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UPTAKE ROUTES AND TOXICOKINETICS OF SILVER NANOPARTICLES AND
SILVER IONS IN THE EARTHWORM *LUMBRICUS RUBELLUS*

Running title: Silver uptake in earthworms occurs mainly via oral exposure

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Abstract: Current bioavailability models, such as the free ion activity model and biotic ligand model, explicitly consider that metal exposure will be mainly to the dissolved metal in ionic form. With the rise of nanotechnology products and the increasing release of metal-based nanoparticles (NPs) to the environment, such models may increasingly be applied to support risk assessment. However, it is not immediately clear whether the assumption of metal ion exposure will be relevant for NPs. Here using an established approach of oral gavage we have conducted a toxicokinetics study to investigate the routes of Ag NP and Ag⁺ ion uptake in the soil dwelling earthworm *Lumbricus rubellus*. Results indicated a significant part of the Ag uptake in the earthworms is through oral/gut uptake for both Ag⁺ ions and NPs. Thus, sealing the mouth reduced Ag uptake by between 40-75%. An X-ray analysis of the internal distribution of Ag in transverse sections confirmed the presence of increased Ag concentrations in exposed earthworm tissues. For the Ag NPs but not the Ag⁺ ions, high concentrations were associated with the gut wall, liver-like chloragogenous tissue and nephridia, which suggest a pathway for Ag NP uptake, detoxification and excretion via these organs. Overall our results indicate that Ag in ionic and NP form is assimilated and internally distributed in earthworms and that this uptake occurs predominantly via the gut epithelium and less so via the body wall. The importance of oral exposure questions the application of current metal bioavailability models, which implicitly consider that the dominant route of exposure is via the soil solution, for bioavailability assessment and modelling of metal-based NPs. This article is protected by copyright. All rights reserved

Keywords: Silver, Nanoparticles, Exposure route, Uptake, XANES

INTRODUCTION

The rapid increase of nanotechnology can be expected to result in increased rates at which engineered nanoparticles (NPs) are entering the environment. The nature of some common nanotechnology products, including cosmetics, textiles and personal care products, means that NPs can be expected to enter wastewater streams, where within sewage systems they may sediment into the sludge material. The deposition of this waste to land provides a route by which these released NPs may enter into soil ecosystems [1-3]. Once in the environment, there is the potential for metal-based NPs or metal ions, that are derived following their solubilisation, to come into contact with organisms and hence to be accumulated [4-7].

The prevailing ecotoxicology paradigm states that for effects to occur it is necessary for the material to be taken up into the body, and ultimately reach a target site. For conventional chemicals, the concepts of toxicokinetics and toxicodynamics are well established as a coherent framework that links exposure to toxic effects [8]. There is reason to expect that this paradigm will be relevant for NPs, although some debate remains concerning the extent to which toxicity may be dependent on the biological interactions resulting once NPs enter tissues and cells. Hence to understand the effects of NPs it is important to understand key aspects governing uptake into exposed organisms under realistic conditions.

For organisms that live in soils, NP exposure can be expected to occur through three main routes. Exposure through air is relevant only for more volatile chemicals and hence for NPs under normal soil moisture conditions is unlikely to be important. For the remaining two routes, namely exposure through contact with and transfer across the skin (dermal) and ingestion and transfer across the gut epithelium (oral), previous studies with conventional chemicals have generally suggested dermal exposure as the dominant route. This includes pesticides and non-polar organic chemicals in studies with woodlice and earthworms [9] and for Cd and Zn metal ions in a classic study by Vijver et

al. [10] that used surgical glue to inhibit soil ingestion. The latter approach allowed for separate analysis of dermal uptake with and without the additional inputs derived from ingestion and is adopted here.

Indications of dermal contact as the dominant route of exposure have underpinned the development of models, such as the free ion activity model (FIAM) [1] and later the biotic ligand model (BLM) [12] and related terrestrial bioavailability methods, that link soil solution chemistry and metal speciation modelling, to passive absorption and surface ligand binding on biological membranes, notably epidermis and gill and thereafter ultimately to toxic effects [11-14]. The successful application of these models to explain the effects of variations in media properties on metal toxicity [15-17] has pointed to the validity of the assumptions inherent in these models, with exposure mainly through external body surfaces. However, although many studies have used the FIAM and BLM to explain metal bioavailability and toxicity, not all research has necessarily supported this conjecture regarding exposure routes. For example, the results of Cain et al. [18] suggested that free ion concentrations accounted for less than 5% of Cd and Cu accumulation in mayflies in aqueous exposure. While the identification of a dependence of uptake on gut physiology and microbiome composition [19,20] also indicates dietary uptake as an important exposure route [21].

Previous studies of soil invertebrate exposure to metal-based NPs have established that these materials can enter into the tissues either as intact particles or following dissociation to ions [6,22,23]. In cases where the uptake of intact NPs is suggested it is, however, currently unclear how these materials enter tissues. This uncertainty currently places limits on the development of a modelling framework that can link NP exposure to uptake and effects, such as for example whether assumption of dermal uptake are valid. To specifically assess the importance of different potential exposure routes of NP uptake, we conducted a study to assess the toxicokinetic patterns of Ag uptake in earthworms exposed to both ionic and NP forms of Ag in soil, using the same oral sealing approaches as used by Vijver et al [10] to quantify the comparative contributions of both the dermal and oral exposure route.

MATERIALS AND METHODS

Material supply and characterisation

Uptake studies were conducted with two chemical forms of silver, Ag NPs and Ag⁺ ions derived from AgNO₃. The Ag NPs used for the study were obtained from NanoTrade Ltd (Prague, Czech Republic). The material had an indicated average particle size of 50 nm and had no coatings or surface modifications. It was supplied as a white odourless dry powder. For initial material characterisation, dispersions of the supplied material (1 mg mL⁻¹) were prepared in distilled water for analysis of particle morphology and size distribution analysis using transmission electron microscopy (TEM). For the analysis, a drop of the water dispersion was deposited on a holey carbon coated Cu TEM grid and dried at room temperature for several hours before examination. The instrument used was a JEOL 2010 analytical TEM incorporating a LaB6 electron gun operated between 80 and 200Kv and equipped with an Oxford Instruments LZ5 windowless energy dispersive X-ray spectrometer. The Ag nitrate salt (AgNO₃, 99% purity) used was purchased from BHD Chemicals (Poole, UK) as a white crystalline powder.

