Quantification of silver partitioning in sub-cellular fractions in earthworms (*Eisenia fetida*) exposed to Ag-NPs, Ag_2S-NPs and $AgNO_3$ overtime

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May, 2019

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Abstract

Silver nanoparticles (Ag-NPs) are one of the most widely used nanomaterials due to their strong anti-microbial characteristics. Silver particles may be released into the environment through sewer sludge and cause potential environmental risks. Therefore, assessing the bioavailability and toxicity of different forms of Ag on soil organisms is required. Recent studies suggested that the toxicity of Ag-NPs is mainly the result of released Ag ions, but it is still unclear if there are some effects due to nano-specific properties. Instead of the total internal Ag concentration in earthworm, internal compartmentalization of metal-NPs in different subcellular fractions over time can give more specific information on the toxicokinetic and accumulation pattern of NPs. This study quantified the toxicokinetic of silver NPs and ions in earthworm Eisenia fetida exposed to the aged form Ag_2S-NPs and pristine Ag-NPs, with an ionic control ($AgNO_3$) by measuring the concentration of Ag present in both the whole worm tissue and 4 subcellular fractions (granules, tissue and cell membrane, organelles, and cytosol). Results showed that the whole body and subcellular accumulation kinetics of Ag in earthworms exposed to Ag-NPs and AgNO3 were similar over time, while the uptake kinetics of the non-soluble Ag_2S-NPs were significantly different, suggesting that earthworms mainly take up Ag-NPs as the dissolved ionic form. Additionally, cytosol subcellular fraction accumulated most detected Ag, which might associate with the binding of Ag with metallothionein enriched in cytosol.

Keywords: Silver nanoparticles, toxicokinetic, subcellular fraction, earthworm

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1. Introduction

Nanotechnology is identified as a key enabling technology that deals with materials ranging from 1-100 nm in at least one dimension[1]. The high surface area to volume ratio of nanoparticles (NPs) leads to high reactivity[2] and give them specific characteristics. Metal NPs is a prominent class of NPs, and silver nanoparticle (Ag-NP) is one of the most widely used nanomaterials[2]. Silver can lead to cell death caused by the uncoupling of oxidative phosphorylation[3], the induction of free radical formation[4] or the interference with cellular respiration and transport of ions across membranes[5]. These effects give Ag a remarkably strong anti-microbial (antibacterial, antifungal and antiviral) characteristic. Besides, nanotechnology gives Ag particles better feasibility and efficiency for their applications in different field such as micro-field[4]. For these reason, Ag-NPs are used in various fields, including pipelines, medical devices, food packaging, room spays, laundry detergents, water purificants, wall paints, washing machine, textiles and fabrics[6]. However, the increasing use of Ag-NPs and their possible release into the environment also raise some concerns about the potential environmental risks.

Under most environment conditions, pristine Ag-NPs will dissolve and oxidize or react with (in)organic ligands[7]. For example, exposure modelling indicated that most Ag-NPs released from domestic and industrial sources will enter sewer systems and wastewater treatment plants (WWTPs) eventually in most cases[8]. In WWTPs, sulfidation of both the Ag-NPs and the released Ag ions occur and result the formation of Ag_2S-NPs[9] which are characterized by low solubility[10]. In non-aerated WWTP tanks with enriched sulfide, more than 90% of Ag-NPs were transformed to Ag_2S within 2 hours[11]. Finally, Ag_2S-NPs present in the WWTPs may enter the environment by the disposal of sewage sludge on the agricultural land. Mueller and Nowack[8] modelled that the addition of sludge from WWTPs to agricultural land would result in an input of 1 µg nano Ag /kg[3] per year. Therefore, Ag_2S-NPs may accumulate in the top-soil and interact with soil organisms. Therefore, in order to investigate the toxicity and toxicokinetic in realistic scenario, it becomes fundamental to study the aged form (Ag_2S-NPs) instead of just pristine form (Ag-NPs) of silver. In this study, earthworms were used as model soil organism which can take up metals via both dermal and dietary route[12] in order to get a better understand the uptake toxicokinetic and accumulation patterns of silver in earthworms.

According to previous in vitro[13-16] and in vivo[17-19] studies, Ag-NPs showed toxicological implications on organisms. However, most of the study are performed with pristine Ag-NPs rather than with the aged forms. Although some studies suggested that the toxicity of Ag-NPs is mainly the result of released Ag ions[2, 20], it is still unclear if there are some effects due to nano-specific properties[21]. Exposure to both ionic Ag and Ag-NPs was shown to lead to similar bioaccumulation kinetics[10] and to mechanistic effects including changes in sugar metabolism and electron transport, protein turnover and DNA conformation on earthworm[22]. However, the potential toxicokinetic of Ag-NPs and Ag ions related to cellular internalization may be different[22] and can be used to explain their dissimilar toxicities. Belated dissolution of NPs is considered as a possible reason to explain the observed lower toxicity of NPs than their ionic counterparts and increased toxicity over time[23, 24]. Study performed by Hayashi et al. [25] reported dissolution of NPs in the intracellular fractions of *E. fetida*. Oxidative dissolution and intracellular release of Ag ions from Ag-NPs could occur at the low lysosomal pH in the cellular environment[25]. Furthermore, macrophages were proved to form a membrane at the interface to stimulate the oxidation on the silver surface and release Ag ions which are readily taken up by the cells[25]. A

mechanism of cellular sequestration decreasing toxicity of metals associated with specific metal-binding proteins in earthworms and other organism has been discussed Metallothioneins (MTs) is a cysteine-rich protein with low molecular weight that can bind with metals (including Ag) through the thiol group of its cysteine residues for detoxification and protection from heavy metal toxicity and oxidative stress[21, 26-32]. Therefore, the induction of MTs can be used as a biomarker for the abnormal presence of metals[26] in organism.

Instead of the total NPs body burdens in earthworm over time, internal compartmentalization of metals NP in different subcellular fractions (cytosol, debris, granules, etc.) can give more specific information on the difference between the toxicokinetic and the accumulation pattern of metal NP and metal ions and help to better understand their toxic effects[26]. Metals will distribute over the different subcellular fractions after entering the organism, accumulate at these compartments over time, and form a specific internal metal pool of metals[26, 27]. Toxicity of metals on earthworms is related with the internal pools which are assumed to be biologically active rather than the total internal metal burden[26]. Therefore, changes in the subcellular partitioning of metals could be indicators of metal toxicological bioavailability for earthworms, and provide better endpoints to identify pertinent chemical proxies of metal availability to earthworms[27, 33, 34]. For instance, the study of Li, et al.[21] shown a clear difference about Ag subcellular partitioning pattern and metabolite profiles between *E. fetida* exposed to Ag-NPs and dissolved Ag ions in water. At the end of 4-days exposure to Ag-NPs, higher amount of Ag was accumulated in the granules and cell membrane fractions than in the microsome and cytosol, whereas the largest proportion of dissolved Ag ions was found to be associated with the fraction containing cytosol.

To investigate the potential different toxicokinetic of nano form of Ag and Ag ions, it is relevant to identify the fate of the three forms of silver (Ag-NPs, Ag_2S-NPs, and $AgNO_3$) within the earthworms by quantification of the metal within the subcellular fractions overtime. Three research questions were defined: are there differences in Ag subcellular distribution among 3 treatment groups (Ag-NPs, Ag_2S-NPs, and $AgNO_3$ exposed worms)? Are there time-dependent differences after 4-days, 7-days, and 14-days exposure? Are there differences of toxicokinetic between aged form of Ag (Ag_2S-NPs), pristine form of Ag (Ag-NPs), and ionic form ($AgNO_3$)?

2. Methodology

2.1. Experiment design

Solutions containing uncoated Ag-NPs, Ag_2S-NPs and $AgNO_3$ were spiked into sieved natural soil to reach a nominal concentration of 15 mg Ag/kg dry weight of soil for all treatments. Four adult earthworms (*E. fetida*) were exposed in a glass jar with lid containing 173 g of contaminated soil and incubated for 14 days. Earthworms were sampled after 4, 7, and 14 days of exposure. After 4 days and 14 days of exposure, 3 jars from each treatment group were sampled. After 7 days of exposure, 4 jars were sampled. Earthworms were collected, homogenized and centrifuged to obtain 4 different subcellular fractions. The concentration of Ag in the whole earthworm tissue and separated subcellular fractions were analysed by inductively coupled plasma–mass spectrometry (ICP-MS) machine following microwave-assisted acid digestion. The differences between treatment groups, separated fractions, and different exposure time were compared and analysed.

