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Are long-term exposure studies needed? Short-term toxicokinetic model predicts the uptake of metal nanoparticles in earthworms after nine months

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ABSTRACT

Uptake of most metal nanoparticles (NPs) in organisms is assumed to be mainly driven by the bioavailability of the released ions, as has been verified in controlled and short-term exposure tests. However, the changeability of NPs and the dynamic processes which NPs undergo in the soil environment, bring uncertainty regarding their interactions with soil organisms over a long period of time. To assess the potential impacts of long-term exposure scenarios on the toxicokinetic of metal NPs, earthworms *Eisenia fetida* were exposed to soils spiked with pristine Ag-NP, aged Ag-NP (Ag_2S-NP) and ionic Ag for nine months, and results were compared to those from a similar short-term (28 days) experiment, conducted under similar conditions. Overall, there were no statistical differences between long-term accumulation patterns in earthworms exposed to pristine Ag-NP and $AgNO_3$, while for Ag_2S-NP, the amount of Ag internalized after 9 months was five times lower than for the other treatments. Average Ag concentrations in soil pore water in all treatments did not change over time, however the soil pH decreased and electrical conductivity increased in all treatments. Metallothionein concentrations in exposed earthworms were not statistically different from levels in untreated earthworms. Finally, the short-term toxicokinetic models predicted the bioaccumulation in earthworms exposed to Ag-NP, $AgNO_3$ after nine months on the whole. Although the bioaccumulation for Ag_2S-NPs was somewhat under-predicted, the rate of accumulation of Ag_2S-NPs is much lower than that of Ag-NPs or $AgNO_3$ and thus potentially of lower concern. Nevertheless, better understanding about the exposure kinetics of Ag_2S-NP would help to address potential nano-specific toxicokinetic and toxicodynamics, also of other sulfidized metal NPs.

1. Introduction

Highlighting communalities and differences between the toxicokinetics and toxicodynamics of metal nanoparticles (NPs) and bulk materials in organisms has been the focus of many studies in the last decade (Unrine et al., 2010; Croteau et al., 2011; Diez-Ortiz et al., 2015). Although this scientific effort advanced our knowledge regarding the behavior of NPs under relatively stable and controlled conditions, it did not resolve how NP transformations under environmentally relevant conditions influence their uptake in organisms, e.g. realistic exposure forms, concentrations and exposure periods. Metal NPs are the most used nanoparticles which are released to soils (Vance et al., 2015). Their behavior in the soil is mainly affected by dissolution and redox reactions (Zhang et al., 2018). In our previous study (Baccaro et al., 2018) we demonstrated that in earthworms exposed to Ag-NP and $AgNO_3$ for 28 days, ~ 85% (average of both treatments) of the accumulated Ag was present in the earthworms as ions while accumulation patterns did not statistically differ between ionic Ag and pristine Ag-NPs. Both treatments showed particulate Ag in the earthworms, resulting from biogenic formation. The bioaccumulation of Ag from Ag_2S-NPs was significantly lower, likely because of their low dissolvability. Although it appears more and more clear that the availability of ions, released from metal NPs may explain most of the uptake (Laycock et al., 2016), still some studies consistently report particulate uptake (Wang et al., 2017; Wagener et al., 2019). In soil, besides dissolution also other processes such as heteroaggregation and adsorption of NPs to soil particles and dissolved organic matter occur (Cornelis et al., 2014). Since such speciation processes can strongly affect long-term bioavailability, they should be studied, using dynamic approaches under long-term exposure conditions (Coutris et al., 2012; Vijver et al., 2018). However, due to difficulties to select standardized tests that allow assessing effects of long-term environmental fate processes on NP bioavailability, it is an urgent priority to study and compare the accumulation of pristine and aged NPs with the ionic form in soil organisms over realistic long-term

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exposure time frames. To address this, in the present study we exposed earthworms *Eisenia fetida* to pristine Ag-NP, aged Ag-NP (Ag_2S-NP) and $AgNO_3$ in natural soil for nine months and quantified and modeled their uptake over time, deriving uptake rate constants for each Ag form. This also aimed to bring to light the impact of the potential dissolution and of other processes occurring in natural soil during a long-time window on the uptake of metal NPs.

Therefore, exposure conditions were monitored over time, for instance soil pore water was extracted to track changes in the more bioavailable metal fraction of the soil (< 0.45 μm) (Peijnenburg et al., 1997) and to assess if pore water Ag concentrations can explain bioaccumulated concentrations in the different Ag forms. Values of pH were also measured over time to assess as a driver of NP-dissolution. Assuming that NPs would release ions over time, metallothionein (MT) levels in the earthworms were quantified at different time points. Indeed, the synthesis of MT has been widely reported for dissolving metals and we hypothesize that an extended dissolution of NPs would be reflected in the prolonged synthesis of MT. (Correia et al., 2002; Amiard et al., 2006). Finally, long-term uptake and elimination rate constants were quantified after nine months, and compared to a short-term experiment (28 days) which used the same experimental conditions, except for the duration (Baccaro et al., 2018). This allowed to conclude whether results of the short-term toxicokinetic study may be applied to predict outcomes of long-term exposure scenarios, which are often more environmentally relevant.

2. Material and methods

2.1. Nanoparticles

The study was conducted with uncoated Ag-NPs (12.3 g Ag L^{-1} in 5.5 mM sodium citrate with 25 mM tannic acid, 47.3 ± 5.3 nm) and Ag_2S-NPs (3.6 g Ag L^{-1}, 5.5 mM Polyvinylpyrrolidone (PVP), 20.3 ± 9.8 nm). The batches of nanoparticles were synthesized by Applied nanoparticles (Barcelona, Spain) and supplied within the EU NanoFase project and similar to the ones used in other studies (Baccaro et al., 2018; Clark et al., 2019). Characterization of Ag_2S-NPs highlighted some aggregation to a certain extent. Particle characterization data are reported in Supplemental material – paragraph S1.

