Quantification and modelling of accumulation kinetics of nanomaterials in soil organisms under environmentally relevant conditions

Marta Baccaro

Propositions

1. Environmental risk assessment of nanomaterials based on their as-manufactured form is inadequate.
(this thesis)

2. The role of organisms in the fate of nanomaterials in the soil is underestimated.
(this thesis)

3. Scientists without education in data science will fail to keep up with new technologies.

4. The economic valuation of the ecosystem services is a useful tool for decision making but ignores the intrinsic value of nature.

5. Having done many trips does not necessarily make you a good traveler.

6. Modern ecocriticism is fundamental for the development of a non-anthropocentric view in young generations.

Propositions belonging to the thesis, entitled

"Quantification and modelling of accumulation kinetics of nanomaterials in soil organisms under environmentally relevant conditions".

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Quantification and modelling of accumulation kinetics of nanomaterials in soil organisms under environmentally relevant conditions

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Table of content

Chapter 1

General introduction

Nano-revolution

Nanotechnology is the design, production and manipulation of any material and technology at the scale of nanometres. An important aspect of nanotechnology is the development of so-called nanomaterials (NMs). NMs often have different properties compared to their corresponding bulk materials due to their small sizes, and their surface can be further functionalized to obtain a higher variety of properties. The advancement of nanotechnology resulted in revolutionary applications of NMs in diverse fields such as communication, medicine, energy production, water treatment, agriculture, textiles and cosmetics [1]. The European Commission identified nanotechnology as one of the key enabling fields for economical and societal development in Europe. The design, the production and the application of NMs are indeed growing economical fields, which is illustrated by the fact that between 2012 and 2015 the number of known products containing NMs present at the European market increased from ~1200 to ~2200 [2]. In spite of their wide use and their benefits like for instance for the treatment of diseases and safer production of food, there is still an urgent need to address the human and environmental risks related to NMs. According to the definition adopted by the European Commission (Recommendation 2011/696/EU) [3], a nanomaterial is "A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm.". This definition only provides a general and incomplete understanding of the term NM. It is generally assumed that NMs may be more toxic compared to their bulk counterparts, which is related to the high surface-to-volume ratio, making NMs potentially highly reactive. However, other properties of NMs have been also identified to be linked to their toxicity such as shape (e.g. spherical nanoparticles (NPs), nano-

plates, nano-rods) surface charge (positive, neutral or negative), roughness, stability (tendency to dissolve, aggregate) and coating (to stabilise or to functionalise) [4-7]. All these properties may influence their behaviour in their respective environments, which in turn however can alter some of the NMs' properties [8, 9]. Depending on whether NMs are released to the water stream, to the air or to the soil, their fate and bioavailability for potential target organisms will be different, depending on compartment specific conditions and processes. From their production to their release in the environment and even until their interaction with the biota, NMs can undergo several transformations providing them with completely new physicochemical and biological identities [10]. It is an interplay between the initial NM characteristics, the acquired NM characteristics and the different environments/conditions in which NMs are released [11, 12]. Transformations of NMs can occur also within the organisms, e.g. in the biofluids influencing their transport through biological membranes (e.g. dermal membranes, gut epithelium), inside the cells. Finally as a result of this, transformations affect the mode of action of NMs [13].

Environmental compartments (air, soil, water) and organisms can be conceptually considered as environmental reactors for NM transformations (Figure 1). As described in Figure 1, for instance NMs could be transformed by an environmental compartment (e.g. soil) into different aged forms. These forms can either stay in the compartment or be transferred to another, e.g. from soil to surface water by surface run-off after rain. Either way, the new, aged form can undergo further transformations. Finally, the transformed NMs can be taken up by environmental organisms, hence the form of the NM that is being accumulated by the organism depends on the initial form of the pristine NMs and the results of the combination of all environmental compartments and transformations (environmental reactors) the NM has passed through. The type of transformation and to which reactor the aged NM will be transferred

depends not only on the characteristics of the NM (innate and acquired) but also on the specific characteristics of the compartments.

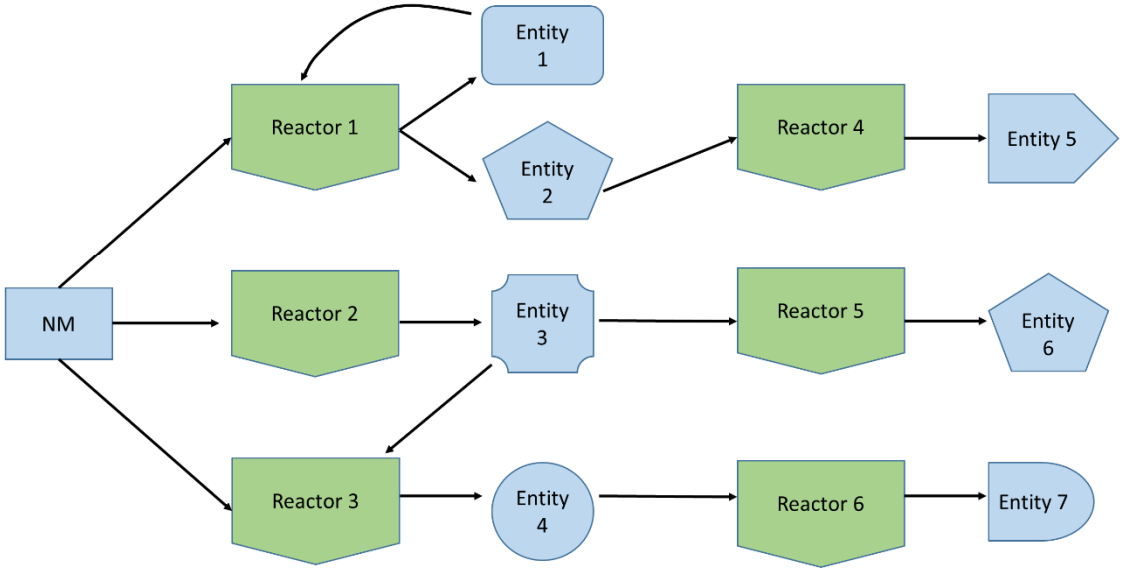

Figure 1. Schematic illustration of the transformation processes which an NM can undergo when it encounters different environmental reactors (combination of environmental compartment/organism and transformation process).

The environment as a reactor for NM transformation

Transformations occurring in the environmental compartments are generally called aging processes.

In natural aquatic and terrestrial systems, NMs interact with diverse dissolved or colloidal compounds, being inorganic (e.g. aluminium phyllosilicates, oxides, hydrous oxides, silicates) or organic (humic and fulvic acids, protein, polysaccharides, nucleic acids), that potentially influence NM homoaggregation (between the same NMs) and heteroaggregation (between NMs and other particles) dynamics and therefore their colloidal stability [14, 15]. As dynamic

reactions, aggregation processes have been shown to be reversible with specific changes in the environmental conditions (e.g. pH or temperature fluctuations) [16-18], so the formed aggregates may disaggregate [19]. For metal NMs such as Ag-, Cu-, CuO-, FeO-, TiO_2-, Al-, AlO-, Zn-, ZnO-, Mn-, Au-, Co-, and CeO_2-NMs, dissolution and complexation with counter-ions appeared to represent the most critical transformations [20, 21]. Release of ions can lead to a decrease of the NP-size in the case of partial dissolution. Ions can be more mobile and bioavailable or they can re-precipitate in association with other elements. NPs can dissolve completely in aqueous media, for instance ZnO-NPs can disappear by total dissolution into ions after a few hours in specific conditions [20].