Soil selection and spiking

The soil used for all Ag uptake kinetic studies was standardised LUFA 2.2 loam sand (LUFA-Speyer, Germany) [24]. Measurement for the single purchased batch used for all studies indicated that this soil had a pH of 5.5 ± 1.1 in a 3:1 water:soil slurry, an organic carbon content of 2.1 ± 0.4 w/w %, a cation exchange capacity of 10 ± 0.5 meq 100 g⁻¹, and a water holding capacity of 55%. The soil was initially screened through a 2 mm mesh to remove any large coarse material and to break up larger aggregates. The batch was then sub-divided into sufficient aliquots for each control, Ag NP spiked and Ag⁺ ion treatment replicate.

The test soils were spiked with the uncoated Ag NPs and Ag⁺ ions (as AgNO₃) at two different nominal concentrations for each of the two silver forms (20 and 100 mg Ag kg⁻¹ for AgNO₃ and 100 and 500 mg kg⁻¹ for Ag NPs). Previous work conducted to assess the toxicity of freshly spiked pristine

Ag NPs and freshly spiked Ag^+ ions have generally shown a greater toxicity of the ionic Ag [25]. For this reason, the toxicokinetics studies were conducted using different exposure concentrations for the two Ag forms. For the putatively less toxic Ag NPs, a higher exposure concentration of $500 \text{ mg Ag kg}^{-1}$ and lower value of $100 \text{ mg Ag kg}^{-1}$ were chosen. For the Ag^+ ions, the highest concentration used was $100 \text{ mg Ag kg}^{-1}$ and the lower value was 20 mg Ag kg^{-1} . The higher concentrations used were selected to provide a relatively high exposure level to ensure detection of uptake. The second concentration was then selected at 20% of this higher value to allow investigation of uptake at levels below those likely to cause overt toxicity. Use of a common value of $100 \text{ mg Ag kg}^{-1}$ dry weight for both forms allowed direct comparison between the two Ag forms. Additional replicates of the unspiked test soils were prepared (control samples). Samples collected from control treatment were used to confirm that all accumulated metal in the spiked soils related to the Ag added and not to assimilation of residual material in the soil.

The same dosing technique was used to spike both silver forms into the test soil. Because there were problems with maintaining a stable dispersion of the Ag NPs in distilled water, direct dry dosing of the powder into the soil was selected as the most suitable option to obtain a homogenous Ag distribution [see 26]. This method was also used to spike the soils with the AgNO_3 . The mixing of the Ag NPs and solid crystalline AgNO_3 was initially into a dried sub-sample of the test soil. These dosed aliquots were then thoroughly mixed with the remaining soil to give the 200 g of spiked soil used for each test replicate. Spiked soils were then wetted with MilliQ water to a soil moisture content of 45% of maximum water holding capacity [27]. After a further mixing, all soils were maintained for an initial period of one week to allow for the initial binding and interactions of the added Ag NPs and ions with soil solid phase and pore water components.

To validate background and added nominal Ag concentrations, samples were taken from a selection of the test replicates over the experimental period. These soil samples (~20 g) were initially oven dried at 80°C and a 100 mg aliquot of this sample digested in 2 ml of a 4:1 mixture of

hydrochloric acid (37% p.a., Baker, Grainger, USA) and nitric acid (65% p.a., Riedel-de-Haen, Seelze, Germany) in closed Teflon® bombs heated at 140 °C for 7 h. Digests were diluted with 8 ml of deionised water and analysed for total Ag by flame atomic absorption spectroscopy (Perkin Elmer AAnalyst 100). For quality assurance purposes, a certified reference material (River Clay, WEPAL-ISE-886) and reagent blanks were also analysed. The averages of measured Ag concentrations were within 25% of the certified reference value (River Clay contains $2.8 \pm 0.4 \text{ mg Ag kg}^{-1}$, silver concentrations in the certified reference material ranged between 2.6 and 3.9 mg Ag kg⁻¹ dry weight). No Ag was detected in the blank samples. The experimental detection limit for the Ag measurements was 0.3 mg kg⁻¹.

Soil pore water samples were also collected for Ag analysis from separately prepared replicate soil samples by centrifugation (J2-HC, Beckman Coulter, California) for all four Ag treatments assessed. A 50 g batch of spiked soils was taken from these four replicates and saturated with 13.5 ml of Milli-Q water and centrifuged for 60 min at 4000 xg [7]. The samples (supernatants) were then acidified and analysed for total Ag concentration by flame atomic absorption spectroscopy (Perkin Elmer AAnalyst 100).

Uptake bioassay and tissue Ag analysis

All earthworms used were morphologically determined as *Lumbricus rubellus* (supplied by Lasebo BV, Nijkerkerveen, The Netherlands). This species was chosen because it is a widely distributed epigeic earthworm species in agricultural soils where it may come into contact with Ag in NP and ionic forms added through routes such as sewage sludge. Further, *L. rubellus* has also been found to be suitable for oral gluing studies [10]. All earthworms were initially maintained in a stock culture on a medium consisting of 1:1:1 mix of composted bark : Spagnum peat : loam soil and supplied *ad libitum* with a combination of horse manure collected from animals grazing uncontaminated pasture, and that had not undergone any recent medication and additional vegetable

peelings supplied as food. Selected earthworms used for the experiment were taken from this stock culture. All earthworms were adults with well-developed clitellum.

Of the earthworms selected for the experiment, half were subjected to oral sealing in order to prevent soil ingestion and, hence, exposure through the digestive tract. Sealing was achieved by covering the mouth parts in the first segment of the earthworm with medical histoacryl glue (Braun Aesculap, Germany). The glue was applied by dipping the earthworm's mouth in the glue as described in detail in the original paper [10]. Following gluing, all sealed and unsealed individuals were placed individually onto the surface of the unspiked, Ag NP and Ag⁺ ion spiked soil. A total of 44 sealed and 44 unsealed earthworms were used for each of these treatments (2 x NP; 2 x ionic; 1 x reference) to provide a sufficient number of individuals for collection of 4 replicate earthworms for each of the 10 exposure times used for the uptake study. Four worms with individual fresh weights ranging from 1.15 to 2.02 g (average \pm SD, 1.62 ± 0.21 g) were placed into glass jars containing about 360 g of dry soil. All containers were checked after 1 h to ensure that the earthworms had burrowed into the test soil. Containers were then incubated at $12^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in a 16 h light, 8 h dark regime for a total of 168 h to allow accumulation of the different Ag forms. Earthworms were not fed during the experiment. Soil moisture loss was checked and if necessary corrected for over the exposure period. Exposures were stopped after 168 h to be sure that we were avoiding the time point from which earthworms could start experiencing physiological changes linked to starvation.