2.2. Materials

Ag-NPs (5.5 mM sodium citrate, 25 mM tannic acid, 47.3 ± 5.3 nm) and Ag_2S-NPs (5.5 mM polyvinylpyrrolidone (PVP), 20.3 ± 9.8 nm) were synthesized by Applied Nanoparticles (Barcelona, Spain). *Figure 1* illustrates TEM images of the nanoparticles stock solution. $AgNO_3$ solution was prepared by dissolving $AgNO_3$ (99.8%, Merck, Darmstadt) in ultrapure water.

Earthworms were supplied by Lasebo (Nijkerkerveen, The Netherlands) and kept in the experimental soil at 20% moisture content (w/w) in incubator at 20 ± 1 °C with 24 hours of light for 1 week prior to the experiments[35].

The soil (pH 5.2 in water, organic matter content 5.4%) was collected from a farm in The Netherlands (Proefboerderij Kooijenburg, Marwijksoord, Netherlands). Air-dried soil was sifted by 5 mm sieve openings before spiking the soil. Homogenous contamination of the exposure soil (15 mg Ag kg^{-1}) was assured by mixing Milli-Q water, soil, and Ag stock solutions by an automatic mixer for 3 minutes. Final moisture content was 20% w/w (~47% water holding content). 173 g spiked soil was added to each glass jar with a lid. 10 jars for each treatment group were prepared. All the jars containing spiked soil were kept in incubator for 24 hours before the earthworm exposure[35].

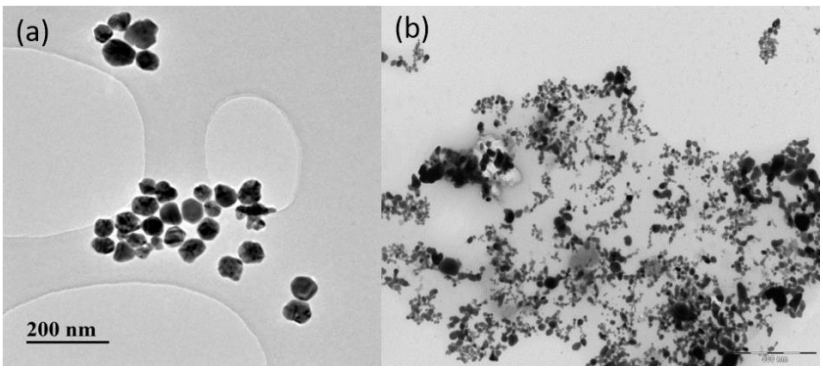

Figure 1. TEM Images of (a) Ag-NPs and (b) Ag_2S-NPs stock solution 3rd Batch.

2.3. Exposure

Earthworms with an average weight of 0.455 ± 0.026 gram per worm (n=177, mean ± standard deviation)were randomly selected, rinsed by Milli-Q water, incubated in petri dishes containing moist filter papers for 24 h to depurate their gut content[35]. Earthworms were then rinsed by Milli-Q water and dried by clean tissue paper. Four adult earthworms were placed in each jar containing spiked soil. Jars were kept in the incubator at 20 ± 1 °C and kept with 20% moisture content during exposure.

2.4. Sampling

At each sampling point (4, 7 and 14 days), earthworms and aliquots of soil were sampled. Jars were emptied separately. Earthworms were collected, rinsed with Milli-Q water, and dried in tissue paper. Earthworms were placed in petri dishes containing moist filter papers and put in incubator at 20± 1 °C for 24 h to depurate their gut content[35]. After depuration, worms were washed, dried, and weighed with empty gut in order to ensure the mean mass loss of worms were not above standard[35]. Earthworms were placed in polypropylene tubes and snap-frozen in liquid nitrogen. All the samples were stored at -80°C freezer for later analysis.

2.5. Subcellular fractionation

A protocol developed by Wallace and Lopez (1996)[36] was adopted to separate different subcellular fractions of earthworm (*Figure 2*) by centrifugation. Earthworms were separated into 4 subcellular fractions including granules fraction (D), tissue, cell membrane, and intact cell fraction (E), organelles fraction (F), and cytosolic fraction (G).

Earthworm samples were thawed and homogenized with a homogenizer (Ultra turrax T25, IKA®-labortechnik, German) for 3 minutes in ice-cold 0.01 M Tris-HCl buffer (pH 7.0, SIGMA-AIDRICH, USA) based on a 1:10 tissue-to-buffer w/v ratio. Homogenates (fraction A) were centrifuged at 10 000 g for 30 min at 4°C to obtain the pellet (fraction B) and the supernatant (fraction C). The supernatant fraction (C) was collected into another centrifuge tube in preparation for ultracentrifugation. The pellet fraction (B) was resuspended in 4 ml of 1 M NaOH (SIGMA-AIDRICH, Sweden) at 70 °C for 1 hour. Pellet (B) was then centrifuged at 10 000 g for 10 minutes at 20 °C to obtain the pellet fraction (D) containing granules, and supernatant fraction (E) containing tissue, cell membranes, and intact cell fractions. To dissolve the pellet, 1 ml of NaOH was added. The supernatant (C) was centrifuged at 41 657 g for 1 hour at 4°C to yield the pellet fraction (F) containing organelles, and the supernatant fraction (G) containing cytosols. To dissolve the pellet, 1 ml of Tris buffer was added.

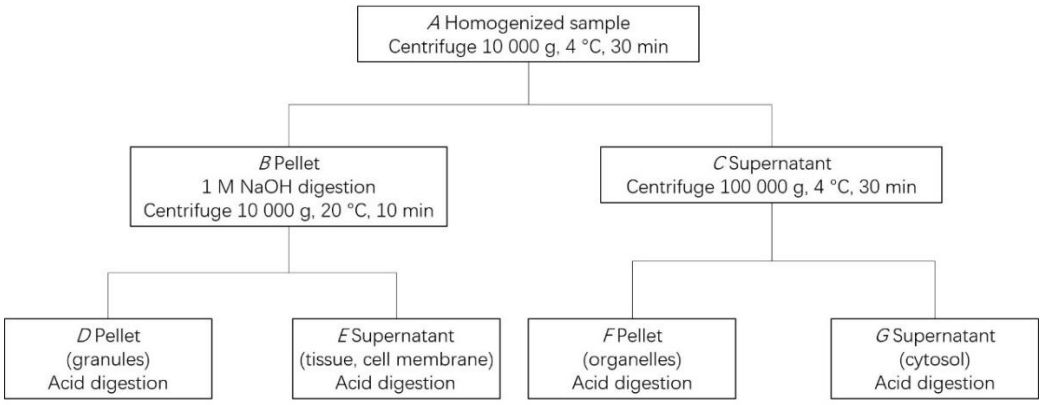

Figure 2. Subcellular fractionation scheme for the determination of Ag in *E. fetida* (adapted from Li et al., 2014[21])

2.6. Extraction and analysis

All subcellular fractions and earthworm tissues were digested and metals were extracted by microwave-assisted acid digestion in aqua regia (1: 3 nitric acid–hydrochloric acid). Prior analysis fractions E and G were concentrated to 1 ml with an automated solvent evaporation system evaporator (TurboVap® LV, Biotage, Sweden). 1 ml of each fraction was added to a Teflon vessel containing 3 ml HCl (37%, Merck, Darmstadt) and 1 ml HNO_3 (69%, Merck, Darmstadt). For the acid digestion of the whole organisms 8 ml of aqua regia was used. External standards of Ag (1000 mg L^{-1} Ag) and blanks were included. Digestion was performed using MARS 5 (microwave system, CEM Corporation) with a temperature ramp from 160 °C (30 min) to 180 °C (30 min). After digestion, samples were diluted properly with Milli-Q water. Rhodium (1000 mg L^{-1} Rh) was added to every sample as the internal standard. Calibration curve was obtained by Ag^{+} standards (0, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 50, 100 µg L^{-1} Ag). The concentrations of Ag were analysed by ICP-MS Nexion 350D (Perkin-Elmer Inc., Waltham, MA).

