the soil samples before and after exposure confirmed relatively high stability of Ag_2S NPs, retaining a similar size and shape. ICP analysis of the earthworms revealed Ag transfer from the soil to the exposed animals. It is worth noting that a trace amount of silver was also detected in control samples of soil and earthworms. However, earthworms exposed to Ag_2S NPs, and $AgNO_3$ contained higher quantities of Ag^+ ions in their bodies than what was detected in the soil after 14 days of exposure (6.4, and 10.8 mg Ag kg^{-1} dry weight, respectively), which suggests the accumulation of Ag (originated from NPs) in earthworms. These results are in accordance with a previous study where the authors observed that earthworms were able to engulf Ag NPs and Ag_2S NPs (Baccaro et al., 2018). The ingestion of various metal NPs, and connected intracellular ions release heighten the damages that produce

oxidative stress, genotoxicity, and cytotoxicity (Gupta et al., 2014; Hayashi et al., 2012; Navarro Pacheco et al., 2021a).

The observed lack of mortality in treated samples after 14 days of exposure was in accordance with Baccaro et al. (2018). Although mortality was not observed, only control earthworms gained weight during 14 days of experiment. In addition, the weight of earthworms exposed to Ag_2S NPs decreased over the 14 days (Fig. 3). The decrease in body weight has already been observed during *in vivo* experiments with metal NPs (e.g., CuO; Tatsi et al., 2018); however, other types of metal NPs, SnO_2 and CeO_2, did not exert any negative effects on body mass. A very probable explanation of the different results can be related to exposure conditions as well as variability caused by the heterogeneity of NPs (Carbone et al., 2016). This could also explain the difference between our results and the weight decrease of 15–17% reported by Curieses Silvana et al. (2017), when earthworms were exposed to Ag NPs and $AgNO_3$ (Curieses Silvana et al., 2017). However, in our study, the treatment with $AgNO_3$ did not affect the weight of earthworms during the exposure. $AgNO_3$ treatment was used to examine the effects of the ions, thus, we suggest that the weight loss would derive from the Ag_2S NPs themselves, and not the Ag^+ ions.

Focusing on the intracellular damage of coelomocytes, the viability, oxidative stress, and immunological parameters were studied. The results of viability assessment showed that the cells were in relatively good shape (Fig. 4A), reaching 71–78% in each treatment (control group, Ag_2S NPs, and $AgNO_3$). A previously published study showed that Ag NPs could decrease cell viability even after 24 h of exposure at the concentration of 0.05 mg kg^{-1} (Curieses Silvana et al., 2017). However, the effects of the most relevant transformation product (i.e., Ag_2S) have not yet been studied (Curieses Silvana et al., 2017). Other *in vitro* studies suggested that the influence of released ions enhances oxidative stress. If cells are not capable to eliminate oxidative stress, they are not viable (Gautam et al., 2018; Navarro Pacheco et al., 2021a). However, if NPs are not able to dissolve and release ions such as TiO_2 NPs, then the cells are viable (Navarro Pacheco et al., 2021b). In our study, the most probable explanation for the cell viability results is the low solubility of Ag_2S NPs, and the low release of Ag^+ ions (Fig. 4A).

Regarding oxidative stress, nitric oxide (NO) is not only a potent antimicrobial agent that is released by the cells when they encounter a pathogen-associated molecular pattern (PAMP), but it can also be generated during stress conditions. This parameter has been extensively studied in crustaceans, and insects because it is a more stable product than reactive oxygen species (ROS; (Swartzwelter et al., 2021). In this study, we observed that coelomocytes exposed to the different treatments (control group, Ag_2S NPs, $AgNO_3$) did not increase nor reduce NO production, although potential NO imbalance was observed in Ag_2S NPs due to the high variances (Fig. 4B). In other studies, the depletion of NO production was observed on coelomocytes from *Metaphire posthuma* exposed to Cu NPs and $CuSO_4$ (Gautam et al., 2018). The differences between both results highlight that the effects of the ions play an important role in NO production because Ag_2S NPs are not strongly soluble, while $CuSO_4$ releases Cu^{2+} immediately. Although NO is in other studies highlighted as a more promising biomarker than reactive oxygen species (ROS), we have observed that NO was not sufficiently produced in *Eisenia andrei*. Further, the addition of L-arginine was needed to increase NO production appropriately for the measurements. Hence, the observed low production of NO in earthworms does not support the use of NO as an oxidative stress biomarker in *E. andrei*.