Because these transformations can change the identity of the NMs and therefore the way they interact with the environment and biota, it is fundamental to characterise both the quality (different forms) and the quantity (amount of the different forms) of an NM when addressing the actual exposure and finally the related environmental risk. In theory, this should be performed by following the environmental transformation kinetics and fate of NMs from their production, going through their use and release in the environment, finally predicting the ultimate NM forms and their bioavailable concentrations for environmental species. In practice, the high variety of NMs, the lack of data about their release and the complexity of the possible transformations hamper filling this data gap for each NM, because it would require a never-ending and unfeasible case by case series of studies. However, a static picture of the NMs form in a specific environmental compartment (e.g. soil) may not be enough for a final identification of the exposure [22]. Altogether this asks for innovative, dynamic approaches in testing and modelling of kinetics of exposure of environmental organisms to relevant forms of NMs.

Biota as a reactor for NM transformation

Transformations take also place once the environmentally aged NMs or the dissolved ions are taken up by the organism. Biocorona formation has been widely reported as the most important issue to address in order to understand the toxicity of NMs [23, 24]. Biocoronas are instantly developed when NMs are exposed to biofluids, such as blood or coelomic fluid, where NMs encounter a plethora of proteins that can bind on their surface, creating a protein coating of which thickness mainly depends on the NM size and chemistry, and the kind of proteins available in the environment [25, 26]. Likely, as part of the detoxification pathway, bio-mediated formation of new nano-objects after exposure to metal ions was observed in rats [27], in plants [28].

Organisms can be considered as NM reactors not only because they can internally transform NMs but also because the organisms' activity within the environmental compartments can have consequences for the fate of the NMs. For instance, vertical displacement of soil by e.g. plants, invertebrates and small mammals has been reported as a considerable process affecting the fate and behaviour of chemicals in soil and sediment [29-31], which likely also applies to NMs in soils and sediments. However, this has not been addressed, yet.

Implication for the exposure assessment

In the last decade a considerable number of studies has been performed to improve understanding of the toxicity of NMs in invertebrates [32-35]. However, reported experiments have always been conducted by exposing organisms to relatively high concentrations (higher than predicted environmental concentrations) of pristine forms of NMs, ignoring the earlier

mentioned potential impact of environmental aging processes on the NMs bioavailability. This approach resulted in an extensive amount of data that may not reflect realistic exposure scenarios, both in exposure levels as in the form of the NM. Under realistic environmental conditions exposure of the organisms to the pristine forms is not likely to occur and therefore their use in ecotoxicological testing may not be predictive of environmental risks. The study of aged NMs is an obvious and essential improvement towards a realistic environmental risk assessment.

Additionally, to date most of the research on risks of NMs is focused on toxicodynamic processes with less consideration for toxicokinetics. However, the uncertainty regarding any potential toxic effect caused by nano-specific properties, which is one of the core questions of nanotoxicology, could be partially extricated by first studying the toxicokinetics of environmentally aged NMs. Furthermore, most of the studies including environmental changes in the forms of NMs were focussed on effect in organism in aquatic systems [36-41], with only a few soil based studies [42, 43].

Research objective of the thesis study

Given the urgent need for assessing the form specific exposure of soil organisms to NMs, the present research aimed to contribute to the exposure assessment of pristine and aged NMs in soil organisms and to evaluate the effect of soil organisms on the fate of aged NMs in the soil under environmentally relevant conditions by addressing the following research objectives:

- Identification and quantification of the transformation processes that mainly influence the uptake of NMs in soil organisms;

- Quantification and modelling of the toxicokinetics (uptake, metabolism and excretion) of pristine and aged NMs in soil invertebrates exposed to environmentally relevant concentrations;
- Assessment of the effect of a prolonged exposure to pristine and aged NMs on their toxicokinetics in soil organisms at environmentally relevant concentrations;
- Assessment of the influence of the activity of invertebrates on the fate of aged NMs in soils.

The model NMs selected for the work are metal based NMs because for these type of NMs sensitive and metal-specific methods (e.g. spICP-MS) are available to quantify their different forms (e.g. ionic and particulate) in complex media like soil and organismal tissues by the application of well-defined specific extraction methods [44-46]. In particular, silver nanoparticles (Ag-NPs) are specifically suitable for different reasons. Ag-NPs are widely produced and applied in everyday goods such as textiles, inks, biomedical devices, personal care products, food packaging and functional ingredients [47, 48] and therefore released in the environment and in particular in the soil, representing a relevant case study. Pristine Ag-NPs have been studied and insights regarding their interactions with soil organisms are available as point of departure. Furthermore, Ag-NPs undergo transformations that represent all the most relevant transformations of NMs in soil, including dissolution, complexation and precipitation. Finally, because of the low Ag natural background, the detection of Ag-NP is possible at relatively low environmental concentrations in complex matrices [44].

The release of Ag-NPs in the soil

Because of the wide use of products containing Ag-NPs, these NMs can be released in the environment. One of the main routes by which Ag-NPs can reach the environment is through their release in wastewater, followed by further transport via the wastewater stream to waste water treatment plants (WWTPs), and their final deposition in sludge. Sludge is the solid or semi solid by-product of WWTPs, rich in phosphorus, nitrogen and organic matter and it may contain contaminants that are not efficiently removed by the biological and mechanical treatments of wastewater in a WWTP. In the case of Ag-NPs, simulation of WWTP processes report that up to 90% of the Ag is retained in the sewage sludge with a near complete sulfidation of the Ag-NPs under both oxic and anoxic conditions within a few hours to days, leading to the formation of nano-sized Ag_2S [49-54]. Use of sludge on agricultural fields as fertilizer, which brings the Ag-NMs into the soil, represents 40% of the final destination of sludge in Europe, which however drastically differs between countries. In some countries like the United Kingdom, Spain and Ireland, the amount of WWTP-sludge used for agriculture was more than 50% of the produced sludge in 2010. However, other countries, like The Netherlands and Greece do not use WWTP-sludge in agriculture [55]. At this moment no field measurements of Ag-NPs or Ag_2S-NPs in sludge or soil are available in literature. A probabilistic material flow analysis approach [56] predicted concentrations of particulate Ag in sludge after WWTP treatments to be around 4 mg kg^{-1} in 2018 with concentrations in the sludge treated soil predicted to increase at a rate of approximately 4 µg kg^{-1} $year^{-1}$. Based on the abovementioned main release route of Ag-NPs, it is the aged form (Ag_2S-NP) that has the highest potential for the interaction with soil organisms in the top soil. The assessment of the risks of the environmentally relevant forms of Ag-NPs (e.g. Ag_2S-NP) in soil organisms represents

therefore a priority. Considering the uptake and the toxicity, the most critical transformation of metal NPs in soil is dissolution [57, 58]. Therefore, assessing the dissolution of the different Ag-NP forms in the soil, their uptake and mobility in the topsoil over time is of high interest. Whether Ag_2S-NPs (or other NMs) are transported from the sludge on the surface of the agricultural fields to deeper layers is also relevant because this may enhance the bioaccessibility of NMs to a broader range of soil organisms while it may decrease the peak concentrations at the surface.