At 0 (i.e., worms taken from batch at the start of exposure), 4, 8, 24, 36, 48, 72, 96, 120, and 168 h after initiation, 4 sealed and 4 unsealed individuals were removed from containers for each of the five treatments. Collected earthworms were rinsed, blotted dry on filter paper and weighed. The earthworms were then kept individually for 36 h in Petri dishes lined with a piece of moistened filter paper to allow them to void their gut content. To restrict coprophagy, the filter paper was changed after 24 h. The amount of excreta produced on filter paper by the sealed earthworms was checked. Only in four cases sealed worms excreted soil particles, those worms were excluded from further analyses. The

earthworms were then snap frozen, freeze-dried for 2 d and the dried tissue weighed. The whole tissue was then digested in 2 ml of a 1:4 mixture of nitric acid (65% p.a.; Riedel-de-Haen) and hydrochloric acid (37% p.a., Baker) in tightly closed Teflon® bombs upon heating in a destruction oven at 140° C for 7 h and Ag concentration measured by flame Atomic Absorption Spectrometry (Perkin Elmer AAnalyst 100). A biological reference material Dogfish Liver, DOLT-4, having a certified concentration of 0.93 ± 0.07 mg Ag kg⁻¹ dry weight was included as quality check. Concentrations measured of this material were between 80 and 120%, with average recovery 97%.

μX-ray fluorescence mapping

Bio-imaging was performed at the Diamond Light Source (UK) using the I18 beamline. Fresh tissue samples taken from the mid-intestinal region of earthworms exposed for 168 h to Ag NPs (500 mg Ag kg⁻¹) and Ag⁺ ions (100 mg Ag kg⁻¹) were processed by overnight fixation in 70% ethanol, embedded in glycol methacrylate resin, and sectioned at 8 μm with a tungsten-coated steel knife.

Sections were mounted on Ultralene® window film 4 μm-thick (SPEX SamplePrep, Metuchen, NJ) stretched across a hole in a plastic slide in order to minimize the Si signal associated with glass substrates. Slides were inserted into the standard I18 sample holder, and imaged ‘externally’ under brightfield conditions for orientation purposes.

X-ray fluorescence (XRF) data was collected using a Si(111) double crystal monochromator and the Kirkpatrick-Baez focusing mirrors, which provided a 3 μm spot size, were also used to remove harmonic contamination. The sample holder was positioned at 45° C to the incident beam. The Ag K-edge at 25.531 eV is above the dynamic range of the I18 beamline and Ag L(III) edge $L\alpha$ line is very close to the Argon $K\alpha$ line hence the Ag L(II)-edge at 3.540 eV was used. However this provides a relatively insensitive signal as the Ag L(III) edge has a low fluorescence yield [28]. Data was recorded using a 4 element Si drifts detector (Hitachi Inc) positioned close to the specimen, whilst the Ar signal (from ambient air) was reduced but not eliminated by enclosing the specimen and detector inside a bespoke bag under flowing He to give a largely He environment during analysis. Two XRF maps over

the same region of the sample were collected using an incident energy below the Ag L(II) edge at 3500 eV and one above the edge at 3580 eV. The signal in the Ag L β energy window in the lower energy map was subtracted from the signal in the higher energy map in PyMca 4.1.1 [29], to remove the signal observed from the Argon K β line and produce the Ag XRF map. Ag XRF maps were acquired but spot μ XANES scans at the Ag L(II)-edge X-ray Absorption Near-Edge Spectroscopy (XANES) spectra are not of sufficient quality to permit spectral analysis due to the relatively low levels signal detection from samples .

Data handling and statistical analysis

Uptake and elimination rate constants were estimated by applying a one-compartment first order kinetics model to the data for the uptake phase, with a constant start value C_0 . This model has been widely used in the ecotoxicological literature as a means to assess uptake and elimination kinetics. The one compartment model fitted to the data took the form:

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

Where t = exposure time (h), C_w = internal concentration in the earthworms ($\mu\text{g g}^{-1}$ dry weight), k_1 = uptake rate constant ($\text{g soil g earthworm}^{-1} \text{h}^{-1}$), k_2 = elimination rate constant (h^{-1}), C_0 = initial concentration in the earthworm (mg kg^{-1} dry weight), and C_{exp} = exposure concentration (mg kg^{-1} dry soil). Two sets of values were calculated for the uptake rate, k_{1-T} was calculated using total measured Ag concentration and k_{1-pw} using silver concentration in the soil pore water. Equations were fitted to the replicate experimental data using least squared regression fitting in SPSS 17.1. From model fits, all parameter values including standard errors and/or confidence intervals and coefficient of determination (r^2) values indicating goodness of fit were derived. Significant differences between uptake rates for sealed and unsealed earthworms were compared using a generalised likelihood ratio test [30].

RESULTS

Material characterization and concentration validation

The TEM analysis of the supplied nanopowder indicated the presence of primary particles in the 50-80 nm range, with a smaller proportion of smaller 10-30 nm particles. These primary particles had often formed into loose agglomerates. These were not, however, stable structures as they were readily disintegrated by exposure to the TEM electron beam during analysis. Further, characterisation of these materials can be found in Diez et al. [31].

Ag concentrations in control soil were below the method detection limit of 0.3 mg kg^{-1} . The average measured Ag concentrations in replicate soil samples from each treatment were $> 85\%$ of nominal values confirming the general efficiency of the spiking procedure (Table 1). Ag concentrations in pore water samples were ten times higher for the soil spiked with ionic Ag than in soil spiked with Ag NPs at the same total nominal Ag concentrations (100 mg kg^{-1}) (Table 1).

Ag uptake in sealed and unsealed earthworms

During the experiments, the burrowing capacity of sealed and unsealed earthworms was similar and they all disappeared into the soil. No obvious visual differences in activity and locomotion were found, although initially the sealed earthworms needed approximately 15 min to begin burrowing. Hence the extent of dermal contact with the soils was not affected, when oral exposure was prevented. No effect on survival was observed during the 96-h exposure period.