2.7. Metallothionein (MT) measurement

A kit developed by IKZUS ENVIRONMENT based on the spectrophotometric method developed by Viarengo, et al.[37] was used to quantify the MT concentration in earthworm. Worms were homogenized on ice in buffer containing sucrose (0.5 M), Tris-HCl buffer (20mM, pH 8.6), leupeptin (0.006mM), phenylmethylsulphonilfluoride (0.5mM, antiproteolytic agents), and β-mercaptoethanol (0.01%, reducing agent) based on a 1:3 tissue-to-buffer w/v ratio. Homogenates were centrifuged at 21,500 g at 4°C for 20 min. 0.3 ml of supernatant (equal to 0.1 g of tissue) were transferred and 0.315ml of absolute ethanol equilibrated at -20°C was added, mixed and centrifuged at 16, 000 g at 4°C for 5 min for purification. The rest of the supernatant was preserved at fridge (2-4°C) for later protein quantification by Pierce method[38]. After centrifugation, all of the supernatant was collected, and 1.5 ml of absolute ethanol equilibrated at -20°C was added, and incubated at -20°C fridge for 30-60 minutes. After incubation, samples were centrifuged at 16, 000 g at 4°C for 5 min. Supernatant was discarded and the pellet was dried under a gently stream of nitrogen for 10 to 20 minutes. The dried samples were resuspended with 50µl buffer containing 25µl NaCl and 25µl HCl containing EDTA (4 mM). 1.95ml of reagent buffer (5,5'-dithiobis-2-nitrobenzoic acid) was added equilibrated at room temperature and mixed with samples. After 2 minutes of incubation at room temperature, samples were centrifuged at 16, 000 g at 4°C for 2 min. The concentration of MT was quantified by spectrophotometric titration of the

sulphydryl residues in samples based on the absorption at 412 nm (ABS^{MT}_{412}) against a blank (25µl NaCl + 25µl HCl containing EDTA (4 mM) + 1.95ml reagent buffer 5,5'-dithiobis-2-nitrobenzoic acid)[29]. Calibration curve was obtained by 4 mM sulfhydryl reference standard solution containing reduced glutathione (GSH) (10, 20, 40, 60, 80 nano moles (nmol) of sulfhydrylic group equivalents).

In order to express the concentration of MT based on the total amount of protein, the amount of proteins present in the homogenised samples was measured by the spectrophotometric method[38]. Calibration curve was prepared by BSA stock solution (2 mg/ml) and demi water (2000, 1500, 1000, 750, 500, 250, 125, 62.5, 31.25, 15.6, 0, 0 µg/ml). Samples were measured at different dilutions. Absorbance was read at 562 nm.

Equations used to calculate the concentration of MTs (nmol MT) per gram of tissue (eqn 1) and the MTs concentration per mg of total protein (eqn 2) were:

$$(nmol\ MT) \cdot g^{-1} of\ tissue = \frac{(nmol\ Cys^{MT})}{0.1\ g \cdot n^{\circ}cys} \quad \text{(eqn 1)}$$

$$(nmol\ MT) \cdot mg^{-1} of\ total\ protein = \frac{(nmol\ Cys^{MT})}{mg \cdot n^{\circ}cys} \quad \text{(eqn 2)}$$

in which 0.1 g is the amount tissue equivalent to 0.3 ml of supernatant used in experiment; $n^{\circ}cys$ represents the number of cysteine residues present in the MTs (20 for earthworms[39]); amount of protein measured in the 0.3 ml supernatant used in experiment.

2.8. Quality control

Earthworm survival rate in all treatment groups (Ag-NP, $AgNO_3$, and Ag_2S-NP) was 95.83% (higher than 90%). The mean mass loss of worms in all treatment groups measured at the end of exposure did not exceed 20% compared with their initial body weight. Therefore, this test fulfilled the requirements of testing of chemicals bioaccumulation in terrestrial oligochaetes[35]. In each sample analysis for Ag concentration, two external standards of Ag (1000 mg L^{-1} Ag), blanks and internal standard rhodium (1000 mg L^{-1} Rh) were added to check the analytical quality of machine. The results shown that 100 ± 30% Ag was recovered on average.

2.9. Statistical analysis

Normality was tested to ensure that all groups of data come from normal distribution. Data are reported as mean ± standard deviation in this report. For parametric data, the differences between treatment groups and exposure time on Ag concentrations, proportion in subcellular fractions, and the MT concentrations (MT nmol mg^{-1} of total protein) in worms were compared by the one-way analysis of variance test (one-way ANOVA) followed by the Tukey HSD post-hoc test. For non-parametric data, the differences were compared by the Kruskal-Wallis one-way ANOVA (k samples) test with all-pairwise multiple comparisons. Differences were statistically considered significant when $p<0.05$.

3. Results and discussion

3.1. Accumulation of Ag in earthworms

Ag accumulated in the tissues of earthworms exposed to different forms of Ag (Ag-NPs, $AgNO_3$, and Ag_2S-NPs) at the same soil concentration (15 mg Ag kg^{-1} dry weight of soil). The concentrations of Ag measured in the whole organisms (mg Ag kg^{-1} wet body weight of worm) showed no significant differences within the same treatment over time (*Table 1* and *Figure 3*). The Ag accumulation kinetics of worms exposed to Ag-NPs and $AgNO_3$ were not significantly different at every time point. However, the concentrations of Ag in earthworms exposed to Ag_2S-NPs were significantly lower than worms exposed to Ag-NPs and $AgNO_3$. After 14-days of exposure, the concentration of Ag accumulated in earthworms exposed to soil spiked with Ag-NPs and $AgNO_3$ were 6.56 ± 1.80 and 6.15 ± 1.09 mg Ag kg^{-1} wet body weight (average ± standard deviation) respectively. More results about the significance between treatment groups and time points are provided in *Appendix 3*.

Table 1. Time dependent concentrations of Ag (mg Ag kg^{-1} wet body weight) measured in the earthworms (*E. fetida*) exposed to soil spiked with AgNPs, $AgNO_3$, and Ag_2S-NPs (mean ± standard deviation; n = number of replicates).

Treatments / *Exposure day*	*Ag-NP*	*$AgNO_3$*	*Ag_2S-NP*
Day 4 (n=3)	3.51 ± 0.86	5.52 ± 0.20	0.33 ± 0.15
Day 7 (n=4)	6.07 ± 1.24	5.25 ± 1.60	0.32 ± 0.11
Day 14 (n=3)	6.56 ± 1.80	6.15 ± 1.09	0.40 ± 0.17

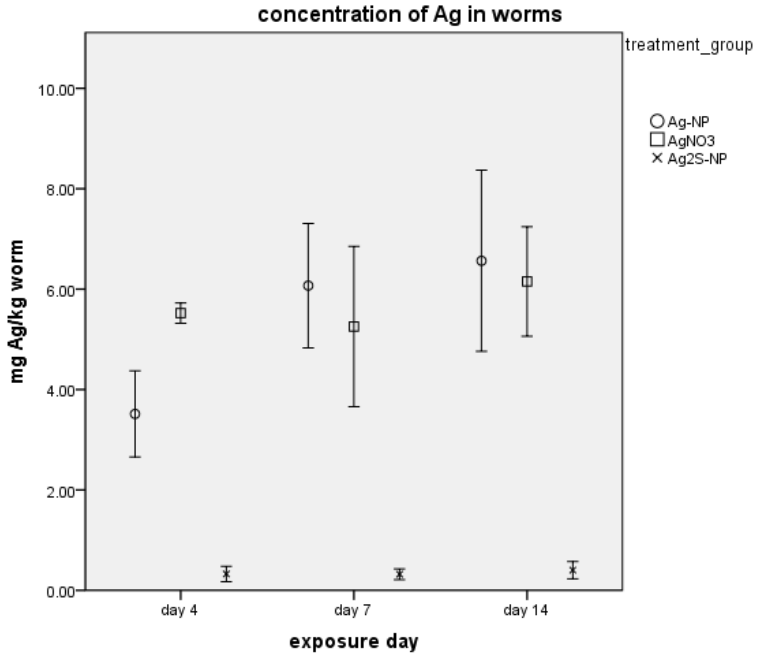

Figure 3. Time dependent concentrations of Ag (mg Ag kg^{-1} wet body weight) measured in the earthworms (*E. fetida*) which were exposed to soil spiked with AgNPs, $AgNO_3$, and Ag_2S-NPs (mean ± standard deviation).

3.2. Kinetics of the subcellular accumulation of Ag in earthworms

The concentrations of Ag in four subcellular fractions were analysed as the concentration of Ag as a proportion of total mass of Ag in whole worms at different time points. The proportion of Ag in different subcellular fractions—granular fractions (D); tissue, cell membrane fractions (E); organelle fractions (F); and cytosolic fractions (G)—at different time points (4, 7 and 14 days) is provided in *Figure 4*. The proportion was obtained by relating the normalized mass of Ag in each subcellular fraction to the total mass of Ag in the earthworms which was calculated by summing the normalized mass of all individual subcellular fractions.

Table 2. Time dependent subcellular distribution of Ag (% of total mass) in the earthworm (*E. fetida*) exposed to AgNPs, $AgNO_3$, and Ag_2S-NPs (mean ± standard deviation; n = number of replicates). Fraction D = granules; fraction E =tissue, cell membrane, and intact cell fractions; fraction F = organelles; fraction G = cytosolic fraction; total mass = total body mass of Ag calculated by summing all subcellular fractions.