Ag-NPs, Ag_2S-NPs and risks for soil organisms: toxicodynamics and toxicokinetics

In the last decade a considerable effort was made in order to evaluate the toxicity of Ag-NPs in several model organisms [59-62]. In particular, a significant amount of studies focussed on the toxicity of NMs in aquatic organisms while only few studies aimed to increase the comprehension regarding soil organisms [63-66]. Generally, the main attempt of those studies was to compare the toxicity of pristine Ag-NPs to the toxicity of Ag ions (from $AgNO_3$) by exposing the organisms to the same Ag concentrations in order to highlight a potential particle-specific toxicity. Studies testing the toxicity of aged, environmental relevant forms of Ag-NPs, like Ag_2S-NPs, are very limited. The few studies conducted report lower toxicity and lower availability of Ag_2S-NPs compared to the pristine NP form, likely related to their lower dissolvability which lead to negligible dissolution rate [41, 43, 67].

In the arthropod *Folsomia candida*, $AgNO_3$ affected survival with an LC_{50} value of 284 mg Ag kg^{-1} dry weight soil and reproduction with EC_{10} and EC_{50} values of 47.6 and 99.5 mg Ag kg^{-1} dry soil, respectively. No effects of Ag-NPs on survival and reproduction were observed up to 673 mg Ag kg^{-1} dry soil [68]. The isopod *Porcellionides pruinosus* avoided soil with spiked

Ag, with EC_{50} values of ~16.0 and 14.0 mg Ag kg^{-1} dry weight soil of Ag-NPs and $AgNO_3$, respectively [69]. In the same study, EC_{50}s for effects on food consumption ratio were 127 and 56.7 mg Ag kg^{-1} for Ag-NPs and $AgNO_3$, respectively. In another study, the earthworm *Allolobophora chlorotica* showed avoidance effects indiscriminately for nano and ionic Ag forms, both occurring at 12.5 mg kg^{-1} dry weight soil [70]. Although the toxicity tests showed that Ag-NPs are most of the times less or sometimes equally toxic as equivalent amounts of Ag ions (from $AgNO_3$), results were dependent on the type of NP coating and varied due to the variable characteristics of the soils used in the studies, while overall no effect of size was reported. Velicogna et al. [71] reported that effects of Ag-NPs and $AgNO_3$ were greater when plants and collembolan were exposed in sandy soil compared to soil with higher silt content. Earthworms (*Eisenia fetida*) showed no size related differences in mortality, growth or reproduction when exposed to Ag-NPs of two different sizes (10 and 30 to 50 nm) [72]. Significant decreases in reproduction were observed in earthworms (*E. fetida*) exposed to 94.2 mg Ag kg^{-1} dry weight soil of $AgNO_3$, but for Ag-NPs coated with oleic acid or polyvinylpyrrolidone this was at 727.6 mg Ag kg^{-1} dry weight soil and 773.3 mg Ag kg^{-1} dry weight soil, respectively [72]. However, behavioural effects such as avoidance and feeding inhibition were reported at far lower exposure concentrations, indicating that behavioural endpoints are more sensitive. Variation in size and surface coating of Ag-NPs was tested exposing *Lumbricus rubellus* [63]. The study provided evidence of the influence of coating of NPs on their reproductive toxicity: Ag-NPs coated with BSA (bovine serum albumin, negatively charged), were most toxic, Ag-NP with PVP (polyvinylpyrrolidone, neutral) intermediate and chitosan coated (positively charged) least toxic. Additionally, a limited effect of size (range of 20–50 nm) in driving uptake and toxicity of the Ag-NPs tested was reported. In a four weeks reproductive study [64] it was reported that in *L. rubellus* 15.4 mg Ag kg^{-1} dry

weight soil of $AgNO_3$ caused a higher inhibition in cocoon production than the same Ag exposure in the form of Ag-NPs. However, tissue injuries were more severe for the Ag-NP treatment compared with the $AgNO_3$ treatment. This was interpreted as an indication that Ag-NPs may prolong the presence of a bioavailable fraction of Ag, which may have effects on the immune system in a long term exposure [73].

Although studies performed with pristine NMs showed uptake and toxicity, the application of such pristine, non-aged NMs may be of little environmental relevance. As described earlier, it became generally clear that properties of NMs change in the environment, and this was also shown for Ag-NMs. The resulting Ag_2S-NPs appeared to be bioavailable [74] and to cause toxicity [75] to plants. Starnes et al. [76] observed effects on gene expression in *Caenorhabditis elegans*. At concentrations corresponding to the EC_{30}, the toxicogenomic responses differed among exposure to $AgNO_3$, Ag-NPs and sulfidized Ag-NPs. The responses to sulfidized Ag-NPs were distinct from $AgNO_3$ while some of the effects of pristine Ag-NPs were similar to the ionic form, suggesting that effects from Ag-NPs is partially due to dissolved silver ions but this seems not to be true regarding the sulfidized Ag-NPs.

Ions or particles?

There is uncertainty discriminating and assigning toxic effects to particulate forms of Ag-NPs or to ions released from the Ag-NPs. The generally observed lower toxicity of Ag-NPs compared to $AgNO_3$ in soil organisms could be due to the lower uptake of particulate forms of metals compared to the ionic forms. Dissolution of the NPs may be too slow to become relevant within experimental time frames. This may especially apply to environmental relevant forms of NPs which are generally hardly dissolving, like Ag_2S-NPs. It is still unknown to which extent

Ag-NPs and Ag_2S-NPs reach the organisms as particles and/or ions. The concept of bioavailability is therefore even more relevant in the case of NPs in different environmental compartment where it more likely that NPs are present as aged NPs than in pristine forms. As the main risk assessment principle states, occurrence of risk from a toxic compound depends on to what extent the organism is exposed. Therefore quantification of NM exposure is a crucial point to address in order to understand risks posed by nano-particulate metals (e.g. pristine versus aged Ag-NPs). Within this framework distinguishing the particulate and ionic exposure resulting from exposure to different Ag-NP forms is also of primary importance.

Earthworm as model organism in ecotoxicology

Earthworms are important organisms for soil formation and its general functioning and fertility [77-80]. Aristotele (300 b.C.) properly called them "the Intestine of the Earth", while in 1881 Darwin published a book called "*The Formation of Vegetable Mould through the Action of Worms, with Observations of their Habits"* on the impacts of earthworms on the formation of soil and soil quality [81]. Indeed, earthworms mix mineral particles with organic matter while ingesting and digesting soil. The formed aggregates improve many of the properties of the soil, e.g. its aeration and water holding capacity, which are also desirable for agriculture practices [82]. Earthworms are sentinel species, used as bioindicators for monitoring the health of soil ecosystems [83]. Additionally, earthworms are prey of a wide variety of terrestrial animals such as birds, amphibian, mammals so that the accumulation of contaminants in earthworms can represent a concern for the predators due to biomagnification of persistent chemicals [84]. Because of their ecological relevance, earthworms represent valuable model species for assessing environmental risks that chemicals may pose to soil organisms [85]. Practically,

earthworms are relatively easy to handle and can be used with common laboratory facilities, hence they have been extensively employed in ecotoxicological testing of chemicals. Over the last decades, standardized guidelines have been developed to test e.g. bioaccumulation (OECD no 317) [86], acute toxicity (OECD no 207) [87], reproductive effects (OECD no 222) [88] and behavioural effects (ISO 17512:1-2008) [89] of chemicals, including NMs, in earthworms [90-92]. Earthworms are also specifically suitable for exposure studies due to their dual uptake routes including 1) dietary uptake via ingestion of the soil including soil particles and soil pore water, 2) dermal uptake via the skin including its pores. However, uncertainties still exist on the predominant route for uptake of NMs in earthworms, with the route being also dependent on the type of chemicals or NMs [93, 94].