Ag concentrations in earthworms exposed to the unspiked soil were low (average Ag concentration was $0.02 \mu\text{g g}^{-1}$ dry weight, d.w.) over the duration of the exposure. This is consistent with the low background Ag levels found in this soil and confirms that the patterns of uptake seen are not the result of the accumulation of any background metal. Ag concentrations in *L. rubellus* exposed to both Ag forms increased with time (Figure 1). For all eight data-sets for sealed and unsealed earthworms at two concentrations of Ag^+ ions and NPs, Ag uptake patterns could be described by the

one compartment model. There was, however, significant inter-individual variation between earthworms. As a consequence the proportion of total variation explained by the model was relatively modest ($r^2 = 0.290 - 0.815$).

For unsealed worms, one compartment model fits indicated that uptake rates (k_{I-T} and k_{I-pw}) were greater at the higher exposure concentrations in earthworms exposed to Ag ions (Table 2), while non significant differences were found when exposed to different concentrations of Ag NPs ($p < 0.05$). The earthworms that were subject to oral sealing with surgical glue also assimilated Ag from both forms of Ag (Figure 1). The Ag uptake seen for the Ag NPs, both with and without the potential for oral exposure, is not in itself indicative of direct NP uptake, because assimilation may be of the ions produced by dissolution. Comparing parameters for the earthworms exposed to 100 mg kg^{-1} of Ag^+ ions and Ag NPs indicated significantly higher uptake rates (k_I) for the ionic treatment (with a $p < 0.05$, Table 2).

Even though there was assimilation of Ag from soil in sealed earthworms, inhibition of ingestion resulted in a substantial reduction in uptake of both Ag forms. Thus after 168 h, Ag tissue concentrations in sealed earthworms were between 40-75% of those in unsealed individuals across both Ag forms and exposure levels (Figure 1). This suggests that for both Ag NPs and Ag^+ ions, soil ingestion is a major route of exposure. Thus in unsealed earthworms, body concentration reached equilibrium after 48-72 h when exposed to Ag^+ ion, but not until after 96 h in Ag NP exposures. In sealed earthworms, tissue Ag levels increased throughout the exposure period and did not reach equilibrium within 96 h for both Ag forms (Figure 1).

Elimination rate constants in all cases were low and close to zero, and they were similar in both sealed and unsealed worms exposed to silver ions and silver nanoparticles at 100 mg kg^{-1} , suggesting that the elimination route might be similar independently of the exposure route.

Ag uptake by both unsealed and sealed earthworms raises questions regarding the target organs for Ag sequestration, and whether Ag was more efficiently accumulated within specific tissues

following experimental exposure for intact Ag NPs or for Ag⁺ ions. To help address these questions, tissues of unsealed earthworms were analysed using synchrotron μ -XRF. The tissue specific *in situ* distribution of Silver (Ag), Sulphur (S) and Phosphorus (P) was derived for unsealed worms exposed to 500 mg kg⁻¹ Ag NPs (Figures 2A & C) and unsealed worms exposed to 100 mg Ag kg⁻¹ of Ag⁺ ions (Figures 2B & D). Alcohol-fixed, methacrylate-embedded transverse sections (10 μ M) were imaged directly using a light microscope and an area identified for μ XRF mapping which included the majority of earthworm organs involved in metal sequestration and excretion (Figures 2A & B; areas showed by the perimeter of the black rectangle). Elemental distributions were derived using μ XRF mapping performed under a helium environment, in order to minimise the interference of Argon K-edge. Elemental fluorescence spectra were model and any residual contribution of Ar removed by spectral subtraction. This enabled us to overlay the distribution of Ag (shown in Blue) with S (green) and separately P (red), which provides us with the anatomical location of where Ag was. In order to confirm the presence of Ag, we XANES scans across the Ag L(II)-edge at specific points within tissues derived from Ag-NP and Ag-Ion (Figures 2C & D). These showed that in NP-exposed unsealed earthworms Ag (L β) fluorescence was observed (after removal of interference from the strong Ar K β signal predominantly in: the chloragogenous tissue within the typhlosole (i) and around the basal intestinal surface (ii); nephridial tubules (iii) and near the base of setae (iv) of the Ag NPs exposed specimen (Figure 2A). Due to the spectral manipulation required to create the fluorescent maps which limited the identification by mapping, the presence of Ag at these probable sequestration sites was confirmed by the acquisition of short XANES scans across the Ag L(II)-edge, and observing the sharp increase in intensity at the characteristic energy of the Ag L(II) edge at these locations as shown in the XANES scans (Figure 2C, spectra i-iv). Fluorescence mapping and XANES scanning under identical micro-focus conditions and in equivalent tissue structures (spectra v-viii, Figure 2D) at locations v-viii in the ion exposed specimen (centre) was unable to detect Ag in the earthworm.

DISCUSSION

The successful application of models such as the FIAM and BLM to explain the effects of variations in media properties on metal toxicity [15-17] has pointed to the validity of the assumptions of these models that relate to the concentration of the ionic form in contact with external surfaces (epidermis, gill). Attempts to apply models such as the BLM that assume dermal interactions with the dissolved metal to explain observed metal uptake and toxicity in soils dwelling organisms have generally been informative [15,16]. Such assessments could be taken as an indication of exposure to the free dissolved ion occurring primarily through contact of external surfaces such as the body wall with the external media: notably the soil solution, because this is an assumption inherent in these models. So far, the application of the FIAM and BLM to explain the effects of metals on soil organisms has not extended to studies with Ag. Further examples of the application of these two models to explain effects of metal and metal oxide based NPs are currently not available. Our studies on the relevance of different exposure routes for Ag ions and Ag NP uptake by earthworms are informative for the validity of such future application.

For geophagous groups such as earthworms, the gut-lining, like the skin, will be in near continuous contact with the soil medium. Hence for these species exposure to metals ions and other metal forms, including intact NPs, from diet is a clear possibility. Such intake via the gut provides the potential for exposure that may be additional to that resulting from dermal contact alone [9]. As many soil organisms live in and also consume the soil (and associated contaminants), disentangling the contribution of different exposure routes to uptake can be challenging. For example, even if gut exposure is actually an important site of uptake, deviations of accumulation rates from those derived from only epidermal exposure will only occur if gut physiology has a substantive influence on the rates of metal ion association with uptake sites or alternatively if the frequency of uptake sites in gut tissues exceeds that of the epidermis. If such differences are not the case, then metal transport over the body wall and gut epithelium will be effectively equivalent.