Treatment group	*Exposure day*	*Subcellular fractions*			
		Fraction D	*Fraction E*	*Fraction F*	*Fraction G*
Ag-NP	Day 4 (n=3)	26±8%	10±2%	11±3%	53±5%
	Day 7 (n=4)	15±5%	17±3%	8±2%	59±3%
	Day 14 (n=3)	38±20%	14±2%	7±3%	41±19%
$AgNO_3$	Day 4 (n=3)	40±8%	25±17%	7±3%	28±21%
	Day 7 (n=4)	13±3%	20±3%	9±3%	58±4%
	Day 14 (n=3)	31±7%	8±2%	8±1%	53±8%
Ag_2S-NP	Day 4 (n=3)	29±24%	12±10%	11±4%	48±31%
	Day 7 (n=4)	45±21%	25±15%	10±5%	19±5%
	Day 14 (n=3)	27±14%	14±4%	36±25%	23±9%

As shown in *Figure 4*, no significant time-dependent difference was observed between the amount of Ag in the subcellular fractions during 14-days exposure in the Ag-NPs and Ag_2S-NPs treatment groups. Only the proportion of Ag accumulated in cytosolic fractions (G) of $AgNO_3$ exposed worms shown significant increase within 14-days exposure. The Ag accumulation kinetics of worms exposed to Ag-NPs and $AgNO_3$ were similar at all subcellular fractions and time points without significant difference. The largest proportion of Ag was associated with the cytosolic fractions (G) in both Ag-NPs and $AgNO_3$ exposed worms. Indeed, in these two treatment groups, the proportions of Ag in tissue and cell membrane fraction (E) and organelle fraction (F) were significantly lower than the proportion of cytosolic fraction (G), except in day 4 in $AgNO_3$ treatment and in day 14 in Ag-NP treatment. In contrast, the Ag accumulation kinetics of worms exposed to Ag_2S-NPs were different with Ag-NPs and $AgNO_3$ treatment groups. The accumulation of Ag in cytosolic fractions (G) in worms exposed to Ag_2S-NPs was significantly lower, and no significant difference was observed between the Ag content quantified in the fractions. The absolute amounts of Ag measured in each fraction and its corresponding proportions are provided in *Appendix 3 and Appendix 4*.

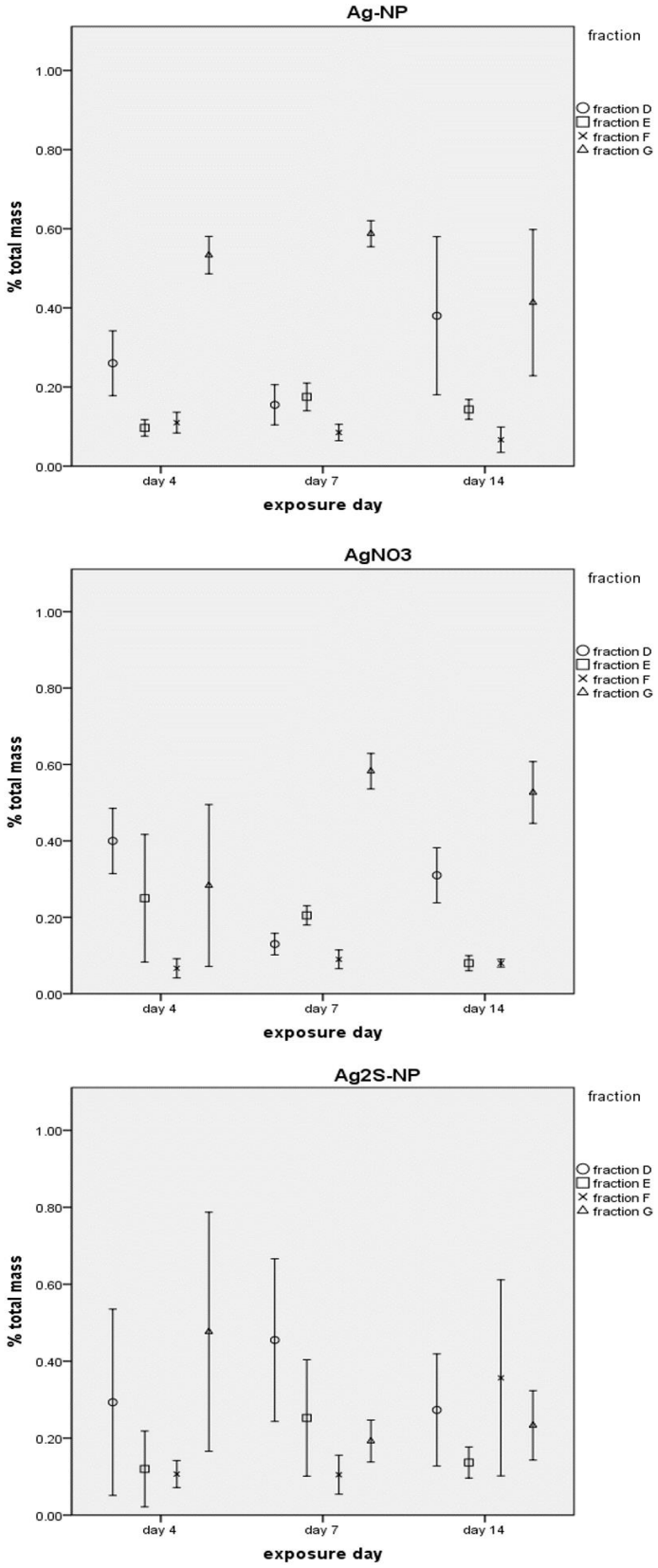

Figure 4. Time dependent subcellular distribution of Ag in the earthworm (*E. fetida*) exposed to (a) AgNPs, (b) $AgNO_3$, and (c) Ag_2S-NPs (mean ± standard deviation). D = granules; E =tissue, cell membrane, and intact cell fractions; F = organelles; G = cytosolic fraction; total mass = total body mass= total body mass of Ag calculated by summing all subcellular fractions.

The similar time-dependent accumulation kinetics of Ag in both whole worm tissues and four subcellular fractions between Ag-NPs and $AgNO_3$ treatment groups indicates that the uptake and accumulation of Ag-NPs mainly occur as the dissolved ionic form rather than nanoparticle form. This is supported by the significantly different Ag uptake kinetics in Ag_2S-NP exposed worms compared with Ag-NPs and $AgNO_3$ exposed individuals, due to the extremely low solubility of Ag_2S-NPs (Ksp =6.3×10^{-50}) [38] caused by the change of Ag surface composition[41]. According to the study of Levard, et al.[41], a significant decrease of released Ag^+ from Ag_2S-NPs was observed by a factor of about 7 (3 ppm) compared with pristine Ag-NPs group (about 20 ppm with an initial Ag-NPs concentration of 1000 ppm) in solution with a S/Ag ratio as low as 0.019. Since most of Ag_2S-NP (about 0.3% at a S/Ag ratio=0.019)[41] maintain nano form, its different uptake kinetics compared with Ag-NPs treatment groups indicates that Ag-NPs were mainly be taken up as the ionic form of Ag^+ released from NPs in worms rather than their nano form. The toxicokinetic of 3 forms of Ag observed in this experiment is consistent with previous study[10] which reported that the uptake and elimination kinetics of Ag in worms exposed to Ag-NPs and $AgNO_3$ were similar, while different with Ag_2S-NP exposed worms. Further study performed by Li, et al.[21] exposed *E. fetida* to Ag-NPs and separated Ag^+ which were released from same amount of Ag-NPs during 96-hous exposure in aquatic environment. The results shown that there was no significant difference of the amount of accumulated Ag in worms exposed to Ag-NPs (10±4.0 nm) and only released Ag^+ indicating the accumulation of Ag in Ag-NPs exposed worms mainly occur as the ionic form. However, this result can only be used as a reference for the exposure in soil environment investigated in this study, because the exposure media (soil, liquid, or air) can heavily influence the uptake and the dissolution kinetics of NPs. Since only about 2% of Ag present in pristine Ag-NPs (initial concentration was 1000 ppm in solution) would finally be released as Ag^+ after reaching equilibrium[41], there is a high potential that non-dissolved Ag-NPs could also affect the kinetics of Ag accumulation in worms.