Based on the before mentioned advantages, earthworms will be used in the current thesis as a model for soil organisms. The species used in the studies are *Eisenia fetida* (or *foetida*) and *Lumbricus rubellus* (both belonging to the family of Lumbricidae). The first species (length 60-120 mm, diameter 3-6 mm) is the most commonly used species in ecotoxicological testing of effects on soil organisms, because it can easily be maintained and bred. It is an epigeic species (surface feeder), commonly distributed in the Palaearctic [95]. *L. rubellus* (length 60-130 mm, diameter 3-4 mm) is an epi-endogeic species (sub-surface feeder), known to burrow down to 25-30 cm deep in the topsoil, and is commonly present in Europe and North America [96]. This species is environmentally more relevant than *E. fetida*, but more difficult to rear, so most tests are performed on wild caught specimens. This makes regular testing difficult, especially during summer and winter. Furthermore the history of wild caught specimens generally is relatively unknown (although exposure to chemicals can be assessed), which may hamper the interpretation of (unexpected) results.

Toxicokinetic studies

Toxicokinetics describe the rates with which a chemical enters the organism body (absorption) and how it is distributed (distribution), metabolized (metabolism) and excreted (excretion), combined also referred to as ADME characteristics. Absorbed doses are related to exposure duration and route of uptake, chemical bioavailability from the media (e.g. soil) and physiology of the organism. Metabolized, stored and excreted amounts are mainly related to the physiology of the organisms and its ability to detoxify and/or excrete the chemical. One approach to study toxicokinetics could be based on static accumulation factors which assume that the exposure concentration of the chemical in the media and the accumulated concentrations of the chemical within the organism reaches an equilibrium. This approach, based on equilibrium partitioning theory, is however not applicable to NMs because they are not thermodynamically stable [97]. In the last decades, dynamic models based on the compartmentalization of the organism have been developed. They may consist of classical one or two compartment models, where fitting of the data to the equations for one or two compartment models defines kinetic parameters. In addition so-called physiologically based kinetic models have been defined in which the compartments and rate constants have physiological meaning such as for example the liver or kidney and kinetic constants for metabolism in the liver or uptake from the intestine. Both approaches allow to simplify the complexity of an organism by defining compartments that are regions of the body with a uniform xenobiotic concentration and the use of kinetic rate constants to quantify the different ADME processes within and between compartments [98]. The simplest model is the classical one compartment model that considers the whole organism as one single compartment which is able to take up the chemical with a specific constant rate (k_1) and excrete the chemical with a specific rate constant (k_2) [99].

Classical toxicokinetic models have been widely used to describe the kinetics of different contaminants in a broad variety of organisms [99-103]. They have also been applied to NMs in different organisms. Tervonen et al. [104] assessed accumulation and depuration rates in daphnia after C_{60} exposure in artificial freshwater. Ramskov et al. [105] reported an elimination rate not statistically different from zero for CuO-NPs in the freshwater gastropod *Potamopyrgus antipodarum*, highlighting the lack of excretion of CuO—NPs and therefore the potential for high bioaccumulation. Diez-Ortiz et al. [106] applied a one compartment toxicokinetic model to *L. rubellus* exposed to Ag-NP and Ag ions in order to identify the predominant uptake route by distinguish the oral from dermal uptake and found that the uptake occurred predominantly via the gut epithelium.

Outline of the thesis

The aim of this thesis was to contribute to the exposure assessment of pristine and aged NMs in soil organisms and to evaluate the effect of soil organisms on the fate of aged NMs in the soil under environmentally relevant conditions. To this end the thesis also aimed to identify and quantify the transformations that mostly affects the NM uptake in soil organisms.

Chapter 1, the introduction chapter, provides background information and definition of the aim of the present thesis.

Chapter 2 describes a short-term (28 days) toxicokinetic study in earthworms exposed Ag-NPs, Ag_2S-NPs, and $AgNO_3$. A one-compartment model is applied to calculate separately the kinetic constants for uptake and excretion of particulate and ions. The uptake of true particles was distinguished from that of the dissolved ions in exposure with pristine and aged Ag-NPs,

in order to establish the role of dissolution for their uptake in the earthworms. Additionally, biotransformation and/or biogenic formation of NPs within the earthworms were identified.

Chapter 3 aims to assess the influence of NP dissolution on the uptake and the extent to which biogenic transformation of NPs occurred within the organisms. A bioaccumulation study is reported in which earthworms were exposed to bimetallic Au core-Ag shell-NP. By assuming that the Ag shell interacts with the soil environment and that the Au core represents a tracker for the uptake in the particular form, dissolution was measured under environmental realistic conditions.

Chapter 4 describes a long-term (270 days) toxicokinetic study performed in in earthworms exposed Ag-NPs, Ag_2S-NPs, and $AgNO_3$. Kinetic rate constants for uptake were calculated. In order to evaluate how adequately the short-term exposure model was able to take into account potential belated dissolution of NPs occurring over a prolonged period of time, toxicokinetic models based on short-term and long-term exposure were compared.

Chapter 5 addresses the potential influence of the activity of earthworms on the fate of Ag_2S-NPs in the soil. To accomplish this, a series of microcosms (soil columns) were used to mimic the disposal of Ag_2S-NP containing sludge and its effect on the burrowing behaviour of earthworms. The relevance of the transport of Ag_2S-NP along the soil columns in presence and absence of artificial rain water by the earthworms was highlighted and the process was modelled for prediction.

Chapter 6 presents a critical discussion of the findings of the studies presented within the current scientific framework and identifies the future perspectives.

Chapter 7 provides a summary of the whole thesis.

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Chapter 2

Ageing, dissolution and biogenic formation of nanoparticles, how do these factors affect uptake kinetics of silver nanoparticles in earthworm?

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Abstract

The soil represents an important environmental compartment that can be regarded as a final sink for metal nanoparticles including silver particles (Ag-NPs). Assessing realistic exposure scenarios, including bioavailability of Ag-NPs for soil organisms requires taking into account that Ag-NPs can undergo physico-chemical transformations, such as sulphidisation, before interacting with organisms. However, differentiating between uptake of true metal NPs and released ions is essential to assess the actual role of these two metal forms in toxicity over time. The present study quantified toxicokinetic rate constants of particulate and ionic Ag in *Eisenia fetida* exposed to soil treated with pristine Ag-NP (50 nm), Ag_2S-NP (20 nm), as an environmentally relevant form, and $AgNO_3$ as an ionic control. Results showed that uptake and elimination rate constants of Ag in earthworms exposed to Ag-NP and $AgNO_3$ were not significantly different from each other, whereas uptake of Ag_2S-NPs was significantly lower. Interestingly, the biogenic formation of particulate Ag (~10 % of the total Ag accumulated over time) in earthworms exposed to $AgNO_3$ led to a kinetic pattern of particulate Ag similar to pristine Ag-NPs. SEM-EDX analysis confirmed the presence of particulate Ag in earthworms exposed to both Ag-NP and $AgNO_3$, showing that these particles were different than those to which earthworms were exposed. We demonstrated that around 85 % of the Ag accumulated in the worms after exposure to Ag-NPs and $AgNO_3$ was present as ions or as particles with size < 20 nm. Additionally, the low accumulation of the non-dissolvable, sulphidised form of nano-Ag, reflecting aged particles in the environment, confirms the importance of ionic uptake of Ag. This study clearly shows that the main form of uptake for Ag in earthworms is the ionic species, which stresses the fundamental need to use environmental relevant forms of metal NPs in performing ecotoxicological tests, because pristine NPs may behave completely different in terms of dissolution.