2.2. Earthworms and experimental soil

Earthworms *Eisenia fetida* were purchased from Lasebo (Nijkerkerveen, The Netherlands). After 2 weeks acclimatization in the experimental soil at 20 °C, 24 h light, earthworms were selected based on their weight, 380 ± 90 mg (average ± standard deviation, n = 336). Before the start of the experiment, earthworm's gut was allowed to empty in petri dish with moist filter paper for 24 h. Natural soil was collected from Proefboerderij Kooijenburg, Marwijksoord, The Netherlands, air dried and sieved (5 mm sieve openings).

2.3. Exposure soil preparation

Each experimental unit consisted of a round glass jar (4.5 cm of diameter, 6 cm height) filled with 90 g of air-dried soil with additional Milli Q water (20% w/w, ~ 47% water holding capacity (WHC)). Soils were spiked with Ag-NPs, Ag_2S-NPs and $AgNO_3$ to reach a nominal concentration of 15 mg Ag kg^{-1} dry weight soil for all treatments. This exposure concentration was chosen considering both modeled environmental Ag-NP concentrations (Gottschalk et al., 2009, 2013; Sun et al., 2014) and concentrations that enable accurate quantification of exposures and uptake. In the control soil, only water was added. Soil, water and Ag were homogeneously mixed by an automatic mixer for 3 min. After filling with the spiked soil, jars were closed with a lid and kept in the incubator at 20 °C for 24 h, before the earthworms were added. Due to the extensive duration of the experiment, in order to avoid production of cocoons and development of the second generation of earthworms, one single adult earthworm was randomly introduced into each experimental unit. A total of 336 jars were prepared (84 jars per each treatment and control). The lid was replaced by a plastic net (125 μm openings) to allow air circulation, however this also induced evaporation of water from the soil. Therefore, every ~ 3 days, jars were weighed and the loss of water was replenished by addition of Milli Q water. Additionally, every two months 1.5 g of oven dry horse manure was added to each jar as feeding. Sampling time points were set at 7, 14, 28, 84, 168, 270 days of exposure. For each time point 12 jars of each treatment and control were sampled, except for the last time for which 24 jars were analyzed. Soil was separately collected in plastic bags and stored in the freezer at − 20 °C. Worms were collected, carefully washed in Milli Q water, dried on an absorbent tissue and transferred to a petri dish with moist paper in the incubator at 20 °C where were allowed to purge their gut for 24 h. After depuration, earthworms were weighed, snap-frozen in liquid nitrogen and chopped for subsequent analysis.

2.4. Electrical conductivity and pH

Soil collected after 7 and 270 days of exposure was analyzed for pH and electrical conductivity (EC). Only soils of the jars where earthworms were found alive were included in the analysis. An aliquot of 10 g of soil was weighted to the nearest 0.01 g in a polyethylene tube and 50 mL of ultrapure water were added (Slavich and Petterson, 1993). The suspension was shaken at room temperature (20 °C ± 1 °C) for one hour after which it was allowed to settle overnight before the measurement (Handy Lab 680FK, SI Analytics, Germany).

2.5. Extraction of soil pore water for Ag analysis

Soil pore water was extracted by adaptation of the method reported in Waalewijn-Kool et al. (2014). Aliquots of wet soil (25 g weighed to the nearest 0.1 g, n = 3) of each treatment of each time point were equilibrated at 100% WHC for 18 h with ultrapure water, assuming their water content at the moment of sampling being 20% w/w. Pore water was extracted in a two-step filtering process. At first, soil was centrifuged through a cell sieve with 40 μm pores (EASYstrainer Cell Sieves, Greiner Bio-One, Germany) at 2000*g* for 20 min (Hermle Z400K, Germany) and the finer soil fraction was collected together with the pore water. This was further filtered through a 0.45 μm cellulose acetate syringe filter (Chromafil, Macherey-Nagel, Germany). In order to avoid the adsorption of Ag (both ionic and particulate) on the surface of the materials used for the extraction, cell sieves and filters were conditioned by soaking them in a solution 0.1 M $CuNO_3$ + H_2O (99.9%, Sigma Aldrich, USA) overnight before use; syringes were rinsed with the same solution. Samples were stored at 4 °C overnight before analysis.

2.6. Extraction and analysis of Ag in earthworms, soil and soil pore water

Soil and earthworm tissues (aliquots of ~0.3 g) were acid digested by microwave treatment (MARS 6, CEM Corporation, USA) in Teflon vessels containing 8 mL of aqua regia (1: 3 nitric acid (69%, Merck, Germany)–hydrochloric acid (37%, Merck, Germany)) in order to extract the Ag (Element, 2007; Makama et al., 2015). Digestion was performed applying a temperature ramp from 160 °C (20 min) to 200 °C (40 min). Nitric acid was added to the soil pore water samples (1:1) two hours before analysis. After proper dilutions, the samples were analyzed by ICP-MS Nexion 350D (Perkin-Elmer Inc., Waltham, USA). The isotopes monitored were silver (*m/z* 107) and rhodium (*m/z* 103). The calibration curve was prepared by diluting Ag^+ (standard stock solution 1000 mg L^{-1} Ag, Merck, Germany) in a matching acid matrix. Rhodium (standard stock solution 1000 mg L^{-1} Rh, Merck, Germany) was used as an internal standard. For every batch of samples, analytical quality was checked by blanks and external standard of Ag^+ (1000 mg L^{-1} Ag, Merck, Germany). The average recovery resulted to be 98 ± 24%. The

limit of detection (LOD) of silver (m/z 107) was calculated as the mean of digested blank + 3 σ blank and resulted to be 0.31 μg L^{-1}. The limit of quantification (LOQ) was calculated as the mean of digested matrix blank + 10 σ blank matrix and resulted to be 0.59 μg L^{-1}.