To tease apart the contributions of different routes for chemical uptake by earthworms, a number of different approaches such as ligature [9] and oral gluing [10] to inhibit feeding have been applied. In the latter seminal study, gluing was suggested as a suitable approach for oral sealing, as it allowed normal burrowing and behaviour in the absence of feeding. This suggestion is confirmed by observations made in the present study. Thus the sealed *L. rubellus* used, showed no physical signs of skin irritation by the procedure and features such as mucus producing did not interfere with the setting of the glue. Further during the experiments, earthworm burrowing behaviour, physical appearance and survival were all normal. Thus, even though the sealing of the anterior end could have been potentially stressful, these effects did not appear to influence key physiological and behavioural parameters that could have affected metal uptake.

This study is to our knowledge the first to separate exposure routes for both Ag ion and also Ag NP (or indeed any nanoparticle) in a soil exposure experiment. As such the study provides a unique view of the role of different exposure routes for two different untested cases. The results for both the Ag ion and Ag NPs point to the relative importance of the intestinal route for Ag uptake, with oral sealing reducing Ag assimilation by between 40-75% in all four cases (2 Ag⁺ ion concentrations, 2 Ag NP concentrations) (Figure 1). The relative contributions of the two exposure routes, most notably the dominance of oral exposure, were similar for whether exposure was to Ag⁺ ions or Ag NPs. As the concentrations used here were above current anticipated environmental concentrations [32], they need to be validated at lower concentrations. However, because no physiological effects were seen it may not be anticipated that behaviour or physiological difference would change uptake patterns at lower exposure concentrations.

The physiological basis for the dominance of Ag assimilation principally through the intestine, rather than the dermal route that appears dominant for other metals [10], still remains open to speculation. This is compounded by the fact that in NP exposure, it is not certain whether accumulation is of intact NPs, released metal ions or both. One contributing factor could be the strong affinity of Ag⁺

for soil organic matter [33]. This results in relatively low concentrations of free Ag^+ ions in soil pore water. When this organic matter is consumed, the presence of surfactant molecules and other conditions within the gut lumen may possibly allow the greater release of Ag into solution that may then be assimilated. Since the stability of Ag NPs in solution can be influenced by the presence of molecules such as surfactant molecules [34,35], similar processes may affect NP assimilation. Alternative suggestions to explain preferential Ag^+ ion and Ag NPs uptake in the gut could include a greater representation of key receptors for Ag such as the Na^+/K^+ ATPase in the gut lining compared to body wall, or a specific speciation chemistry of Ag under the specific redox and solute conditions associated with the earthworm gut and its microbial community that particularly facilitate uptake.

To confirm whether the measured total Ag concentration in earthworms is actually internalized by cells and tissues, and not merely associated by adsorption onto dermal and gut epithelial surfaces, *in situ* Ag distribution mapping using a synchrotron-beam micro-focus imaging and analysis was carried out on unsealed earthworms exposed to the highest concentrations of Ag^+ ions and Ag NPs. The fate of assimilated Ag within earthworms showed a qualitative difference depending on the chemical form of Ag to which they were exposed. Ag was detectable in setae ('hair') follicles, chloragogenous tissue and nephridia after 168 h exposure to Ag NPs, but could not be detected in any tissue of Ag^+ ion exposed earthworms. The difference is not easy to explain, especially since our bulk chemistry observations indicate that Ag is more readily accumulated when the exposure is to Ag^+ ions compared to Ag NPs.

Our micro-focus observations on Ag NP exposed earthworms suggest that the mechanism, if not the anatomical route, of Ag NP uptake could differ from that of Ag^+ ions. Ag was not adsorbed onto epidermal and epithelial surfaces. Indeed, the highly focal distribution of Ag in NP-exposed earthworm tissues implies that the metal was internalized mainly, if not exclusively, to local hotspot, potentially as intact NPs via endocytosis pathways in intestinal epithelia. This finding could support the identification of the gut as the major route of Ag uptake by earthworms as derived from the toxicokinetics studies of oral sealed and unsealed earthworms. This aspect, however, requires further confirmation from

additional imaging and mechanistic studies. Whether the detection of Ag near the base of the setae is also indicative of a hitherto unsuspected route of trans-epidermal uptake, or a means of NP excretion analogous to the recently identified hair follicle route in mammals [36], also warrants further study.

The identification of the gut as the dominant route of uptake of Ag as ion and NPs has a number of specific implications for the way these chemicals can be handled within risk assessment. A current focus in metal and NP ecotoxicological research is to understand how environmental conditions such as pH and organic matter, and also for NPs relevant processes such as aggregation and dissolution, are related to toxicity. Models of ionic metal toxicity, such as the BLM, have been used to account for soil and water chemistry influences on toxicity and it has been proposed that such models could be useful for predicting the toxicity of metals and metal oxide NPs [37]. The BLM relies on a mechanistic assumption that the toxic effect is driven by the concentration of the free ion, as derived from measurement, that is able to bind to a relevant receptor (the “biotic ligand”) after the competing effects of other cations on receptor occupancy have been taken into account. For NPs subject to dissolution, the relevance of the BLM for dissolved ions has been suggested. However, application for intact NPs themselves has yet to be demonstrated [38]. Principles for the application of the BLM implicitly assume exposure via the soil solution principally via external organs such as the body wall and respiratory surfaces. The results presented here however, suggest that dermal exposure may not be the only, or even most important, route of exposure for both Ag⁺ ions and Ag NPs for earthworms. Inclusion of exposure and subsequent tissue distribution through the gut is also necessary to fully account for exposure effects on toxicity. Given that the gut surface, like the skin, is in near continuous contact with the soil, this consideration may not actually require extensive modification of the BLM if the physicochemical conditions for uptake across both are similar. However, if gut chemical conditions or physiology are different from those of the bulk soil and skin, then this would require specific consideration in exposure modelling. Regardless of the actual mechanism(s) involved, our results show

that dietary uptake of NPs is a route of exposure that should be considered in risk assessments, bioavailability and modelling of metal-based NP.

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Data availability—Data, associated metadata, and calculation tools are to be requested from the authors (mdiez@leitat.org).