The phenoloxidase activity of coelomocytes was measured after exposure to Ag_2S NPs, and $AgNO_3$. Phenoloxidase is an enzyme that produces melanin (an antimicrobial product). Melanin and lipofuscin are components of the brown bodies found in earthworms, which lead to the scavenging of free radicals, and the neutralization or encapsulation of foreign material (Valembois et al., 1994). In earthworms, the pattern recognition receptor (PRR) called coelomic cytolytic factor (CCF) has been discovered (Prochazkova et al., 2020). CCF activates the prophenoloxidase cascade (proPO) leading to the cleavage of inactive prophenoloxidase to its active state phenoloxidase (PO; Bilej et al., 2010). Active PO catalyzes both hydroxylation, and oxidation of phenols to quinones, which are subsequently polymerized to melanin. Melanin exerts various biological activities important in defense reactions in many invertebrates (e.g., insects, arthropods, and crustaceans; Cerenius and Söderhäll, 2021; Swartzwelter et al., 2021). However, the phenoloxidase activity in earthworms is lesser in comparison with arthropods (Procházková et al., 2006). The results obtained in this study showed that Ag_2S NPs, and $AgNO_3$ did not activate the prophenoloxidase cascade (Fig. 4C), which is more responsive to pathogens, like bacteria, parasites, or viruses (Cerenius and Söderhäll, 2021). The result is also in concordance with NO production because if free radicals are not produced, there is no need to produce melanin to scavenge them. However, *M. posthuma* earthworms exposed to Cu NPs and $CuSO_4$ showed an increase in phenoloxidase activity when the earthworms were exposed to both materials. Further, the solution of $CuSO_4$ had a stronger effect than nanoparticles (Gautam et al., 2018). Thus, in this study, the nanoparticles could have been eliminated in another way, or the immune system of the earthworms was not affected. Similar to NO production, the low solubility of Ag_2S NPs, and their slow release of Ag^+ ions could explain the observed results.