Introduction

In the last decades the impressive progress of nanotechnology has led to a broad use of engineered nanoparticles (ENPs) in a large variety of applications. Their potential release in the environment however draws the attention to potential hazards and risks for organisms. Metal nanoparticles (NPs) represent a prominent class of ENPs, subject of numerous toxicological studies since the early 2000s [1]. Since then a huge number of studies have assessed the toxicity of metal NPs in different model systems, some *in vivo* [2-5] and also *in vitro* [6, 7]. Meanwhile, others have started to examine mode of action, internalisation and distribution [8-10]. Progressively, the understanding that ENPs not only have peculiar characteristics but also that they undergo diverse transformations in the environmental compartments and within organisms, has led to the need of more advanced and realistic testing approaches. Characterization of actual exposure and quantification of NPs bioaccumulation including toxicokinetics became fundamental in order to properly interpret toxicological data. Some studies [11-14] have focused on uptake kinetics, however, the tissue concentrations were generally quantified as total metal content (nanoparticles and ions), and not NP-specific. Dissolution is considered a crucial reaction for the study of toxicity of metal NPs: Ions were generally found to be more toxic than the particulate form in organisms [11, 15]. Other studies report NPs causing as much effect as the ionic form of the same metal [16], or results varied related to the species, endpoints tested, exposure time and matrix [2, 17-19]. Additionally, NPs can represent a source of ions in the environment and in biological targets over time. Differentiating and quantifying kinetic rates related to different forms of metal, resulting from metal-NPs, is therefore essential to assess the actual time related role of particles and ions in toxicity. Thus, studies focussing on model metal NPs are needed for the understanding of

general behaviour of metal NPs. Silver nanoparticles (Ag-NPs) are suitable for this purpose because 1) they have extensively been studied in toxicological studies, 2) they can be detected and quantified at relatively low concentrations, and 3) they are widely used in goods (*e.g.* paints, cosmetics, textiles, food packaging, medical devices [20]). Pristine, non-functionalised Ag-NPs dissolve in the environment, and are therefore an excellent model to study the uptake of NPs and its associated ionic forms [21]. Ag_2S-NP, formed in waste water treatment plants (WWTP), represents an environmentally relevant form of Ag-NP in soil because of the disposal of sewage sludge onto fields [22]. Depending on the removal efficiency of the WWTP, up to 90% of the Ag is indeed retained in the sewage sludge due to mechanical and biological treatment [23]. During the anaerobic phase of treatment in WWTP, Ag-NPs are mostly transformed into non-soluble Ag_2S-NPs because of the high concentration of bisulfide (HS^-) [24, 25]. Predicted Ag_2S-NP concentrations in sewage sludge were reported to be in the order of magnitude of mg Kg^{-1} [26]. In contrast to pristine Ag-NPs, Ag_2S-NPs hardly dissolve, which was confirmed in studies on the fate of Ag-NP in soil reporting that, Ag_2S-NPs remained the dominant species after 6 months of simulated composting [27] and 7 months in soil [28], showing persistence and low dissolution. Furthermore, Ag-NPs undergo numerous environmental transformations and it is unlikely that soil organisms are exposed to pristine NPs under natural conditions [29, 30]. In order to assess uptake kinetics of different forms of Ag in earthworms, an environmentally relevant soil organism commonly used in toxicity testing, *Eisenia fetida*, was exposed to pristine, uncoated Ag-NPs (50 nm), Ag_2S-NPs (20 nm) and $AgNO_3$ in natural soil and as well as to clean soil for 28 days in order to quantify particulate and ionic toxicokinetics of the three different forms. Accumulated Ag, both particulate and total content in earthworms, was quantified by spICP-MS and ICP-MS, respectively. Uptake and excretion rate constants were modelled for both particulate and ionic forms using a single compartment toxicokinetic model

[31]. Additionally, identification of NPs was performed by Scanning Electron Microscopy coupled Energy dispersive X-ray (SEM-EDX) in order to examine elemental composition of particles present in the earthworm tissue. Based on the results, an overall interpretation of the different routes of uptake of Ag-NPs and ions will be presented.

Materials and Methods

NPs and characterisation

The study was conducted with uncoated Ag-NPs, Ag_2S-NPs and $AgNO_3$ (99.8 %, Merck, Darmstadt). Ag-NPs (12.3 g Ag L^{-1} in 5.5 mM sodium citrate, 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) were synthesised by Applied Nanoparticles (Barcelona, Spain). Particles synthesis and characterization data are reported in Supplementary Information - S1. Size characterisation by TEM (Transmission Electron Microscopy) was performed by Applied Nanoparticles (Barcelona, Spain) and Oxford Materials Characterisation Service (University of Oxford, UK) (Figure 1). The use of chemically synthesised Ag_2S-NPs allowed the application of known exposure concentration, while reflecting environmentally relevant forms of Ag-NPs in soil amended with sewage sludge.

Earthworms breeding

Earthworms (*Eisenia fetida*) were chosen as a model organism because of their considerable ecological functions. *E.fetida* is a well-studied species, widespread in temperate regions of the world. Earthworms, supplied by Lasebo (Nijkerkerveen, The Netherlands), were kept in the same soil used for the experiments (Table S2) in an incubator at 20 ± 1°C with 24 hours light

for approximately 1 month prior to the experiments and fed with horse manure from an organic farm (Bennekom, The Netherlands) once every 2 weeks.

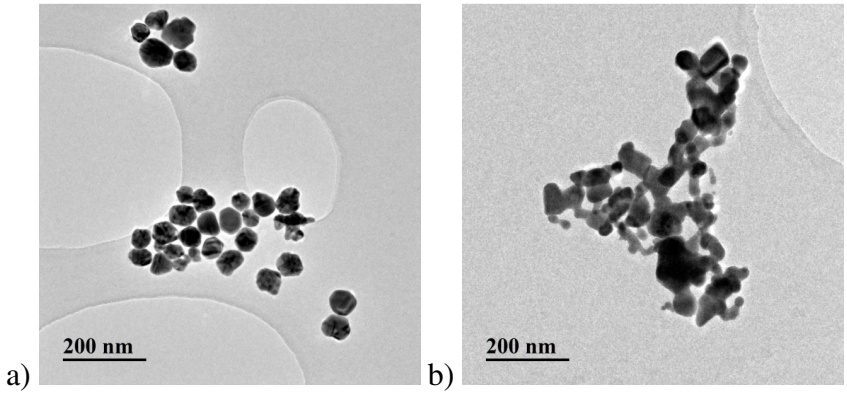

Figure 1. TEM images of a) Ag-NPs stock solution, b) Ag_2S-NPs stock solution.