REFERENCES

1. Kiser MA, Westerhoff P, Benn T, Wang Y, Perez-Rivera J, Hristovski K. 2009. Titanium nanomaterial removal and release from wastewater treatment plants. *Environ Sci Technol* 43:6757-6763.
2. Johnson AC, Bowes MJ, Crossley A, Jarvie HP, Jurkschat K, Juergens MD, Lawlor AJ, Park B, Rowland P, Spurgeon D, Svendsen C, Thompson IP, Barnes RJ, Williams RJ, Xu N. 2011. An assessment of the fate, behaviour and environmental risk associated with sunscreen TiO₂ nanoparticles in UK field scenarios. *Sci Total Environ* 409:2503-2510.
3. Colman BP, Arnaout CL, Anciaux S, Gunsch CK, Hochella MF, Jr., Kim B, Lowry GV, McGill BM, Reinsch BC, Richardson CJ, Unrine JM, Wright JP, Yin L, Bernhardt ES. 2013. Low concentrations of silver nanoparticles in biosolids cause adverse ecosystem responses under realistic field scenario. *PLOS One* 8:e57189.
4. Coutris C, Hertel-Aas T, Lapied E, Joner EJ, Oughton DH. 2012. Bioavailability of cobalt and silver nanoparticles to the earthworm *Eisenia fetida*. *Nanotoxicology* 6:186-195.
5. Kool PL, Ortiz MD, van Gestel CAM. 2011. Chronic toxicity of ZnO nanoparticles, non-nano ZnO and ZnCl₂ to *Folsomia candida* (Collembola) in relation to bioavailability in soil. *Environ Pollut* 159:2713-2719.
6. Unrine JM, Hunyadi SE, Tsyusko OV, Rao W, Shoults-Wilson WA, Bertsch PM. 2010. Evidence for bioavailability of Au nanoparticles from soil and biodistribution within earthworms (*Eisenia fetida*). *Environ Sci Technol* 44:8308-8313.
7. Heggelund L, Diez-Ortiz M, Spurgeon DJ, Svendsen C. 2013. Soil pH effects on the comparative toxicity of dissolved zinc, non-nano and nano ZnO to the earthworm *Eisenia fetida*. *Nanotoxicology* 5:559-572
8. Jager T, Albert C, Preuss TG, Ashauer R. 2011. General unified threshold model of survival - a toxicokinetic-toxicodynamic framework for ecotoxicology. *Environ Sci Technol* 45:2529-2540.

9. Jager T, Fleuren R, Hogendoorn EA, De Korte G. 2003. Elucidating the routes of exposure for organic chemicals in the earthworm, *Eisenia andrei* (Oligochaeta). *Environ Sci Technol* 37:3399-3404.
10. Vijver MG, Vink JPM, Miermans CJH, Van Gestel CAM. 2003. Oral sealing using glue: a new method to distinguish between intestinal and dermal uptake of metals in earthworms. *Soil Biol Biochem* 35:125-132.
11. Morel FMM, Hering JG. 1983. *Principles of Aquatic Chemistry*. John Wiley & Sons, New York, USA.
12. Di Toro DM, Allen HE, Bergman HL, Meyer JS, Paquin PR, Santore RC. 2001. Biotic ligand model of the acute toxicity of metals. 1. Technical basis. *Environ Toxicol Chem* 20:2383-2396.
13. Lofts S, Spurgeon D, Svendsen C. 2005. Fractions affected and probabilistic risk assessment of Cu, Zn, Cd, and Pb in soils using the free ion approach. *Environ Sci Technol* 39:8533-8540.
14. Lofts S, Criel P, Janssen CR, Lock K, McGrath SP, Oorts K, Rooney CP, Smolders E, Spurgeon DJ, Svendsen C, Van Eeckhout H, Zhao FZ. 2013. Modelling the effects of copper on soil organisms and processes using the free ion approach: Towards a multi-species toxicity model. *Environ Pollut* 178:244-253.
15. Thakali S, Allen HE, Di Toro DM, Ponizovsky AA, Rooney CP, Zhao FJ, McGrath SP, Criel P, VanEeckhout H, Janssen CR, Oorts K, Smolders E. 2006. Terrestrial biotic ligand model. 2. Application to Ni and Cu toxicities to plants, invertebrates, and microbes in soil. *Environ Sci Technol* 40:7094-7100.
16. Le TTY, Peijnenburg W, Hendriks AJ, Vijver MG. 2012. Predicting effects of cations on copper toxicity to lettuce (*Lactuca sativa*) by the biotic ligand model. *Environ Toxicol Chem* 31:355-359.
17. Lofts S, Spurgeon DJ, Svendsen C, Tipping E. 2004. Deriving soil critical limits for Cu, Zn, Cd and Pb: a method based on free ion concentrations. *Environ Sci Technol* 38:3623-3621.
18. Cain D, Croteau M-N, Luoma S. 2011. Bioaccumulation dynamics and exposure routes of Cd and Cu among species of aquatic mayflies. *Environ Toxicol Chem* 30:2532-2541.

19. Simkiss K, Watkins B. 1990. The influence of gut microorganisms on zinc uptake in *Helix aspersa*. *Environ Pollut* 66:263-271.
20. Hopkin SP. 1990. Species-specific differences in the net assimilation of Zn, Cd, Pb, Cu and Fe by the terrestrial isopods *Oniscus asellus* and *Porcellio scaber*. *J Appl Ecol* 27:460-474.
21. Poteat MD, Buchwalter DB. 2014. Four reasons why traditional metal toxicity testing with aquatic insects is irrelevant. *Environ Sci Technol* 48 887–888.
22. Hooper HL, Jurkschat K, Bailey J, Morgan AJ, Lawlor AJ, Spurgeon D, Svendsen C. 2011. Comparative chronic toxicity of nanoparticulate and ionic zinc to the earthworm *Eisenia veneta* in a soil matrix. *Environ Int* 37, 1111-1117.
23. Novak S, Drobne D, Goobic M, Zupanc J, Romih T, Gianoncelli A, Kiskinova M, Kaulich B, Pelicon P, Vavpetic P, Jeromel L, Ogrinc N, Makovec D. 2013. Cellular internalization of dissolved cobalt ions from ingested CoFe₂O₄ nanoparticles: *In vivo* experimental evidence. *Environ Sci Technol* 47:5400-5408.
24. Løkke H, Van Gestel CAM. 1998. *Handbook of Soil Invertebrate Toxicity Tests*. John Wiley, Chichester.
25. Shoultz-Wilson WA, Reinsch BC, Tsyusko OV, Bertsch PM, Lowry GV, Unrine JM. 2011. Effect of silver nanoparticle surface coating on bioaccumulation and reproductive toxicity in earthworms (*Eisenia fetida*). *Nanotoxicology* 5:432-444.
26. Waalewijn-Kool PL, Ortiz MD, Van Straalen NM, Van Gestel CAM. 2013. Sorption, dissolution and pH determine the long-term equilibration and toxicity of coated and uncoated ZnO nanoparticles in soil. *Environ Pollut* 178:59-64.
27. OECD. 2004. Guideline for the testing of chemicals No. 222. Earthworm reproduction test (*Eisenia fetida*/*Eisenia andrei*). Organisation for Economic Co-operation and Development, Paris.