In this study, the induction of metal-binding MTs was used as a biomarker for the dissolution kinetics of Ag-NPs and release of Ag^+ in worms over time[26]. MT concentration measured in worms exposed to Ag-NPs and $AgNO_3$ spiked soil are provided in

Table 3 and *Figure 5*. After 7-days and 14-days of exposure, 0.24 ± 0.05 and 0.60 ± 0.06 nmol MTs per mg of total protein were detected in worms exposed to Ag-NP, and 0.43 ± 0.18 and 0.48 nmol MTs per mg of total protein were detected in worms exposed to $AgNO_3$, respectively. A significant increasing was observed in the Ag-NP treatment group between 7-days and 14-days exposure, indicating that belated dissolution of Ag-NPs and gradual releasing of Ag^+ might have occurred in worms. Besides, the dominate accumulation of Ag in cytosolic fraction (G) in both Ag-NPs and $AgNO_3$ exposed worms was observed, which might be caused by the binding of Ag with MTs which is enriched in cytosolic fraction and associated with detoxification mechanism[21, 32]. Other kinds of protein such as Glutathione transferase (GST) isoforms, a major cytosolic aminopeptidase in the earthworms, was also reported as the dominate protein in earthworm (*Lumbricus rubellus*) cytosols which constitutes the detoxification mechanism of metals after exposure[42, 43]. Those metal-binding enzymes might keep Ag in the cytosol fraction of worms for series of detoxification reactions, which could explain the dominate accumulation of Ag in cytosolic fractions (G) in worms exposed to Ag-NPs and $AgNO_3$ observed in this study. The significantly lower amount of Ag in cytosolic fractions (G) in Ag_2S-NPs exposed worms indicates that these MTs enriched in cytosol might mainly bound with ionic form of Ag rather than the nano form.

Table 3. Time dependent concentrations of Ag ((nmol MT) · mg^{-1} protein) measured in the *E. fetida* exposed to soil spiked with Ag-NPs and $AgNO_3$ (mean ± standard deviation; n = number of replicates). * indicates that group only had two replicate data, so no standard deviation exists.

Treatments / *Exposure day*	*Control group*	*Ag-NP*	*$AgNO_3$*
Day 7 (n=3)	0.27 ± 0.09	0.24 ± 0.05	0.43 ± 0.18
Day 14 (n=3)	0.41 ± 0.24	0.60 ± 0.06	0.48 *

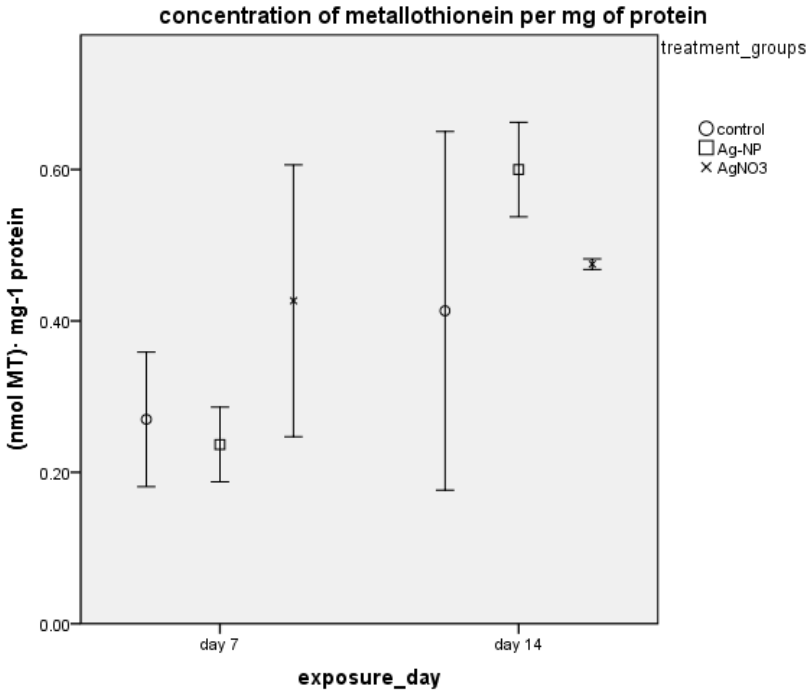

Figure 5. Time dependent concentrations of Ag ((nmol MT) · mg-1 protein) measured in the *E. fetida* exposed to soil spiked with Ag-NPs and $AgNO_3$ (mean ± standard deviation) after 7-days and 14-days exposure.

Another explanation for the enrichment of Ag in cytosolic fractions (G) observed in this study could be the intracellular kinetics of Ag-NPs and released Ag^+. A well-represented transcriptome model for *E. fetida* developed by Novo et al.[22] indicated that Ag-NPs exposed worms caused a significant enrichment for genes associated with endocytosis and cilia structures which were possibly related with the entering of NPs into cells and tissues. Macrophages were proved to form a membrane at the interface of Ag-NPs to stimulate the oxidation on the silver surface and release Ag ions which are readily taken up by the cells[25]. NPs were observed be associated with cytoskeleton or vacuoles[44] confirming that cytosolic fractions (G) might contain the largest proportion of Ag in worms exposed to Ag-NPs, which was consistent with our results.

4. Conclusion

In this study, similar uptake and accumulation kinetics were observed in earthworms exposed to Ag-NPs and $AgNO_3$ contaminated soil. In contrast, the uptake of Ag_2S-NP, which has an extreme low dissolution rate and mainly presents as nanoparticle form, was significantly lower and the accumulation kinetics were different compared with the Ag-NPs and $AgNO_3$ treatment groups. These results suggest that the uptake and accumulation of Ag-NPs in earthworms mainly occur as the dissolved ionic form rather than the nano form of Ag. Distribution of Ag among subcellular fractions in earthworms indicated that most amount of Ag accumulated in the cytosolic fraction in worms exposed to Ag-NPs and $AgNO_3$, which might be caused by the binding of Ag with metallothionein which are present in the cytosol of earthworms. In addition, the Ag accumulation in cytosolic fraction of worms exposed to Ag_2S-NPs was not higher than other subcellular fractions as the other two treatment groups, suggesting that metallothionein might mainly bind with Ag^+ in worms rather than NPs. Further studies are needed to investigate the concentration of metallothionein present in Ag_2S-NPs exposed worms to better understand if metallothionein can also bind with nanoparticles. Shorter exposure (such as 48-hours and 72-hours) can also be included in both Ag and metallothionein concentration measurement, because not much time-dependent changing after 4-days exposure was observed in this study. Main changes of the Ag and metallothionein concentration might occur within 4 days.

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Appendix

Appendix 1. The result of normality test about the normalized mass of Ag (mg Ag/mg wet weight of worm) in whole body tissues and subcellular fractions (fraction D = granules; fraction E = tissue, cell membrane, and intact cell fractions; fraction F = organelles; fraction G = cytosolic fraction) of worms which were exposed to 3 types of spiked soil (AgNPs, $AgNO_3$, and Ag_2S-NPs) of all time points (4-days, 7-days, and 14-days).

Treatment group	Fraction	Exposure day	P (Shapiro-Wilk)	Normal distribution
AgNP	Fraction D	Day 4	0.649	Yes
		Day 7	0.440	Yes
		Day 14	0.142	Yes
	Fraction E	Day 4	0.341	Yes
		Day 7	0.389	Yes
		Day 14	0.828	Yes
	Fraction F	Day 4	0.353	Yes
		Day 7	0.887	Yes
		Day 14	0.286	Yes
	Fraction G	Day 4	0.365	Yes
		Day 7	0.981	Yes
		Day 14	0.005	NO
AgNO3	Fraction D	Day 4	0.796	Yes
		Day 7	0.213	Yes
		Day 14	0.615	Yes
	Fraction E	Day 4	0.715	Yes
		Day 7	0.453	Yes
		Day 14	0.664	Yes
	Fraction F	Day 4	0.835	Yes
		Day 7	0.713	Yes
		Day 14	0.862	Yes
	Fraction G	Day 4	0.749	Yes
		Day 7	0.835	Yes
		Day 14	0.064	Yes
Ag2S-NP	Fraction D	Day 4	0.301	Yes
		Day 7	0.291	Yes
		Day 14	0.797	Yes
	Fraction E	Day 4	0.465	Yes
		Day 7	0.193	Yes
		Day 14	0.050	Yes
	Fraction F	Day 4	0.775	Yes
		Day 7	0.520	Yes
		Day 14	0.146	Yes

	Fraction G	Day 4	0.484	Yes
		Day 7	0.165	Yes
		Day 14	0.846	Yes
	Treatment group	Exposure days	P (Shapiro-Wilk)	Normal distribution
Whole worm tissues	AgNP	Day 4	0.144	Yes
		Day 7	0.193	Yes
		Day 14	0.761	Yes
	AgNO3	Day 4	0.209	Yes
		Day 7	0.597	Yes
		Day 14	0.110	Yes
	Ag2S-NP	Day 4	0.891	Yes
		Day 7	0.694	Yes
		Day 14	0.360	Yes

Appendix 2. The result of normality test about the concentration of MT per mg of protein of worms which were exposed to 2 types of spiked soil ($AgNO_3$, and Ag_2S-NPs) of all time points (7-days, and 14-days).