Soil preparation and exposure

The soil (pH 5.2 in water, organic matter content 5.4 %, Supplementary Information - Table S2) was collected from an uncontaminated farm in The Netherlands (Proefboerderij Kooijenburg, Marwijksoord, The Netherlands) [32], air-dried and sifted (5 mm sieve openings) before use. Each experimental unit, consisting of a glass jar with a lid was prepared with 450 g of air-dried soil with additional water (20 % w/w; ~47 % Water Holding Capacity (WHC)). Soils were spiked with Ag-NPs, Ag_2S-NPs and $AgNO_3$ initially to reach a nominal concentration of 15 mg Ag kg^{-1} for all treatments, although actual concentrations differed (see Results and Discussion). The nominal concentration of 15 mg Ag kg^{-1} was chosen to assure a reliable analytical detectability, while being still environmentally relevant. Soil, water and added Ag were homogeneously mixed by an automatic mixer for 3 minutes. Every jar was prepared individually. 24 hours after spiking the soil, 7 adult *E. fetida* earthworms with an average weight of 0.245 ± 0.058 g per worm were randomly introduced into every experimental

unit. For each time point, 4 jars were prepared (n=4). Samples in the uptake phase were collected at day 7, 14, 21 and 28. After 28 days of exposure, the worms of the remaining 16 jars were transferred to jars with clean, not spiked soil (n=4 per each time point) for depuration phase. Samples were further collected after 35, 42, 49 and 56 days.

Sampling

At each time point of both uptake and depuration phase, 4 jars were separately emptied. Aliquots of soil were sampled and stored in polypropylene tubes at -80˚C after snap-freezing in liquid nitrogen. Worms were collected, carefully washed in deionized water, dried in a double layer of tissue paper and placed in glass Petri dishes in the incubator at 20˚C. Worms were allowed to purge their guts for 24 hours because, although it may be that residuals of soil still remain in the gut after 48 h, a prolonged period of starvation may result in the excretion of metal from earthworm tissue. Furthermore, in our experience > 95 % of the gut content is depurated in the first 24 hours [33]. After depuration, worms were washed again in deionized water, dried and snap-frozen in liquid nitrogen and homogenised by cooled mortar and pestle in order to obtain a fine powder. Worms from each jar were pooled. Immediately after homogenisation, samples were extracted and analysed for different forms of Ag. Soil pore water was obtained by equilibrating 24 g of wet soil treated for 28 days following saturation 100% of WHC [34]. After 24 hours of equilibration, soil was centrifuged through glass wool at 2000 g for 35 min (Hermle Z400K, Germany). The collected water was filtered through 0.45 µm cellulose acetate syringe filter (Chromafil, Macherey-Nagel, Germany). Glass wool and filters were conditioned by soaking them in a solution 0.1 M of $CuNO_3 + H_2O$ (99.9 %, Sigma Aldrich) overnight before use, in order to avoid adsorption of Ag on the surface of glass fibres and filter.

Extraction and analysis of Ag particulate form

Extraction of particulate Ag from worm tissue, soil and pore water was performed by an alkaline extraction using a tetramethylammoniumhydroxide solution (TMAH, 25 % in H_2O, Sigma Aldrich) [35]. An aliquot of powdered worm tissue or soil was weighed to the nearest mg (total ~ 0.4 g) and mixed 1:20 (w/v) with TMAH 20 %. To analyse pore water, 1 mL was mixed with 4 mL of the reagent. All samples were sonicated using the ultrasonic bath for 30 minutes and incubated at room temperature overnight. The next day, samples were again sonicated for 30 minutes. Since concentrations of particulate Ag in the worm tissues were unknown, different dilutions were tested before the analysis (data not reported). In all the cases a dilution factor of 10 000 times was optimal for the detection and quantification of particulate Ag in worm tissue. Proper dilution was reached by two steps dilution 1:400 TMAH 20% - sample followed by dilution 2:50 TMAH 20 % - sample in 50 mL Milli-Q water. Samples were analysed by Perkin-Elmer Nexion 350D (PerkinElmer Inc., Waltham, MA) operating in single particle mode (spICP-MS). The components of the instrument were: low-pressure nebulizer µflow-54 PFA-ST-4414 MicroFlow, standard demountable quartz torch, quartz 2.0 cassette injector, quartz cyclonic spray chamber, platinum sampler, skimmer cone with aluminium hyper skimmer, automatic sampler. The plasma power was set as 1600 W, the nebulizer gas flow was optimised daily between 1.03-1.06 L min^{-1} and sample flow rate was determined daily and ranged between 0.300-0.400 mL min^{-1}. Transport efficiency was calculated using Au-NPs 60 nm NIST (reference material 8013) which was 3-4 %. The data were acquired with a dwell time of 3 ms for 60 s resulting in 20000 readings per sample at *m/z* ratio of 107 for silver. The sampling system was rinsed with a 3 % HNO_3 (Merck, Darmstadt) solution before and in between running each sample. The calibration curve (0, 0.5, 1 and 2.5 µg L^{-1}) was prepared from a Ag^+ standard solution in matching matrix (standard stock solution 1000 mg L^{-1} Ag, Merck, Darmstadt). The

linear regression correlation coefficient (R^2) of calibration curves resulted to be 0.976 ± 0.025 (average ± standard deviation). The limit of detection (LOD) was 2 ng L^{-1} and the limit of quantification (LOQ) was 3 ng L^{-1}. LOD and LOQ were determined as number of particles in blank matrix + 3σ blank matrix and blank matrix + 10σ blank matrix and transformed to mass concentration, respectively.

SpICP-MS data processing

The data acquired from the instrument were further processed in Microsoft Excel (2016) by the Single Particle Calculation tool (WFSR – Wageningen Food Safety Research, Wageningen, The Netherlands)[36]. It allows the calculation of number and mass concentration, size and size distribution of the nanoparticles in aqueous suspension. The setting of the threshold of the signal intensity, which distinguishes the detection of a particle from the dissolved analyte and background signal was reached by application of μ + 5σ method [37, 38], where μ is the mean of the data set and σ is the standard deviation. The data points exceeding a μ + 5σ value are considered NPs and are removed from the data set. This process is repeated multiple times until no data are removed from the data set. Determination of NPs mass and size distribution is based on the assumption that particles are perfectly spherical in shape and that all the detected particles are either Ag-NPs or Ag_2S-NPs based on the exposure. The size detection limit was 20 nm, therefore particles smaller than this diameter are included in the ionic fraction.

Extraction and analysis of total Ag

Extraction of total Ag (ionic Ag and nano-Ag) from soil, worm tissues and pore water was performed as microwave-assisted acid digestion in aqua regia (1:3 Nitric Acid-Hydrochloric Acid). An aliquot of samples was weighed (~0.3 g of wet soil, worms or 1 mL of pore water)

and placed in Teflon vessels with 6 mL HCl 37 % (Merck, Germany) and 2 mL HNO_3 69 % (Merck, Germany). Digestion was performed by MARS 5 (microwave system, CEM corporation, USA) applying a temperature ramp from 160˚C (20 min) to 200˚C (40 min). After proper dilutions, samples were analysed by ICP-MS Nexion 350D (Perkin-Elmer Inc., Waltham, MA). Isotopes monitored were silver (*m/z* 107) and rhodium (*m/z* 103). A calibration curve was performed using concentrations of Ag^+ (standard stock solution 1000 mg L^{-1} Ag, Merck, Darmstadt) in matching matrix. Rhodium (standard stock solution 1000 mg L^{-1} Rh, Merck, Darmstadt) was used as internal standard. The limit of detection (LOD) of silver (*m/z 107*) was calculated as mean digested blank + 3σ blank and resulted to be 0.12 µg L^{-1}. The limit of quantification (LOQ) was calculated as mean digested matrix blank + 10σ blank matrix and resulted to be 0.14 µg L^{-1}.