28. Mosselmans JFW, Quinn PD, Dent AJ, Cavill SA, Moreno SD, Peach A, Leicester PJ, Keylock SJ, Gregory SR, Atkinson KD, Rosell JR. 2009. I18-the microfocus spectroscopy beamline at the Diamond Light Source. *J Synchrotron Radiat* 16:818-824.
29. Sole VA, Papillon E, Cotte M, Walter P, Susini J. 2007. A multiplatform code for the analysis of energy-dispersive X-ray fluorescence spectra. *Spectrochimica Acta Part B: Atomic Spectroscopy* 62:63-68.
30. Sokal RR, Rohlf FJ. 1994. *Biometry: Principles and Practice of Statistics in Biological Research*, Third Edition edition. Freeman & Co, London, UK.
31. Diez-Ortiz M, Lahive E, George S, Ter Schure T, Van Gestel CAM, Jurkschat K, Svendsen C, Spurgeon DJ. A novel aging paradigm for silver nanoparticles in soil with long-term incubation increasing toxicity. *Environ Pollution (under review)*
32. Gottschalk F, Sonderer T, Scholz RW, Nowack B. 2009. Modeled environmental concentrations of engineered nanomaterials (TiO₂, ZnO, Ag, CNT, Fullerenes) for different regions. *Environmental Science & Technology*; 43: 9216-9222.
33. Coutris C, Joner EJ, Oughton DH. 2012. Aging and soil organic matter content affect the fate of silver nanoparticles in soil. *Sci Total Environ* 420:327-333.
34. Chappell MA, Miller LF, George AJ, Pettway BA, Price CL, Porter BE, Bednar AJ, Seiter JM, Kennedy AJ, Steevens JA. 2011. Simultaneous dispersion-dissolution behavior of concentrated silver nanoparticle suspensions in the presence of model organic solutes. *Chemosphere* 84:1108-1116.
35. Ruge CA, Schaefer UF, Herrmann J, Kirch J, Canadas O, Echaide M, Perez-Gil J, Casals C, Muller R, Lehr CM. 2012. The interplay of lung surfactant proteins and lipids assimilates the macrophage clearance of nanoparticles. *Plos One* 7:e40775.
36. Kempson IM, Chien C-C, Chung C-Y, Hwu Y, Paterson D, de Jonge MD, Howard DL. 2012. Fate of intravenously administered gold nanoparticles in hair follicles: Follicular delivery, pharmacokinetic interpretation, and excretion. *Adv Healthcare Materials* 1:736-741.

37. Fabrega J, Luoma SN, Tyler CR, Galloway TS, Lead JR. 2011. Silver nanoparticles: Behaviour and effects in the aquatic environment. *Environ Int* 37:517-531.
38. Choi O, Cleuenger TE, Deng B, Surampalli RY, Ross L, Jr., Hu Z. 2009. Role of sulfide and ligand strength in controlling nanosilver toxicity. *Water Research* 43:1879-1886.

Figure 1. Total Ag concentrations in tissues ($\mu\text{g g}^{-1}$ dry weight) of *Lumbricus rubellus* exposed to Ag^+ ions (AgNO_3 , exposure concentration: 20 and 100 mg kg^{-1} dry weight) and Ag NPs (exposure concentration: 100 and 500 mg kg^{-1} d.w.) in LUFA 2.2 loam sand soil (points indicate measured concentrations for individual earthworms; dashed and smooth lines indicate one-compartment model fit to Ag uptake by unsealed and sealed worms, respectively). Note differences in scales of vertical axes.

Figure 2. μXRF maps and Ag L(II)-edge spectra derived from histological sections of Ag-NP and Ag ions exposed earthworms. Sections were generated from alcohol-fixed, methacrylate-embedded, transverse sections (TS) of mouth-unsealed earthworms (*Lumbricus rubellus*) exposed to 500 mg Ag kg^{-1} applied in the form of Ag NPs (A & C) and 100 mg Ag kg^{-1} in the form of Ag^+ ions (B & D). For each of the treatments we have provided unstained light microscopic image of the TS labelling all the major anatomical features of the earthworm (key given below). Also we have included binary overlays of Sulphur (green) and Silver (Blue), and Phosphorus (Red) and Silver (Blue), as well as Silver alone (Blue). The relative fluorescence of each element is represented on a log scale between 0-140,000 RFU with scale bar for the individual elements provided at the base of each image. The locations used for Ag L(II)-edge spectra are labelled (i)-(iv) shown in Panel C for AgNP sample and (v)-(viii) shown in Panel D for the Ag-Ion sample. Anatomical labels used include: ep = epithelium, cm = circular muscle, lm = longitudinal muscle, ch = chloragogenous tissue; ge = gut (intestinal) epithelium; gl = gut lumen; lm = longitudinal muscle; np = nephridial tubule profiles (in coelomic cavity); ty = typhlosole fold (with enclosed chloragog).

Table 1. Nominal, measured and pore water concentrations of Ag in soils sampled at the start of the earthworm uptake experiment with ionic Ag and Ag NPs. Mean values \pm standard deviation (SD) are shown.