		P (Shapiro-Wilk)	Normal distribution
Day 7	Control	.367	Yes
	Cd	.228	Yes
	AgNP	.220	Yes
	AgNO3	.340	Yes
Day 14	Control	.097	Yes
	Cd	.142	Yes
	AgNP	.578	Yes
	AgNO3		NO

Appendix 3. The results of one-way ANOVA followed by Tukey HSD post-hoc test in comparison of normalized mass of Ag (μg Ag/g wet weight of worm) detected in whole body tissues of worms which were exposed to spiked soil (AgNPs, AgNO3, and Ag2S-NPs) of all time points (4-days, 7-days, and 14-days). Positive confidence interval means that measured mass of Ag is higher in first factor, and vice versa.

Treatment group	Exposure days	Mean Difference (I-J)	Std. Error	P value	Significant difference	95% Confidence Interval of difference
AgNP	Day 4 VS Day 7	-2.55493	1.02457	0.093	No	-5.5723 to 0.4625
	Day 4 VS Day 14	-3.05001	1.09531	0.062	No	-6.2758 to 0.1757
	Day 7 VS Day 14	-0.49507	1.02457	0.881	No	-3.5125 to 2.5223
AgNO3	Day 4 VS Day 7	0.26798	0.91753	0.954	No	-2.4342 to 2.9702
	Day 4 VS Day 14	-0.63022	0.98088	0.802	No	-3.5190 to 2.2585
	Day 7 VS Day 14	-0.89820	0.91753	0.612	No	-3.6004 to 1.8040
Ag2S-NP	Day 4 VS Day 7	0.00614	0.10828	0.998	No	-0.3128 to 0.3250

	Day 4 VS Day 14	-0.07593	0.11576	0.795	No	-0.4169 to 0.2650
	Day 7 VS Day 14	-0.08207	0.10828	0.739	No	-0.4010 to 0.2368
Exposure days	Treatment group	Mean Difference (I-J)	Std. Error	P value	Significance	95% Confidence Interval of difference
4 days exposure	AgNP VS $AgNO_3$	-2.00532	0.36857	0.007	Yes	-3.3034 to -0.7130
	AgNP VS Ag_2S-NP	3.19059	0.36857	0.001	Yes	1.8925 to 4.4829
	$AgNO_3$ VS Ag_2S-NP	5.19591	0.36857	<0.001	Yes	3.9007 to 6.4911
7 days exposure	AgNP VS $AgNO_3$	0.81469	0.82594	0.603	No	-1.4913 to 3.1207
	AgNP VS Ag_2S-NP	5.74876	0.82594	<0.001	Yes	3.4427 to 8.0548
	$AgNO_3$ VS Ag_2S-NP	4.93406	0.82594	0.001	Yes	2.6280 to 7.2401
14 days exposure	AgNP VS $AgNO_3$	0.41157	0.99760	0.912	No	-2.6494 to 3.4725
	AgNP VS Ag_2S-NP	6.16176	0.99760	0.002	Yes	3.1008 to 9.2227
	$AgNO_3$ VS Ag_2S-NP	5.75019	0.99760	0.003	Yes	2.6893 to 8.8111

Appendix 4. The results of one-way ANOVA followed by Tukey HSD post-hoc test in comparison of the proportion (%) of Ag in subcellular fractions (fraction D = granules; fraction E = tissue, cell membrane, and intact cell fractions; fraction F = organelles; fraction G = cytosolic fraction) relating to the total normalized mass of Ag in whole worms exposed to spiked soil (AgNPs, $AgNO_3$, and Ag_2S-NPs) of all time points (4-days, 7-days, and 14-days). Positive confidence interval means that measured mass of Ag is higher in first factor, and vice versa. * indicates that group of data did not come from normal distribution. Therefore, Kruskal-Wallis one-way ANOVA (k samples) multiple comparisons of all pairwise were run to run to determine the significance.

Treatment group	Fraction	Exposure days	Mean Difference (I-J)	Std. Error	P value	Significant difference	95% Confidence Interval of difference
AgNP	D	Day 4 VS Day 7	0.10662	0.09174	0.510	No	-0.01636 to 0.3768
		Day 4 VS Day 14	-0.12033	0.09807	0.476	No	-0.4092 to 0.1685
		Day 7 VS Day 14	-0.22696	0.09174	0.095	No	-0.4971 to 0.0432
	E	Day 4 VS Day 7	-0.07498	0.02141	0.024	Yes	-0.1380 to -0.0119
		Day 4 VS Day 14	-0.04283	0.02289	0.217	No	-0.1102 to 0.0246
		Day 7 VS Day 14	0.03215	0.02141	0.347	No	-0.0309 to 0.0952
	F	Day 4 VS Day 7	0.02188	0.02068	0.567	No	-0.0390 to 0.0828
		Day 4 VS Day 14	0.03980	0.02211	0.238	No	-0.0253 to 0.1049
		Day 7 VS Day 14	0.01792	0.02068	0.677	No	-0.0430 to 0.0788
	*G	Overall test			0.066	No	
AgNO3	D	Day 4 VS Day 7	0.26834	0.04704	0.002	Yes	0.1298 to 0.4069
		Day 4 VS Day 14	0.09040	0.05029	0.238	No	-0.0577 to 0.2385
		Day 7 VS Day 14	-0.17793	0.04704	0.017	Yes	-0.3165 to -0.0394

	E	Day 4 VS Day 7	0.04807	0.06940	0.775	No	-0.1563 to 0.2525
		Day 4 VS Day 14	0.16853	0.07420	0.126	No	-0.0500 to 0.3870
		Day 7 VS Day 14	0.12046	0.06940	0.258	No	-0.0839 to 0.3249
	F	Day 4 VS Day 7	-0.02028	0.01752	0.512	No	-0.0719 to 0.0313
		Day 4 VS Day 14	-0.01469	0.01872	0.724	No	-0.0698 to 0.0405
		Day 7 VS Day 14	0.00559	0.01752	0.946	No	-0.0460 to 0.0572
	G	Day 4 VS Day 7	-0.29613	0.09490	0.039	Yes	-0.5756 to -0.0166
		Day 4 VS Day 14	-0.24425	0.10145	0.105	No	-0.5430 to 0.0545
		Day 7 VS Day 14	0.05188	0.09490	0.851	No	-0.2276 to 0.3314
Ag2S-NP	D	Day 4 VS Day 7	-0.16051	0.15662	0.586	No	-0.6218 to 0.3008
		Day 4 VS Day 14	0.01895	0.16744	0.993	No	-0.4742 to 0.5121
		Day 7 VS Day 14	0.17946	0.15662	0.519	No	-0.2818 to 0.6407
	E	Day 4 VS Day 7	-0.12881	0.08628	0.350	No	-0.3829 to 0.1253
		Day 4 VS Day 14	-0.01553	0.09224	0.985	No	-0.2872 to 0.2561
		Day 7 VS Day 14	0.11328	0.08628	0.433	No	-0.1408 to 0.3674
	F	Day 4 VS Day 7	0.00553	0.10762	0.999	No	-0.3114 to 0.3225
		Day 4 VS Day 14	-0.24720	0.11505	0.149	No	-0.5860 to 0.0916
		Day 7 VS Day 14	-0.25273	0.10762	0.114	No	-0.5697 to 0.0642
	G	Day 4 VS Day 7	0.28379	0.13375	0.155	No	-0.1101 to 0.6777
		Day 4 VS Day 14	0.24378	0.14298	0.269	No	-0.1773 to 0.6649
		Day 7 VS Day 14	-0.04000	0.13375	0.952	No	-0.4339 to 0.3539
Exposure day	Fraction	Treatment group	Mean Difference (I-J)	Std. Error	P value	Significant difference	95% Confidence Interval of difference
4-days exposure	D	AgNP VS $AgNO_3$	-0.13788	0.12676	0.555	No	-0.5268 to 0.2511
		AgNP VS Ag_2S-NP	-0.03218	0.12676	0.965	No	-0.4211 to 0.3568
		$AgNO_3$ VS Ag_2S-NP	0.10570	0.12676	0.698	No	-0.2833 to 0.4946
	E	AgNP VS $AgNO_3$	-0.15033	0.09149	0.300	No	-0.4311 to 0.1304
		AgNP VS Ag_2S-NP	-0.02369	0.09149	0.964	No	-0.3044 to .2570
		$AgNO_3$ VS Ag_2S-NP	0.12664	0.09149	0.406	No	-0.1541 to 0.4074
	F	AgNP VS $AgNO_3$	0.03969	0.02556	0.334	No	-0.0387 to 0.1181
		AgNP VS Ag_2S-NP	-0.00153	0.02556	0.998	No	-0.0799 to .0769
		$AgNO_3$ VS Ag_2S-NP	-0.04122	0.02556	0.311	No	-0.1196 to 0.0372
	G	AgNP VS $AgNO_3$	0.24852	0.17758	0.399	No	-0.2964 to 0.7934
		AgNP VS Ag_2S-NP	0.05741	0.17758	0.945	No	-0.4875 to 0.6023
		$AgNO_3$ VS Ag_2S-NP	-0.19112	0.17758	0.561	No	-0.7360 to 0.3538