2.7. Metallothionein analysis

Semi-quantification of MT was performed according to the protocol of Viarengo et al. (1997) with minor modifications. Briefly, earthworms were homogenized in three volumes of 20 mM Tris–HCl buffer, pH 8.6 containing 0.5 M sucrose, 0.006 mM leupeptine, and 0.5 mM phenylmethylsulfonylfluoride as antiproteolytic agents and 0.01% β-mercaptoethanol as a reducing agent using an automatized glass tissue homogenizer (B. Braun, Melsungen, Germany). Homogenate was centrifuged at 21,500*g* for 40 min at 4 °C. An aliquot (200 μL) of the resulting supernatant was collected for protein content analysis by Pierce assay kit (Thermo Fisher, Waltham, USA). Another aliquot (300 μL) was used for two consecutive ethanol precipitations. Absolute ethanol equilibrated at − 20°C (315 μL) was added to the supernatant. Centrifugation was run at 16,000*g* for 5 min at 4 °C. The supernatant was collected and mixed with 1.5 mL of absolute ethanol (−20°C). After incubation at − 20 °C for 60 min and centrifugation under the same condition, the supernatant was discarded. The obtained pellet, containing MT, was dissolved by adding 25 μL of 0.25 M NaCl and 25 μL of a mixture of 4 mM EDTA/1 M HCl. A volume of 1.95 mL 2 M NaCl containing 0.43 mM DTNB (5.5-dithiobis-2-nitrobenzoic acid) buffered with 0.2 M Na-phosphate, pH 8, was then added to the sample. Samples were vortexed, centrifuged at 16,000*g* at 4 °C for 2 min, transferred in triplicate to microplates and the absorbance was measured at 412 nm against a blank by a spectrophometer (SpectraMax iD3, Molecular Devices, USA). MT concentrations were quantified as sulhydrylic group equivalents by using a calibration curve of reduced glutathione (GSH). The curve represented 10–80 nano moles of sulfhydrylic group equivalents. Final data were expressed in μg MT mg^{-1} of proteins, considering that each MT in the earthworm contains twenty cysteine residues and assuming the metallothionein molecular weight being 6000 daltons (Sutherland and Stillman, 2011). Metallothionein concentrations quantified in earthworms exposed to Cd (30 mg kg^{-1} dry weight soil) with the same experimental condition for 21 days were set as positive control (Stürzenbaum et al., 2001; Calisi et al., 2014).

2.8. Modeling of uptake and elimination kinetic rate constants

The internal concentrations of Ag in the earthworms were fitted with a one compartment model for Ag-NP, Ag_2S-NP and $AgNO_3$. Similar models are widely used to model the toxicokinetics of metals in model organisms (Ardestani et al., 2014). In the present study, the model used allowed the calculation of the uptake kinetic constant (k_1), elimination kinetic constant (k_2) as proposed in van den Brink et al. (2019). The model is described by the Eq. (1),

$$C_{int} = C_0 + k_1/k_2 * C_{exp} * (1 - e^{-k_2 * t}) \quad (1)$$

where C_{int} is the internal metal concentration in the earthworms (mg kg^{-1} wet body weight), C_0 the initial metal concentration in the earthworms (mg kg^{-1} wet body weight) which in the present work is assumed to be equal to 0, k_1 the uptake rate constant (mg Ag kg dry soil mg Ag^{-1} kg^{-1} wet body weight day^{-1}), k_2 the elimination rate constant (day^{-1}), C_{exp} the measured exposure total metal concentration (mg kg^{-1} dry soil), t the exposure time (day). Standard error and 90% confidential intervals of all parameters were calculated. Kinetic rate constants of the different treatments were compared.

2.9. Comparison of the uptake kinetic rate constants between short-term and long-term exposure

In order to assess if the kinetic rate constants derived for the short-term exposure were able to predict the bioaccumulation of all three forms of Ag after nine months, the kinetic rate constants k_1 and k_2 of the short-term experiments were used to calculate the Ag concentrations in earthworm after 270 days of exposure. Kinetic rate constants k_1 and k_2 from short- and long-term toxicokinetic studies were compared between exposure scenarios.

2.10. Statistical analysis

Differences in mortality and weight change amongst treatments were tested by Fisher exact test and one-way ANOVA followed by Tukey multiple comparisons, respectively ($p < 0.05$). Differences in the accumulation in earthworms amongst treatments and within the same treatment over time were tested by one-way ANOVA followed by Tukey multiple comparisons ($p < 0.05$). The differences in pH and EC amongst treatments were assessed by one-way ANOVA followed by Tukey multiple comparisons ($p < 0.01$). The differences in pH and EC within the same treatment between 7 and 270 days were assessed by one-way ANOVA followed by t-test ($p < 0.01$). Statistical significance amongst the concentrations of Ag in soil pore water were tested by Bonferroni's multiple comparison following one-way ANOVA ($p < 0.001$). Differences between concentrations of MT in exposed earthworms and control were tested by Dunnett's multiple comparison following one-way ANOVA ($p < 0.05$). Difference between the positive control (Cd) and the control was tested by t-test ($p < 0.05$). To determine the significance of differences in kinetic rate constants k_1 and k_2 amongst treatments and between short- and long-term exposure, a t-test was used. All the analyses were performed by GraphPad Prism 7.0.

3. Results

3.1. Earthworm mortality and loss of weight

At the last sampling dates (day 168 and day 270) missing (decomposed) earthworms were recorded. At day 270, less than 30% of control and Ag_2S-NP exposed earthworms were still alive, which may have been related to natural mortality since no toxicity was observed at the earlier sampling dates. For Ag-NP and $AgNO_3$ exposure the survival was somewhat higher, being 75% and 62.5%, respectively. There are statistical differences in the survival rates between control and Ag_2S-NP exposed groups on the one hand and Ag-NP and $AgNO_3$ exposed groups on the other (Fig. 1a, Tables S2). Weight change of alive earthworms (percentage of the difference between the weight at the sampling time and the weight at the start of the exposure) is reported in Fig. 1b. A substantial loss of weight was recorded for all the treatments from day 84 day onwards, reaching a − 50% change in weight on average at day 270. However, no statistical difference was found between treatments (Tables S3).