Quality Control

Earthworm survival after 28 days of control exposure was > 90 % in all treatments, fulfilling requirements of the validity of bioaccumulation tests of chemicals in terrestrial oligochaetes (OECD n. 317, 2010) [39]. For every batch of samples, analytical quality was checked by blanks and an external standard of Ag^+ (1000 mg/L^{-1} Ag, Merck, Darmstadt), obtaining an average recovery of 100 ± 25 %. Spike tests of Ag-NP and Ag_2S-NP measured by spICP-MS in soil after TMAH extraction showed 53% and 87% recovery, respectively. In the case of spiked worm tissue the recovery was 92% for Ag-NP and 93% for Ag_2S-NP. Recovery was calculated based on stock solution concentration. TMAH was tested for inducing dissolution and particle formation (Supplementary Information - S5).

Calculation of uptake and elimination kinetic rate constants

Internal concentrations of particulate Ag (nano-Ag ≥ 20 nm) and total Ag content (ionic Ag and nano-Ag dissolved by acid digestion) in *E. fetida* were fitted with a one compartment model for Ag-NPs, $AgNO_3$ and Ag_2S-NPs specifically. This model is widely used to model toxicokinetics of metals in model organisms [31]. In the present study, equation 1 and 2 were used for NP and ions as two different species for the calculation of the uptake rate constant value (k_1) and the elimination rate constant value (k_2):

$$C_{int} = C_0 + k_1/k_2 * C_{exp} * (1 - e^{-k_2 * t}) \qquad 0 \leq t \leq t_n \qquad (1)$$

$$C_{int} = C_0 + k_1/k_2 * C_{exp} * (e^{-k_2 * (t - t_n)} - e^{-k_2 * t}) \qquad t > t_n \qquad (2)$$

where C_{int} is the internal metal concentration in the earthworms (mg Ag kg^{-1} wet body weight), C_0 the initial metal concentration in the earthworms (mg Ag 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 Ag kg^{-1} dry soil), t the exposure time (days) and t_n the last day of uptake phase. Equation 1 is used for the uptake phase and equation 2 for the elimination phase. In particular, k_1 and k_2 were calculated with data from the uptake and elimination phases together.

Results and discussion

Characterisation of exposure soil

Concentrations of total (ionic Ag and nano-Ag dissolved after acid digestion) and particulate Ag (nano-Ag ≥ 20 nm, measured by spICP-MS) were quantified in soil at four time points during the exposure phase (7, 14, 21 and 28 days) (Figure 2). For Ag-NPs, Ag_2S-NPs and $AgNO_3$ the actual total Ag concentrations in soil were respectively 9.0 ± 1.4, 3.7 ± 1.1, 9.3 ± 0.6, mg Ag kg^{-1} dry weight soil (average ± standard deviation), while actual particulate Ag concentrations were 2.0 ± 1.5, 4.5 ± 1.3, 2.5 ± 2.0 mg Ag-kg^{-1}dry weight soil (average ± standard deviation), respectively. Significant differences between particulate and total Ag concentration confirmed dissolution of Ag-NP and $AgNO_3$, but not of Ag_2S-NPs. Furthermore, there were no significant differences between concentrations of Ag in Ag-NP and $AgNO_3$ treated soils, neither for particulate nor total Ag (Supplementary Information - Table S3a). Hence, in the soil, pristine Ag-NPs dissolve in pore water but the absence of significant differences between total and particulate concentrations for Ag_2S-NP indicates that Ag was particulate, demonstrating its relative stability in soil (Supplementary Information - Table S3a). Our results are in agreement with previous studies [22, 28], in particular with Wang et al. [40], who reported Ag_2S-NP still being stable after an incubation in soil of 400 days, regardless of different pH ranges (5.4-7.1). In earthworms, metal uptake, including Ag, can take place via the dermal route as pore water solutes and via the dietary route as pore water solutes and complexes of metals present in the exchange fraction [41, 42]. Pore water metal concentration has been found to be well correlated to the internal concentration in earthworms [43]. As reported in Diez-Ortiz et al. [44], the concentration of particulate Ag and the release of Ag ions into pore water are highly important for understanding soil biota uptake.

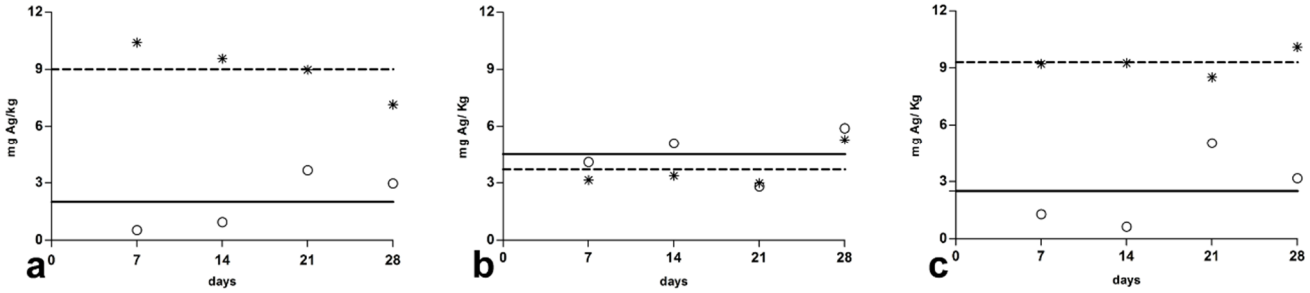

Figure 2. Concentrations of particulate Ag (◦; continuous line) and total Ag content (*; dashed line) in samples of soil of Ag-NPs (a), Ag_2S-NPs (b) and $AgNO_3$ (c) exposure at four time points during uptake phase of the experiment. Lines represent the average value.

Table 1. Overview of the concentration of Ag in soil and pore water (mean ± standard deviation; n=number of replicates); values with the same letters within a column are not significantly different from each other.

	Soil* (mean±SD)	Pore water* (filtered over 0.45 µm)	
	Total Ag content [a] mg Ag kg^{-1} (n=4)	Total Ag content [a] µg Ag L^{-1} (n=9)	Particulate Ag [b] µg Ag L^{-1} (n=3)
Ag-NP	9.0 ± 1.4 B	40.5 ± 5.4 A	0.08 ± 0.02 B
Ag_2S-NP	3.7 ± 1.1 A	<LOD**	0.04 ± 0.04 A
$AgNO_3$	9.3 ± 0.6 B	37.9 ± 5.4 A	0.04 ± 0.03 A

*from soil treated for 28 days

**LOD= 0.12 µg L^{-1}

[a] total Ag measured by ICP-MS

[b] particulate Ag measured by spICP-MS

To address this, total Ag and particulate Ag concentration in pore water samples extracted from soil treated with Ag-NP, Ag_2S-NP and $AgNO_3$ for 28 days, were analysed (Table 1). Results showed that soil pore water only contained 0.21 % and 0.19 % of the total Ag in the soil for Ag-NP and $AgNO_3$ treated soil, respectively (calculated based on the assumed total amount of Ag in 20 g of dry soil used to extract the soil pore water). For Ag_2S-NPs the total concentrations were below the limit of detection (LOD= 0.12 µg L^{-1}), indicating very low release of Ag to the

pore water, again implying low solubility [45-47]. Particulate Ag represented 0.2 % and 0.1 % of the total Ag measured in pore water from Ag-NPs and $AgNO_3$ treated soil. The relatively low concentrations of total Ag suggest the low release of Ag in the pore water of which most seemed to be ionic or at least below 20 nm. Furthermore, the low relative particulate concentration in case of Ag-NP and $AgNO_3$ exposure, indicates relatively high dissolution in pore water [29, 30, 48].