7-days exposure	D	AgNP VS $AgNO_3$	0.02383	0.08999	0.962	No	-0.2274 to 0.2751
		AgNP VS Ag_2S-NP	-0.29932	0.08999	0.022	Yes	-0.5506 to -0.0481
		$AgNO_3$ VS Ag_2S-NP	-0.32315	0.08999	0.015	Yes	-0.5744 to -0.0719
	E	AgNP VS $AgNO_3$	-0.02728	0.06319	0.903	No	-0.2037 to 0.1491
		AgNP VS Ag_2S-NP	-0.07751	0.06319	0.468	No	-0.2539 to 0.0989
		$AgNO_3$ VS Ag_2S-NP	-0.05024	0.06319	0.715	No	-0.2267 to 0.1262
	F	AgNP VS $AgNO_3$	-0.00247	0.02438	0.994	No	-0.0705 to 0.0656
		AgNP VS Ag_2S-NP	-0.01788	0.02438	0.751	No	-0.0859 to 0.0502
		$AgNO_3$ VS Ag_2S-NP	-0.01541	0.02438	0.807	No	-0.0835 to 0.0527
	G	AgNP VS $AgNO_3$	0.00592	0.03122	0.980	No	-0.0813 to 0.0931
		AgNP VS Ag_2S-NP	0.39471	0.03122	<0.001	Yes	0.3075 to 0.4819
		$AgNO_3$ VS Ag_2S-NP	0.38879	0.03122	<0.001	Yes	0.3016 to 0.4760
14 days exposure	D	AgNP VS $AgNO_3$	0.07285	0.12089	0.824	No	-0.2981 to 0.4438
		AgNP VS Ag_2S-NP	0.10709	0.12089	0.668	No	-0.2638 to 0.4780
		$AgNO_3$ VS Ag_2S-NP	0.03424	0.12089	0.957	No	-0.3367 to .4052
	E	AgNP VS $AgNO_3$	0.06103	0.02457	0.104	No	-0.0144 to 0.1364
		AgNP VS Ag_2S-NP	0.00361	0.02457	0.988	No	-0.0718 to 0.0790
		$AgNO_3$ VS Ag_2S-NP	-0.05742	0.02457	0.126	No	-0.1328 to 0.0180
	F	AgNP VS $AgNO_3$	-0.01479	0.12075	0.992	No	-0.3853 to 0.3557
		AgNP VS Ag_2S-NP	-0.28852	0.12075	0.118	No	-0.6590 to 0.0820
		$AgNO_3$ VS Ag_2S-NP	-0.27373	0.12075	0.137	No	-0.6442 to 0.0968
	*G	AgNP VS $AgNO_3$	-0.11909	0.10425	0.526	No	-0.4390 to 0.2008
Treatment group	Exposure day	Fraction	Mean Difference (I-J)	Std. Error	P value	Significant difference	95% Confidence Interval of difference
AgNP	Day 4	D VS E	0.16164	0.04080	0.018	Yes	0.0310 to 0.2923
		D VS F	0.15470	0.04080	0.022	Yes	0.0241 to 0.2853
		D VS G	-0.27213	0.04080	0.001	Yes	-0.4028 to -0.1415
		E VS F	-0.00694	0.04080	0.998	No	-0.1376 to 0.1237
		E VS G	-0.43378	0.04080	<0.001	Yes	-0.5644 to -0.3031
		F VS G	-0.42684	0.04080	<0.001	Yes	-0.5575 to -0.2962
	Day 7	D VS E	-0.01997	0.02540	0.859	No	-0.0954 to 0.0554
		D VS F	0.06996	0.02540	0.072	No	-0.0054 to 0.1454

		D VS G	-0.43228	0.02540	<0.001	Yes	-0.5077 to -0.3569
		E VS F	0.08992	0.02540	0.018	Yes	0.0145 to 0.1653
		E VS G	-0.41231	0.02540	<0.001	Yes	-0.4877 to -0.3369
		F VS G	-0.50223	0.02540	<0.001	Yes	-0.5776 to -0.4268
	Day 14	D VS E	0.23914	0.11278	0.226	No	-0.1220 to 0.6003
		D VS F	.31483	0.11278	0.089	No	-0.0463 to 0.6760
		*D VS G			0.910	No	
		E VS F	0.07569	.11278	0.905	No	-0.2855 to 0.4369
		*E VS G			0.141	No	
		*F VS G			0.013	Yes	
AgNO3	Day 4	D VS E	0.14919	0.11526	0.591	No	-0.2199 to 0.5183
		D VS F	0.33227	0.11526	0.078	No	-0.0368 to 0.7014
		D VS G	0.11427	0.11526	0.758	No	-0.2548 to 0.4834
		E VS F	0.18309	0.11526	0.436	No	-0.1860 to 0.5522
		E VS G	-0.03492	0.11526	0.990	No	-0.4040 to 0.3342
		F VS G	-0.21800	0.11526	0.303	No	-0.5871 to 0.1511
	Day 7	D VS E	-0.07108	0.02220	0.033	Yes	-0.1370 to -0.0052
		D VS F	0.04365	0.02220	0.253	No	-0.0222 to 0.1096
		D VS G	-0.45019	0.02220	<0.001	Yes	-0.5161 to -0.3843
		E VS F	0.11473	0.02220	0.001	Yes	0.0488 to 0.1806
		E VS G	-0.37912	0.02220	<0.001	Yes	-0.4450 to -0.3132
		F VS G	-0.49385	0.02220	<0.001	Yes	-0.5597 to -0.4279
	Day 14	D VS E	0.22732	0.04551	0.005	Yes	0.0816 to 0.3731
		D VS F	0.22718	0.04551	0.005	Yes	0.0814 to 0.3729
		D VS G	-0.22038	0.04551	0.006	Yes	-0.3661 to -0.0746
		E VS F	-0.00013	0.04551	1.000	No	-0.1459 to 0.1456
		E VS G	-0.44769	0.04551	<0.001	Yes	-0.5934 to -0.3020
		F VS G	-0.44756	0.04551	<0.001	Yes	-0.5933 to -0.3018
Ag2S-NP	Day 4	D VS E	0.17013	0.16590	0.740	No	-0.3611 to 0.7014
		D VS F	0.18535	0.16590	0.690	No	-0.3459 to 0.7166
		D VS G	-0.18255	0.16590	0.699	No	-0.7138 to 0.3487
		E VS F	0.01522	0.16590	1.000	No	-0.5160 to 0.5465
		E VS G	-0.35268	0.16590	0.224	No	-0.8839 to 0.1786
		F VS G	-0.36790	0.16590	0.198	No	-0.8992 to 0.1634
	Day 7	D VS E	0.20184	0.09543	0.203	No	-0.0815 to 0.4852
		D VS F	0.35140	0.09543	0.014	Yes	0.0681 to 0.6347
		D VS G	0.26175	0.09543	0.074	No	-0.0216 to 0.5451
		E VS F	0.14956	0.09543	0.431	No	-0.1338 to 0.4329
		E VS G	0.05991	0.09543	0.921	No	-0.2234 to 0.3432

		F VS G	-0.08965	0.09543	0.785	No	-0.3730 to 0.1937
	Day 14	D VS E	0.13566	0.12534	0.709	No	-0.2657 to 0.5370
		D VS F	-0.08079	0.12534	0.915	No	-0.4822 to 0.3206
		D VS G	0.04229	0.12534	0.986	No	-0.3591 to 0.4437
		E VS F	-0.21644	0.12534	0.371	No	-0.6178 to 0.1849
		E VS G	-0.09337	0.12534	0.876	No	-0.4947 to 0.3080
		F VS G	0.12308	0.12534	0.763	No	-0.2783 to 0.5245

Appendix 5. The results of one-way ANOVA followed by Tukey HSD post-hoc test in comparison of the MT concentration per mg of total protein present in worms exposed to spiked soil (AgNPs, AgNO3, and Ag2S-NPs) of all time points (4-days, 7-days, and 14-days). Positive confidence interval means that measured mass of Ag is higher in first factor, and vice versa. * indicates that group of data did not come from normal distribution. Therefore, Kruskal-Wallis one-way ANOVA (k samples) multiple comparisons of all pairwise were run to determine the significance.