3.2. Accumulation of Ag in earthworms

Actual exposure concentrations in soil were determined to be 13.2 ± 0.3, 15.9 ± 1.6, 12.5 ± 0.7 mg Ag kg^{-1} dry weight for soils spiked with Ag-NP, Ag_2S-NP and $AgNO_3$, respectively (mean ± standard deviation). Ag in soil of control jars was randomly measured and resulted to be 0.03 ± 0.01 mg Ag kg^{-1} soil dry weight (average ± standard deviation, n = 6). Internalized Ag concentrations in the depurated earthworms are shown in Fig. 2 (for details see Table S4). Concentrations of Ag in earthworms exposed to Ag-NP and $AgNO_3$ did not show any statistical difference at any time point (Tables S5), while concentrations of Ag in earthworms exposed to Ag_2S-NP were significantly lower than in earthworms exposed to either Ag-NP or $AgNO_3$ at

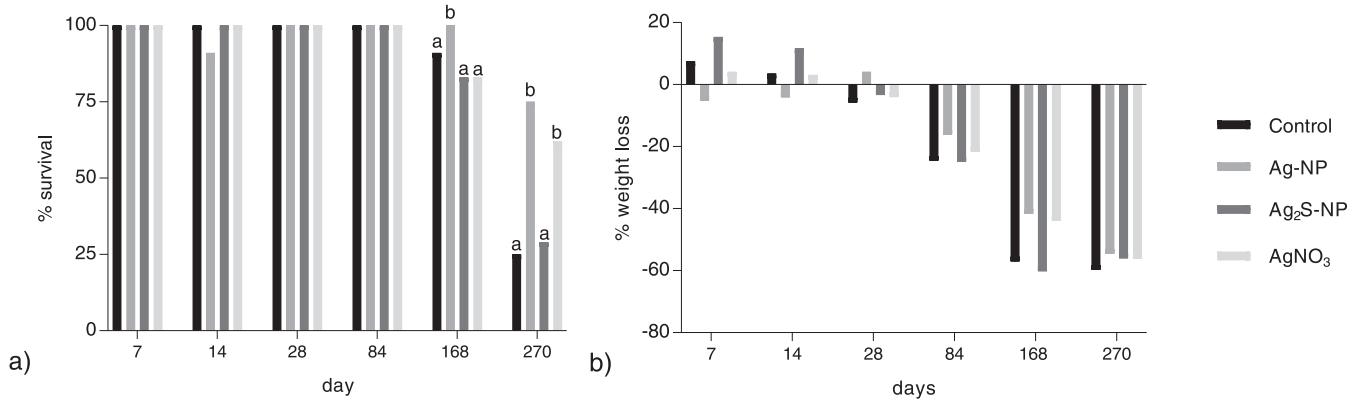

Fig. 1. Percentage of survival (a) and weight loss (b) of the survivals compared to time 0 (n = 12 for all treatments and time point except for day 270 for which n = 24).

all the time points. When testing differences amongst the time point data of the same treatment, a significant increase occurred in all silver exposures. However, no statistically difference could be detected between 168 days and 270 days exposure in all treatments (Tables S6).

3.3. Change of pH and electrical conductivity in the soil

Soil spiked with Ag_2S-NP had a lower pH than the other treatments after 7 days of incubation (Fig. 3), probably due to the release of the sulfur. At day 270, the pH values of all treatments were statistically lower than the ones at day 7. The final pH in the soils did not differ between treatments anymore, all had decreased to approximately a pH of 5.5–5.6.

At day 7 electrical conductivity (EC) in control and $AgNO_3$ soils was highly variable with average ± standard deviation values equal to 154.25 ± 133.85 and 93.69 ± 72.04 μS cm^{-1}, respectively. However, no statistical differences were apparent between the treatments. After 270 days of incubation, EC significantly increased in all the treatments between 182.93 ± 49.76 and 215.71 ± 29.32 μS cm^{-1}. As for the pH, the change of EC led to no statistically different values for all the treatments at day 270. Results of the statistical analysis are reported in the Tables S7 and S8.

3.4. Soil pore water over time

Ag concentrations in soil pore water extracted from control soils (n = 3) were below LOD. Ag concentrations in soil pore water extracted from spiked soils did not significantly differ among treatments and within the same treatment over time, and these results are shown in Fig. 4 (statistical analysis in Tables S9 and S10).

3.5. Metallothionein quantification

The concentrations of metallothioneins in the earthworms unexposed and exposed to Ag-NP, Ag_2S-NP, $AgNO_3$ did not differ significantly from the control with the exception of the MT concentration in the earthworms exposed to $AgNO_3$ for 6 months (Fig. S11). Earthworms exposed to cadmium (positive control) showed higher MT levels than unexposed earthworms except for the control at day 84 (Tables S11).

3.6. Toxicokinetic rate constants and comparison between short- and long-term models

Table 1 shows the uptake kinetic rate constant (k_1) and the elimination kinetic rate constant (k_2) in earthworms exposed to Ag-NP, Ag_2S-NP and $AgNO_3$ for 28 days (short-term) (Baccaro et al., 2018) and nine months (long-term) (present study). The table also includes the kinetic bioaccumulation factor (BAF_k) calculated according to the OECD no 317 (OECD, 2010) as $BAF_k = k_1\, k_2^{-1}$ and corrected for dry body weight (dry body weight = 16% wet body weight) (Hidalgo et al., 2017). Comparison between the uptake kinetic rate constants (k_1) of the different treatments in the long-term study showed significant differences between Ag-NP and Ag_2S-NP and between $AgNO_3$ and Ag_2S-NP while no statistically significant differences were found amongst the three elimination kinetic rate constants k_2. Comparison between the uptake kinetic rate constants of the long-term model and the ones of the short-term model highlighted statistical differences. In particular, both uptake and elimination kinetic rate constants k_1 and k_2 between long-term and short-term experiment were statistically different for all the treatments. Results of the statistical analysis are reported in Tables S11. Confidential intervals for the regression lines were calculated for both models (long- and short-term exposure) and results are plotted in Fig. 5. BAF_k values in the long-term exposure showed higher bioaccumulation for Ag-NP and $AgNO_3$ than Ag for $_2S$-NP. These differences between treatments were found with the same of order of magnitude also in the short-term exposure.