Another biomarker of oxidative stress is malondialdehyde (MDA), a byproduct of lipid peroxidation. MDA production in earthworms exposed to Ag_2S NPs, and $AgNO_3$ did not differ from the control group, similarly to the NO production. MDA is produced by the interaction of free radicals with lipids with subsequent binding to nucleotides resulting in DNA damage or mutations (Ayala et al., 2014; Stara et al., 2021). Recent studies have already demonstrated that metal NPs can induce oxidative stress as well as increase MDA production in earthworm coelomocytes. For example, nZVI NPs induced ROS generation and increased the levels of MDA in the first hours of coelomocytes exposure, while opposite effects occurred when coelomocytes were exposed to TiO_2 NPs (Navarro Pacheco et al., 2021b; Semerad et al., 2020). The increased MDA production induced DNA damage in coelomocytes exposed to CuO NPs (Navarro Pacheco et al., 2021a). Despite the known negative effects of the imbalanced metabolism of ROS induced by metal NPs, the biomarkers used in the present study did not increase significantly after the 14-day-long exposure, and, therefore, oxidative stress did not occur. Although effects caused by $AgNO_3$ were expected, the results showed that $AgNO_3$ did not cause any oxidative stress nor changes in cell viability. Probably the uptaken amount of Ag^+ ions was not enough to exert affects (EC50 reproduction is 42–46.9 mg kg^{-1} in soils; Schlich et al., 2013) and Ag^+ ions could be transformed into particulate Ag, then, allowing earthworms to excrete it (Baccaro et al., 2018). There is also another mechanism that could decrease the effects of Ag^+ ions from $AgNO_3$ which is represented by metallothioneins. These cysteine-rich metal-binding proteins produced by earthworms can suppress oxidative stress. Metallothioneins were found upregulated when earthworms were exposed to $AgNO_3$ (Tsyusko et al., 2012; Garcia-Velasco et al., 2019). According to Bourdineaud et al. (2021), oxidative stress was confirmed for Au, and Ag NPs, where Ag NPs were the strongest oxidant (Bourdineaud et al., 2021). Although Ag NPs are potent oxidants that induce strong oxidative damage, their transformed product, Ag_2S NPs, did not induce oxidative stress in the earthworms during the 14 days of exposure. Many recent studies focusing on toxicity testing of metal NPs have shown that dissolved metal ions could play an important role in the adverse effects, including oxidative stress (Navarro Pacheco et al., 2021a; Semerad et al., 2020). The lack of oxidative stress-related damage could be explained by the low solubility of Ag_2S NPs. Several studies have shown that Ag_2S NPs are quite stable so the lack of toxicity derives from their stability, and the low release of Ag^+ ions (Baccaro et al., 2018; Schultz et al., 2018). However, toxic effects could occur in longer-term exposure (more than 14–28 days) of the earthworms, as we have observed that the animals can ingest the nanoparticles (Table 1). These retarded toxicity effects of Ag_2S NPs were suggested by Wang et al., 2015, 2017. They observed that Ag_2S NPs

required longer periods to cause damage due to their low solubility and slow release of Ag^{+} ions. Thus, similar mechanisms could occur in the earthworms, where Ag_2S NPs would remain in the animal, and the effects would develop later. Finally, light irradiation could be another reason why no oxidative stress occurred, and no toxicity effects were observed in our study. As we mentioned above, the exposure conditions can affect how NPs develop their toxicity. TiO_2 NPs, and Ag_2S NPs enhanced their harmful effects to sea urchin, and Raji human lymphoma cells, respectively, when the nanoparticles were irradiated with UV light (Reeves et al., 2008; Wang et al., 2019). However, the conditions of our study (lack of UV light) would not make Ag_2S NPs more active; therefore, harmful effects were not observed.

5. Conclusion

The exposure of earthworms to the transformation product of Ag NPs (Ag_2S NPs) and their ionic form ($AgNO_3$) showed that the cellular response of earthworms was not activated, even though some loss of weight was detected after 14 days of exposure to 5 mg Ag kg^{-1} soil. It has been proven that *E. andrei* earthworms can engulf the Ag_2S NPs; however, the ingestion, and presence of silver did not cause any major damages to the studied cells or animals since the viability of coelomocytes was comparable to the control. Further, no significant increase in oxidative stress biomarkers was observed, including NO and MDA production. The low solubility of these nanoparticles under exposure/environmental conditions is probably one of the main reasons for the minimal impact on earthworms during the tested 14-day period. However, further studies conducted for longer periods should be performed to assess potential risks connected with Ag_2S, and other transformation products of Ag NPs. The present study demonstrates and highlights the need for a deeper exploration of the environmental fate and toxicity to assess the real risks associated with anthropogenic NPs and show the possibilities of coelomocyte use for *in vivo/in vitro* toxicity testing.

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CRediT authorship contribution statement

Conceptualization, N.I.N.P. and P.P.; methodology and data curation, N.I.N.P., J.S., J.D., M.B.F., J.F. and M.P.; writing—original draft preparation, N.I.N.P., J.S.; writing—review and editing, N.I.N.P., J.S., P. P., and A.R.; supervision, A.R. and P.P.; project administration, P.P., and T.C.; funding acquisition, P.P., and T.C. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare the research was conducted in the absence of commercial or financial relationships that could lead to a potential conflict of interest.

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