Treatment groups	Compared groups	Mean Difference (I-J)	Std. Error	P value	Significant different	95% Confidence Interval of difference
Day 7	Control vs Cd	0.05405	0.09695	0.999	No	-0.2846 to 0.3927
	Control vs AgNP	0.03455	0.09695	1.000	No	-0.3041 to 0.3732
	Control vs $AgNO_3$	-0.15807	0.09695	0.727	No	-0.4967 to 0.1806
	Cd vs AgNP	-0.01950	0.09695	1.000	No	-0.3581 to 0.3191
	Cd vs $AgNO_3$	-0.21212	0.09695	0.409	No	-0.5508 to 0.1265
	AgNP vs $AgNO_3$	-0.19262	0.09695	0.520	No	-0.5313 to 0.1460
Day 14	Control vs Cd	-0.20391	0.09695	0.454	No	-0.5425 to 0.1347
	Control vs AgNP	-0.18791	0.09695	0.548	No	-0.5265 to 0.1507
	Control vs $AgNO_3$	-0.06335	0.10839	0.999	No	-0.4420 to 0.3153
	Cd vs AgNP	0.01600	0.09695	1.000	No	-0.3226 to 0.3546
	Cd vs $AgNO_3$	0.14055	0.10839	0.887	No	-0.2381 to 0.5192
	AgNP vs $AgNO_3$	0.12455	0.10839	0.935	No	-0.2541 to 0.5032
Control	7 days vs 14 days exposure	-0.14317	0.09695	0.808	No	-0.4818 to 0.1955
Cd	7 days vs 14 days exposure	-0.40112	0.09695	0.015	Yes	-0.7398 to -0.0625
AgNP	7 days vs 14 days exposure	-0.36562	0.09695	0.030	Yes	-0.7043 to -0.0270
AgNO3	7 days vs 14 days exposure	-0.04846	0.10839	1.000	No	-0.4271 to 0.3302

Appendix 6. Data management plan MSc Thesis Yiming Liu

Heading	**Implementation**
Organizational context	Name: Yiming Liu Registration number: 950106523080 Supervisors: - Dr.ir. NW (Nico) van den Brink - Marta Baccaro File path: W:\PROJECTS\TOX_Research-data\Nico van den Brink\Marta Baccaro\Yiming Liu
Short description of your research	This study aims to investigate the potential different toxicokinetic of nano form of Ag and Ag ions, it is relevant to identify the fate of the three forms of silver (Ag-NPs, Ag_2S-NPs, and $AgNO_3$) within the earthworms *Eisenia fetida* by quantification of the metal within the subcellular fractions for 14 days.
Data management rules (who is responsible for what?)	The MSc student herself (Yiming) is in principle responsible for the primary storage of the data generated within the thesis according to the data management plan of the Division of Toxicology. Details of the experiments are described in the lab journal. These descriptions should be linked to the raw data files that are produced, using the names of the raw data files. For the data that are used in the thesis report (e.g. data underlying the Figures, Tables, etc), data management tables will be created in Word that indicates where the data can be found. This Word document will be checked by the supervisor (Marta Baccaro) and is stored on W:\PROJECTS\TOX_Research-data\Nico van den Brink\Marta Baccaro\Yiming Liu The lab journal will be handed over to the supervisor at the end of the project.
What type of research data will be produced?	1. Results of silver quantification in ICP-MS. 2. Results of metallothionein quantification by spectrophotometer: a. R: Excel b. P: Analysis results 3. Total Silver quantification in ICP-MS. a. R: Excel b. P: Analysis results Presented data • Thesis report 4. A lab journal will be kept to record the experiments throughout the project.
Software choices	• Raw data ❖ ICP-MS read-outs, Excel ❖ Spectrophotometer read-outs, Excel • Processed data ❖ Statistics; Excel Presented data: Word & PowerPoint
File name and description	• 20190213_weights_Yiming

	❖ Weights of earthworm samples • 20190131_N1054_Yiming_process ❖ Raw data of Ag concentration for 7-days exposed worms • 20190214_N1054_Yiming_process ❖ Raw data of Ag concentration for 4-days and 14-days exposed worms • 20190219_N1054_Yiming_process ❖ Raw data of Ag concentration in whole worms • 20190327_MT_4days ❖ Raw data of metallothionein concentration for 4-days exposed worms • 20190412_MT_7days ❖ Raw data of metallothionein concentration for 7-days exposed worms • 20190417_MT_14days ❖ Raw data of metallothionein concentration for 14-days exposed worms • 20190328_data analysis_Ag conc._Yiming ❖ Data analysis of Ag concentration in worms • 20190418_data analysis_MT conc._Yiming ❖ Data analysis of metallothionein and protein concentration in worms
What is the amount of the data, and how will the amount increase in time.	ca. 1 Gb
Sharing and ownership	The data will be shared in accordance to the "Data management plan of the Division of Toxicology, Wageningen University" Data obtained at the RIKILT will be stored and owned by the RIKILT, but the place of storage will be provided in the data management table.
Documentation and data management table	For data used in the thesis report (e.g. data underlying Figures, Tables, etc.), a data management table will be created in Word indicating where data can be found. This Word document will be checked by the supervisor (Marta Baccaro) and is stored at W:\PROJECTS\TOX_Research-data\Nico van den Brink\Marta Baccaro\Yiming Liu A lab journal will be kept to record the experiments throughout the project.
Short term storage	Short-term storage will be on Yiming her personal WUR account (M-drive) or on computers that are linked to equipment at which data has been obtained.
Long term storage	The data underlying the thesis report (e.g. Figures, Tables and calibration curves), will be stored together with the data management tables at: \PROJECTS\TOX_Research-data\Nico van den Brink\Marta Baccaro\Yiming Liu These data will be transferred to a massive storage location after the project for long term storage.

Table 1: Raw files (20181214_R_Liu200_2):

Name	Yiming Liu
Registration number	950106523080
Start date of thesis	20191101
Supervisor 1	Nico van den Brink
Supervisor 2	Marta Baccaro
File path	W:\PROJECTS\TOX_Research-data\Nico van den Brink\Marta Baccaro\Yiming Liu

File name	Device	Description	Processed Y/N
20190213_weights_Yiming		Weights of earthworm samples	Y
20190131_N1054_Yiming_process	ICP-MS Nexion 350D	Raw data of Ag concentration for 7-days exposed worms	Y
20190214_N1054_Yiming_process	ICP-MS Nexion 350D	Raw data of Ag concentration for 4-days and 14-days exposed worms	Y
20190219_N1054_Yiming_process	ICP-MS Nexion 350D	Raw data of Ag concentration in whole worms	Y
20190327_MT_4days		Raw data of metallothionein concentration for 4-days exposed worms	Y
20190412_MT_7days		Raw data of metallothionein concentration for 7-days exposed worms	Y
20190417_MT_14days		Raw data of metallothionein concentration for 14-days exposed worms	Y

YYYYMMDD: year-month-day, INI: unique wur-name (louis001), X: experiment number (1, 2, 3, etc.)

Table 2: Processed files (20181214_P_Liu200_2):

Name	Yiming Liu
Registration number	950106523080
Start date of thesis	20191101
Supervisor 1	Nico van den Brink
Supervisor 2	Marta Baccaro
File path	W:\PROJECTS\TOX_Research-data\Nico van den Brink\Marta Baccaro\Yiming Liu

File name*	Program	Based on raw file(s)	Description	Presented in Figure
20190328_data analysis_Ag conc._Yiming	Excel	20190131_N1054_Yiming_process 20190214_N1054_Yiming_process 20190219_N1054_Yiming_process	Combined ICP-MS data and graphs of worms	3 & 4
20190418_data analysis_MT conc._Yiming	Excel	20190327_MT_4days 20190412_MT_7days 20190417_MT_14days	Combined data of metallothionein and protein concentration in worms	5
20190418_P_Ag in whole worms	Spss	20190219_N1054_Yiming_process	Output: plot Ag concentration in whole worms	3
20190418_P_Ag in fractions_Ag-NP group	Spss	20190219_N1054_Yiming_process 20190131_N1054_Yiming_process 20190214_N1054_Yiming_process 20190219_N1054_Yiming_process	Output: plot Ag concentration in subcellular fractions of worms exposed to Ag-NPs over time	4
20190418_P_Ag in fractions_$AgNO_3$ group	Spss	20190219_N1054_Yiming_process 20190131_N1054_Yiming_process 20190214_N1054_Yiming_process	Output: plot Ag concentration in subcellular fractions of worms exposed to $AgNO_3$ over time	4

		20190219_N1054_Yiming_process		
20190418_P_Ag in fractions_Ag_2S-NP group	Spss	20190219_N1054_Yiming_process 20190131_N1054_Yiming_process 20190214_N1054_Yiming_process 20190219_N1054_Yiming_process	Output: plot Ag concentration in subcellular fractions of worms exposed to Ag_2S-NPs over time	4
20190418_P_MT	Spss	20190327_MT_4days 20190412_MT_7days 20190417_MT_14days	Output: plot metallothionein and protein concentration in worms exposed to Ag-NPs, $AgNO_3$, and Ag_2S-NPs over time	5

*date is the first date you start processing these data.