4. Discussion

4.1. Experimental conditions

It is known that population densities affect growth and development of earthworms due to the competition for food and space while the costs of reproduction limit the energy available for new tissue formation (Klok, 2007). According to this, it was expected that, since the worms were kept individually in the experiment, their weight would increase. However, they lost weight over time and this cannot be related to metal exposure since also the untreated earthworms lost weight, to an extent that was not statistically different from that observed for the exposed worms. A hypothesis to explain this observation is that the decrease in biomass could have been caused by aging of the earthworms, considering that the earthworms selected at the beginning of the experiment were clitellated adults with weight between 240 and 650 mg, however of undefined age. This could be supported by the high variance of the weight change amongst earthworms within the same sampling time (data not shown). Another explanation may be a lack of nutrients. Feed was limited to 1.5 g of oven dry horse manure every two months. This resulted in an amount that fulfilled the typical consumption rate of organic matter in earthworm (Whalen and Parmelee, 1999). OECD no 317 suggests an addition of a higher amount of organic matter if artificial soil is used, however no details are provided in the case of natural soil as used in the current study. The above-mentioned feed amount was chosen in order to avoid an increase of the amount of organic matter in the soil over time, potentially affecting the bioavailability of Ag, due to

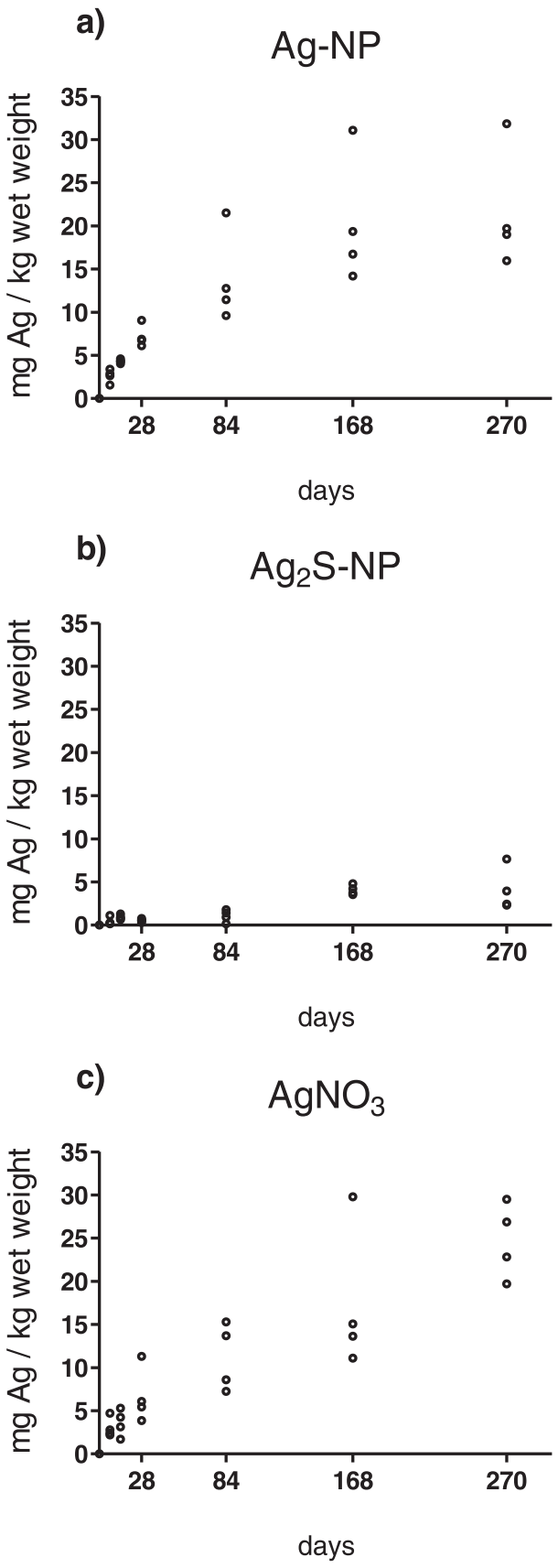

Fig. 2. Time-dependent accumulation of Ag in earthworm *E. fetida* exposed to 15 mg Ag kg^{-1} dry weight soil of a) Ag-NPs, b) Ag_2S-NPs and c) $AgNO_3$ in natural soil.

the interaction between the increased organic matter and the NPs (Klitzke et al., 2015). Addition of high amount of organic matter may also have altered the pH of the soil. Indeed, it has been reported that manure can increase the soil pH by a unit on average and can act like a buffer, also changing the pH-dependent bioavailability of metals (Whalen et al., 2000). In the present study, the pH decreased which could have been due to the activity of the earthworms. In line with our results, Atiyeh et al. (2000) found that the presence of *E. fetida* led to a decreases of pH in cow manure and proposed that it may be due to the production of fulvic and humic acids during decomposition.