Ag compound	Ag nominal (mg kg ⁻¹)	Total actual [Ag] (mg kg ⁻¹)	% Recovery	Ag pore water (mg L ⁻¹)
AgNO ₃	20 (n=3)	23.5 \pm 2.48	117 %	0.03 \pm 0.01
	100 (n=3)	101 \pm 4.42	100 %	0.26 \pm 0.02
AgNPs	100 (n=6)	84.9 \pm 33.8	85.0 %	0.024 \pm 0.001
	500 (n=6)	431 \pm 115	86.3 %	0.044 \pm 0.001

Table 2. Uptake and elimination rate constants (related to total (k_{1-T} and k_{2-T}) and soil pore water (k_{1-pw}) silver concentrations) estimated from one-compartment model fits of a time series of total body Ag concentrations measured in the earthworm *Lumbricus rubellus* exposed to Ag⁺ ions and Ag NPs in LUFA 2.2 loam sand soil. The 95% confidence intervals are shown in brackets. Coefficient of determination (r^2) describes variance in earthworms body concentrations of Ag explained by the fitted model.

Ag nominal [$\mu\text{g g}^{-1}$]	Treatment	k_{1-T} (g soil earthworm ⁻¹ hour ⁻¹)	k_{2-T} (h ⁻¹)	k_{1-pw} (g soil g earthworm ⁻¹ hour ⁻¹)	r^2
20 mg kg ⁻¹ AgNO ₃	Unsealed	0.03 ^a (0.02 - 0.04)	0.031 (0.01 - 0.05)	21.0 ^a (12.3-29.8)	0.705
	Sealed	0.0012 ^b (0.0005 - 0.002)	0 (-)*	0.83 ^b (0.32 - 1.33)	0.631
100 mg kg ⁻¹ AgNO ₃	Unsealed	0.008 ^{c, I} (0.004 - 0.011)	0.022 (0.009 - 0.035)	2.94 ^{c, I} (1.73 - 4.15)	0.706
	Sealed	0.001 ^{b, I} (0.001-0.001)	0.001 (0 - 0.006)	0.51 ^b (0.32-0.69)	0.815
100 mg kg ⁻¹ AgNPs	Unsealed	0.002 ^{a, II} (4x10 ⁻⁴ -0.004)	0.026 (0 - 0.055)	10.5 ^{a, II} (2.09-18.8)	0.550
	Sealed	1.2x10 ⁻⁴ ^{b, II} (15x10 ⁻⁵ -2.3*10 ⁻⁴)	0 (-)*	0.53 ^b (0.06-0.98)	0.637
500 mg kg ⁻¹ AgNPs	Unsealed	0.015 ^a (0.002-0.033)	0.160 (0 - 0.350)	177 ^c (21-376)	0.290
	Sealed	3x10 ⁻⁴ ^b (4.3x10 ⁻⁵ -0.001)	0.0063 (0 - 0.029)	2.99 ^b (0.49-6.49)	0.637

* Data did not allow calculating reliable 95% confidence intervals.

^{a, b, c} Indicates significant differences between k_1 values for each compound for the different treatments (sealed and unsealed) and different exposure concentrations according to a generalized likelihood-ratio test ($\chi^2(1) > 3.84$; $p < 0.05$).

^{I, II} Indicates significant differences between k_1 values for each exposure concentration according to a generalized likelihood-ratio test ($\chi^2(1) > 3.84$; $p < 0.05$).

Figure 1.

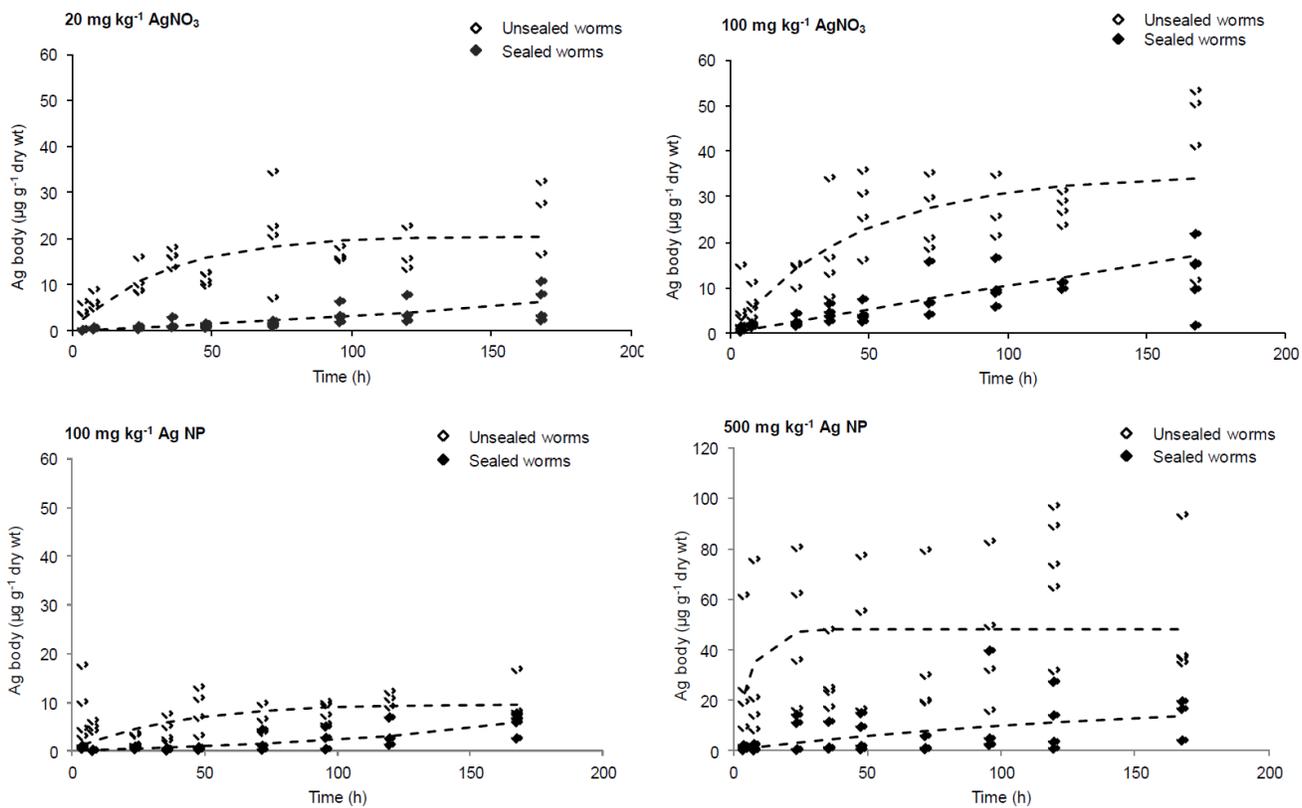


Figure 2.