4.2. Bioavailability of Ag and accumulation in earthworms

A decrease in pH could lead to a tendency toward dissolution of Ag-NPs over time (Spurgeon et al., 2006). SSekine et al. (2017) reported that a lower pH in soil increased dissolution of CuO-NPs, which resulted in CuO-NPs having a similar behavior to that of dissolved Cu within three days. In the present study, a similar scenario was reflected in the statistically significant increase of electrical conductivity in all soils where Ag was present for 270 days, which suggests an increased number of ions and electrolytes, also in the sulfidized Ag-NP samples. Indeed, Sekine et al. (2017) also demonstrated that in the long term (more than four months) the speciation of the different forms of Cu (Cu ions, CuO-NPs and sulfidized CuS-NPs) was similar for all treatments. In the present study, concentrations of Ag in pore water matched with this picture. After 270 days the average total Ag pore water concentrations of the different treatments were not significantly different from the ones at day 7. However, although data for Ag-NP and Ag ions showed no differences in the level of bioaccumulation, in the case of Ag_2S-NPs the accumulation was five times lower which would suggest that Ag was more available in the case of pristine NPs than for aged NPs. We argue that while for $AgNO_3$ ions reached a steady state within the different phases of the soil (Rieuwerts et al., 1998), in the case of pristine NPs dissolution took place fast in the initial phase (from hours to days) which was characterized by a fast release of ions. Afterwards, the process was slowed down by aging of the NP surface in the soil. This has been reported in studies which aimed to assess the dissolution of pristine As-NPs in controlled conditions. Mittelman et al. (2013) tested the dissolution as function of pH and dissolved oxygen in sand columns in 48 h. Results showed that dissolution decreased over time because of oxidation of the surface of NPs. Molleman and Hiemstra (2017) identified the mechanism explaining initial high rates of release. Formation and growth of shallow pits at the surface was proposed as a mechanism that is activated by low solution concentrations of Ag^+ and high proton activity. Liu et al. (2010) corroborated the importance of the surface processes in environmental surroundings. In that study, the dissolution rate of Ag-NPs in the soil was slowed down by thiol and citrate ligand binding and formation of sulfidic coatings within 24 h. The surface of Ag_2S-NPs is aged, covered (for the majority) by sulfur, therefore Ag_2S-NPs are not expected to undergo the first phase of fast dissolution but rather keep on slowly and regularly releasing Ag ions during nine months. This release would be possible due to the presence of some not fully sulfidized particle areas and/or due to the regular addition of water that may have enhanced dissolution. The new fluxes of water may have caused a change in redox condition and influenced the dissolution of NPs and the remobilization of ions weakly attached to the soil particles (Rieuwerts et al., 1998). Indeed, the stability of Ag_2S-NPs has been recently reconsidered because studies showed that Ag_2S-NPs can dissolve in the aquatic environments (Shi et al., 2018; He et al., 2019). Beside different dissolution rates, the Ag concentrations of Ag in the soil pore water are the result of adsorption and complexation of the ions to soil particles which constantly remove Ag^+ ions from the soil solution (Bradl, 2004; Molleman and Hiemstra, 2017).

Therefore, rather than the Ag concentration in the pore water, bioavailability appeared to be governed by fluxes of released and re-adsorbed Ag ions in the soil solution. The rates of release and re-

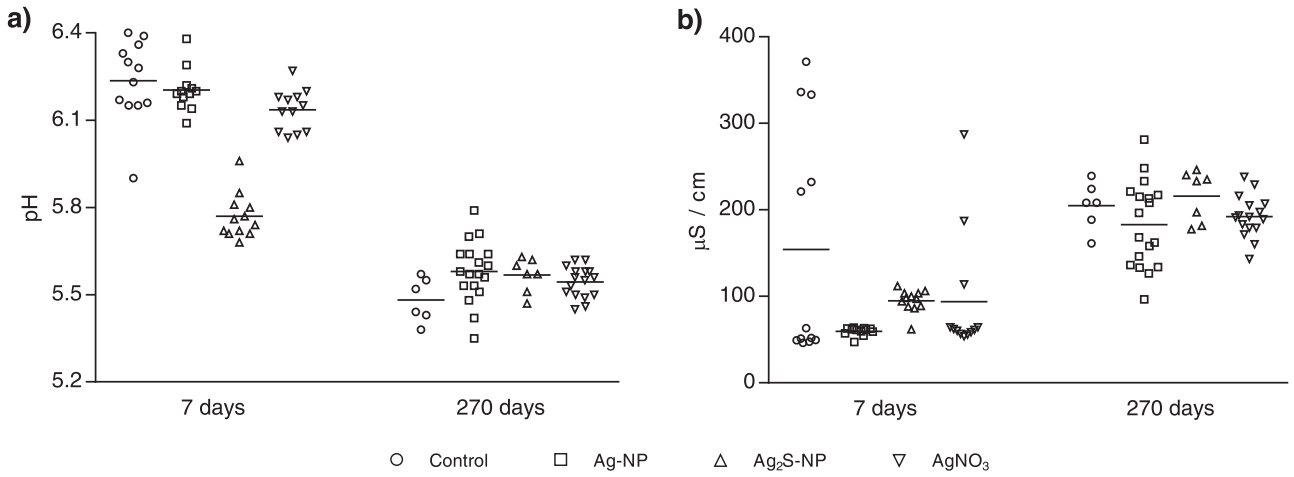

Fig. 3. a) pH and b) electrical conductivity (µS cm^{-1}) values of control soils and soils (n = 12) spiked with 15 mg Ag kg^{-1} of Ag-NP, Ag_2S-NP and $AgNO_3$ and incubated for 7 and 270 days.

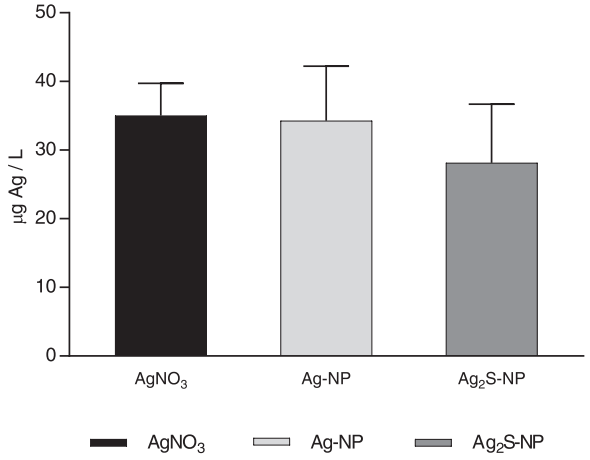

Fig. 4. Average Ag concentrations in soil pore water extracted in soil aliquots of all the treatments at all time points (mean ± standard error, n = 18).

Table 1
Overview of uptake (k_1) and elimination (k_2) kinetic rates in earthworm *E. fetida* exposed to Ag_2S-NPs, Ag-NPs and $AgNO_3$ for nine months (long term exposure, n = 24) and 28 days (short term exposure, n = 32), mean value ± standard error. Parameters with the same lowercase letter are not statistically different amongst treatments within the same exposure time ($p > 0.05$). Parameters with the same capital letter are not statistically different between the long-term and short-term exposure of the same treatment ($p > 0.05$).

	k_1 (mg Ag kg dry soil mg Ag^{-1} kg^{-1} wet body weight day^{-1}) ± SE	k_2 (day^{-1}) ± SE	BAF_k[a]
Long-term exposure			
Ag_2S-NP	0.002 ± 0.001 a, A	0.004 ± 0.003 c, C	3.1
Ag-NP	0.022 ± 0.005 b, A	0.013 ± 0.004 c, C	10.6
$AgNO_3$	0.014 ± 0.003 b, A	0.006 ± 0.003 c, C	14.6
Short-term exposure			
Ag_2S-NP	0.008 ± 0.002 a, B	0.064 ± 0.020 c, D	0.8
Ag-NP	0.061 ± 0.019 b, B	0.040 ± 0.013 c, D	9.5
$AgNO_3$	0.055 ± 0.007 b, B	0.044 ± 0.018 c, D	7.8

[a] Calculated as k1/k2 and corrected assuming dry body weight = 16% wet body weight.

adsorption are dependent on the Ag form, type of soil and its conditions (e.g. moisture content) which are likely not to be static. Comparison between the data of the current study with our previous bio-accumulation study with exposure during 28 days (Baccaro et al., 2018), which did not include addition of water during the experiment, shows that the Ag body burden after 7, 14 and 28 days was not statistically different in the case of Ag-NP and $AgNO_3$ while for Ag_2S-NP uptake is slightly lower in the absence of addition of water (Table S13). When assuming that most uptake is ionic (Baccaro et al., 2018), this would suggest that the Ag_2S-NPs released more ions under conditions with additions of water and are more unstable under these conditions (Li et al., 2015), hence may be reactive to changes in the environmental conditions such as rain fall over a long time.

4.3. MT-induction

Metallothionein (MT) measurement did not show any difference between exposed and control earthworms, and also did not vary over time. It is known that MT induction can be related to sources of stress other than uptake of metals (Hamer, 1986; Kägi, 1991; Kagi, 1993; Jacob et al., 1999). In particular, freezing conditions and starvation have been reported to induce synthesis of MT in earthworm also when they were not exposed to metals (Fisker et al., 2016; Homa et al., 2016), both of which did not happen in the current experiments. Similarly, in the present study it is possible that isolation and weight loss may have played a role in increasing the baseline of MT concentrations in the earthworms, also in the control treatment. Other studies have measured MT in earthworms exposed to Ag-NP by the same spectrophotometric method after shorter exposure time. Patricia et al. (2017) reported MT concentrations statistically higher in earthworms exposed to 0.05 mg Ag-NP kg^{-1} dry weight soil than in unexposed earthworms after 3 days. However, MT concentrations decreased almost to the baseline of the control group after 14 days of exposure. However, another study (Bourdineaud et al., 2019) did not find any statistical difference between MT concentrations in earthworms exposed for 10 days to ionic and particular silver at 2–10–50 mg Ag kg^{-1} dry weight soil and the control group. Based on the above-mentioned studies and others (Tsyusko et al., 2012; Hayashi et al., 2013), it is possible that if any MT induction occurred due to the presence of the metal, it took place within the first days (1–4 days) of exposure, which may have been missed in the current study.

4.4. Comparison between toxicokinetic rate constants

Comparison between the uptake kinetic rate constants amongst

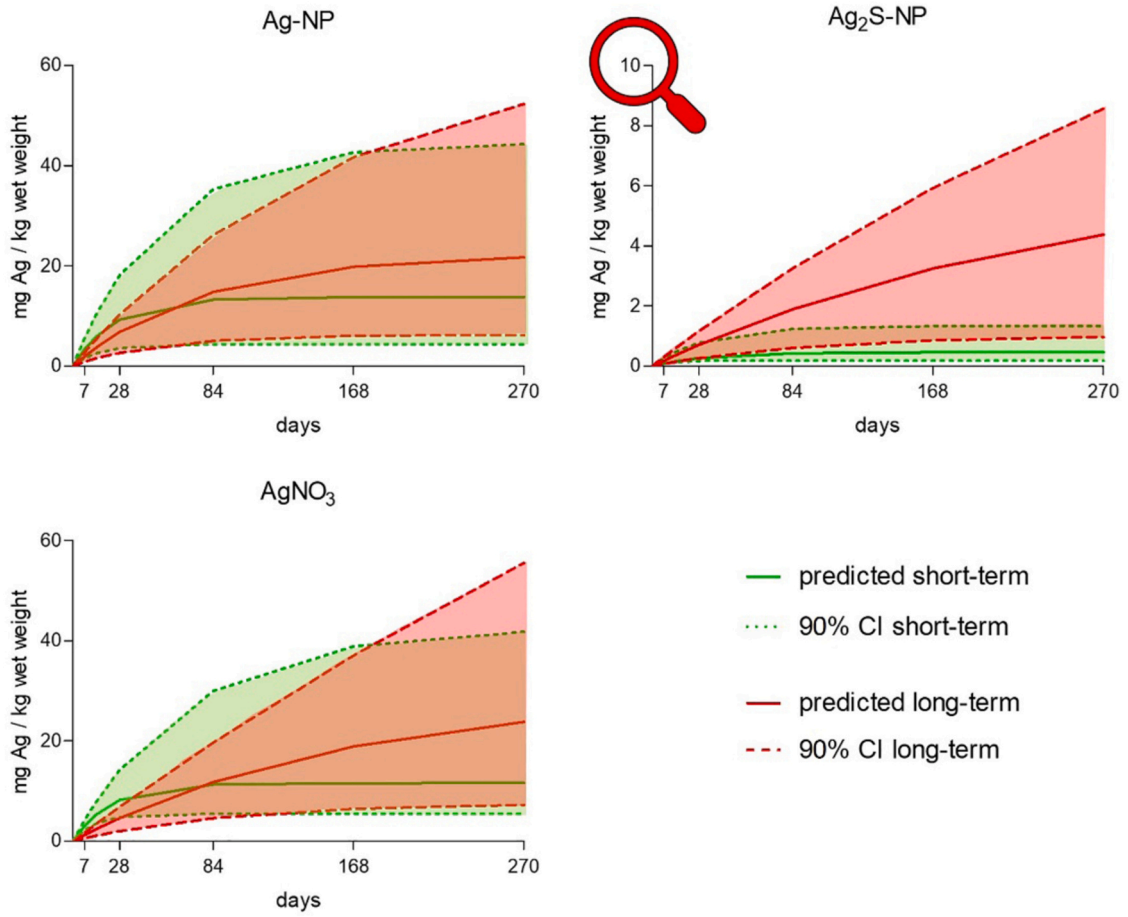

Fig. 5. Comparison between the model calculated with the kinetic rate constants derived from the experimental data of the present study (red lines) and the prediction based using the kinetic constants from the model of a short-term bioaccumulation test (green lines) (Baccaro et al., 2018). Full lines represent the prediction and dashed lines represent the relative 90% confidential interval. Note the different Y axis in the figure for the Ag_2S-NPs.

treatments of the long-term exposure study did not show any statistical difference between Ag-NP and $AgNO_3$ while the uptake rate constant k_1 of Ag_2S-NP was statistically lower compared to both other treatments. This is in line with the fact that the bioaccumulation is similar for Ag-NP and $AgNO_3$ and lower for Ag_2S-NP. The fact that relatively similar values of k_2 adequately described the elimination of all forms of Ag tested, indicates that the elimination is similar regardless of the form to which the earthworms were exposed and is mainly influenced by the uptake. Comparison between the kinetic rate constants of long- and short-term exposure experiments did highlight significant differences (Table 1). Kinetic rate constants k_1 and k_2 of all the treatments were statistically lower in the long-term experiment. The overall trend showed that the kinetic rates constants in the long-term experiment take better into account the late slow dissolution and that they are not over-influenced by the fast and high initial dissolution (Molleman and Hiemstra, 2017). When tracing the confidentiality intervals of long- and short-term models in the graphs (Fig. 5), it appeared clearer that although a slower uptake, an even slower elimination led to a higher upper confidential interval of the long-term prediction compared to the upper confidential interval of the short-term prediction after 270 days. However, for Ag-NP exposure and $AgNO_3$ the short-term model prediction covers most of the area drawn by the experimental data of accumulation after exposure over nine months, in particular 85% and 75% of the area for Ag-NP and $AgNO_3$, respectively. For Ag_2S-NPs exposure, the model derived from the short-term kinetic rate constants somewhat under-predicted the bioaccumulation in the long-term exposure. This difference is likely to be a consequence of the different availability of the Ag ions in the Ag_2S-NPs compared to the other treatments over time, due to the addition of water during exposure as discussed in the paragraph 4.2. The short-term kinetic rate constants took into account the initial fast dissolution of the pristine Ag-NP and did not take into account the late dissolution of Ag_2-NPs.

The lower uptake of Ag_2S-NPs compared to that of Ag-NPs and the late dissolution of Ag_2S-NPs during nine months are relevant findings for the risk assessment of Ag-NPs and its aged form, Ag_2S-NP. Therefore, a better quantification of the dissolution kinetics of Ag_2S-NP is needed to provide parameters improving the model predictions for aged sulfidized NPs. Nevertheless, the bioaccumulation of Ag_2S-NPs after nine months of exposure was five times lower than that of the pristine and the ionic form which confirms that Ag_2S-NPs are less bioavailable and therefore their potential uptake is conservatively covered by assuming uptake of ionic Ag. Future research studies are needed to relate Ag_2S-NP uptake at environmentally relevant exposure conditions and timings to potential adverse effects. Such further studies could also include a possible role of bioaccumulation of the NPs in the earthworms into a fraction not available for subsequent excretion (van den Brink et al., 2019).

5. Conclusion

Accumulation kinetics of Ag in Ag-NP and $AgNO_3$ exposed earthworms did not statistically differ after nine months exposure suggesting that Ag-NPs dissolved and the released Ag ions are accumulated similarly as Ag ions from $AgNO_3$. In Ag_2S-NP exposed earthworms the internalized concentrations were five times lower compared to the other treatments. The Ag concentrations in pore water measured at the sampling time points did not reflect the uptake pattern since a lower Ag

concentration in pore water would be expected for the Ag_2S-NP exposure group. Therefore, a better understanding of the dissolution kinetics of Ag_2S-NP in soil is necessary to improve the prediction of their accumulation patterns in earthworms. Metallothionein concentrations in exposed earthworms were not different from the control group. The kinetic rate constants derived from a short-term exposure model of a previous study mostly predicted the bioaccumulation in earthworms exposed to Ag-NP and $AgNO_3$ for nine months. However, the parameters derived from the short-term model underestimated the bioaccumulation in the long-term exposure for Ag_2S-NPs, likely because the short-term exposure experiment did not account for the continued dissolution of Ag_2S-NP over a longer period of time. Overall, the concentrations of Ag accumulated in the earthworms exposed to Ag_2S-NPs were low and their potential presence in the soil environment should be of more concern than that of Ag ions only when nano-specific toxic effect are identified.

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CRediT authorship contribution statement

Marta Baccaro: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing - original draft, Writing - review & editing; **Johannes H.J. van den Berg**: Investigation, Resources; **Nico W. van den Brink**: Conceptualization, Methodology, Formal analysis, Writing - review & editing, Supervision.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112371.

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