Comparative kinetics of Ag-forms specific bioaccumulation in earthworms

Earthworms exposed to all Ag forms accumulated Ag in their tissues in detectable concentrations. In Figure 3, time-resolved kinetics of uptake and elimination of particulate Ag and total Ag in earthworm tissue exposed to Ag-NP, Ag_2S-NPs and $AgNO_3$ are provided. An increase of Ag concentrations, both particulate and ionic, occurred during 28 days of exposure to Ag-NP, Ag_2S-NPs and $AgNO_3$. Similarly, Ag concentrations decreased during the 28 days clearance period. There were no significant differences between Ag-NP and $AgNO_3$ exposed worms when comparing their particulate concentrations and total Ag concentrations at the different time points. However concentrations of both particulate and ionic Ag in earthworms exposed to Ag_2S-NPs were significantly lower when compared to Ag-NP and $AgNO_3$ exposed worms (Supplementary Information - Table S3b), even considering that actual exposure concentrations of Ag_2S-NPs in the soil were almost three times lower. Even when taking into account a 5% residual gut content in depurated worms (measured to be approximately 2 mg per worm), the total Ag_2S-NPs particulate concentrations due to this soil may account for just 6 % of Ag (in worms exposed for 28 days).

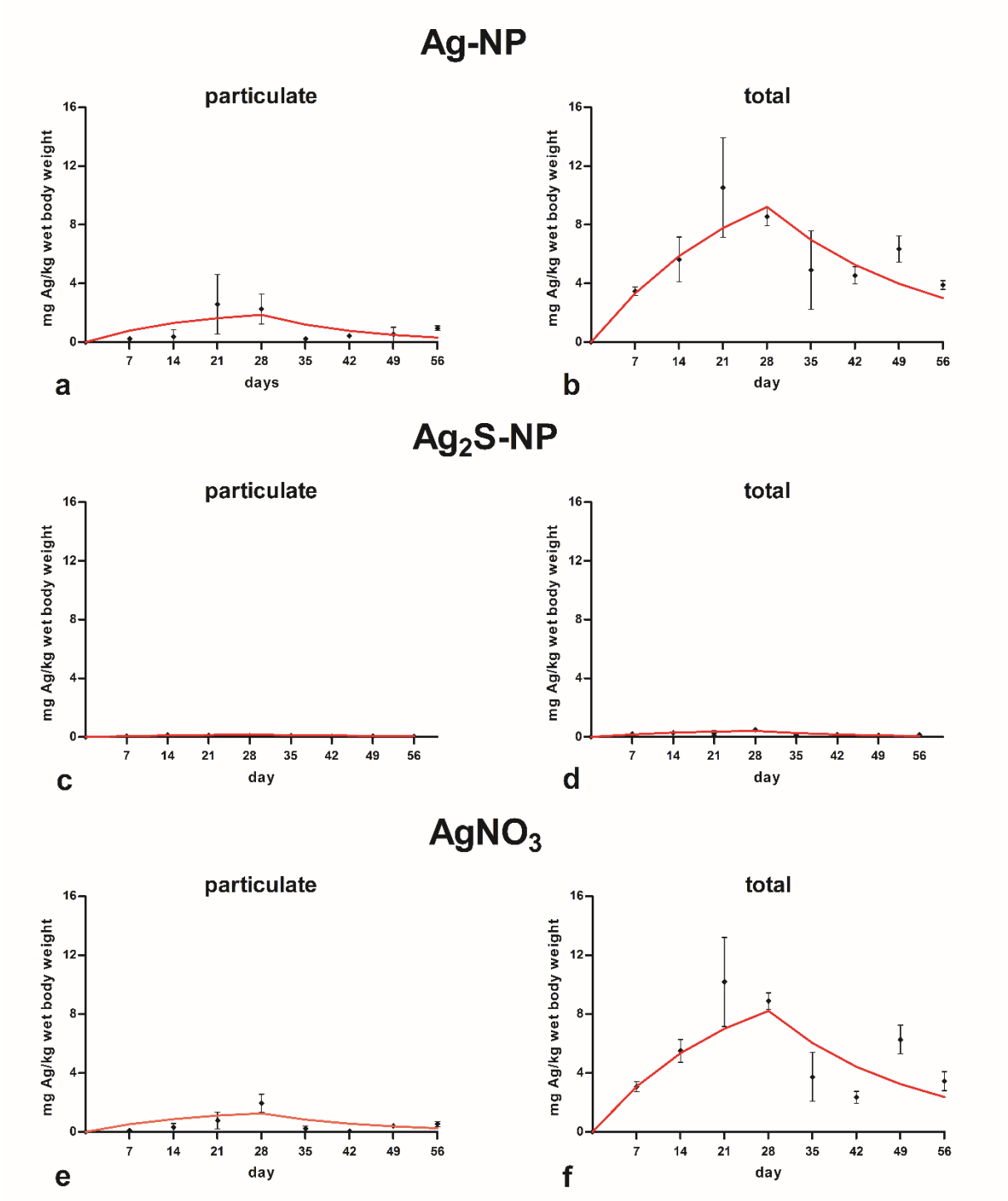

Figure 3. Kinetics of Ag accumulation of particulate Ag (a, c, e) and total Ag (b, d, f) in earthworm tissue exposed to Ag-NP, Ag_2S-NPs and $AgNO_3$ respectively (◆ average of experimental replicates (n=4), bars represent standard deviation, – modelled)

Uptake and Elimination kinetic rate constants of Ag-NPs, $AgNO_3$ and Ag_2S-NPs

An one-compartment model was used for the calculation of uptake rate constant (k_1) and elimination rate constant (k_2) for the different forms of Ag [31]. For the purpose of modelling, actual total Ag concentrations in soil and worm concentrations expressed as wet weight were used. Ag-form specific k_1 and k_2 related to the three different Ag exposure scenarios are reported in Table 2. The table also includes bioaccumulation factor (BAF) calculated as BAF = $k_1\ k_2^{-1}$ and corrected for dry body weight (dry body weight=16 % wet body weight) [49]. As shown in Figure 3, the time dependent uptake and elimination patterns were very similar for Ag-NP and $AgNO_3$ exposure scenarios. Rate constants related to these exposures did not differ significantly from each other and total uptake rate constants are higher than the ones of the particulate form, indicating that uptake occurred mainly as ionic form (including particles < 20 nm) regardless of exposure (Table 2). For Ag_2S-NP uptake rate constants are significantly lower than the ones from the other forms. In contrast, elimination rate constants (k_2) were not different between Ag forms. Studies on ZnO-NPs found indistinguishable differences between uptake of ionic Zn and ZnO-NPs in *L. rubellus* based on total metal measurements [12, 41]. Another study on Ag showed different uptake kinetic rate constants in *L. rubellus* exposed to $AgNO_3$ and Ag-NPs [13]. Based on dry weight (assuming 16 % dry weight) and on hourly time base, those rate constants were within the same range as in the current study for $AgNO_3$ and at the upper limit of the range for Ag-NP. Velicogna et al. [14] reported higher BAF for Ag-NP than $AgNO_3$ in *E. fetida*, 0.98 and 0.71 g dry soil g^{-1} dry tissue, respectively, similar to the present study. The same study reported a much lower BAF (0.12 g dry soil g^{-1} dry tissue) for earthworms exposed to Ag-NP in the presence of biosolid where some degree of sulphidisation may have occurred.

Table 2. Overview of uptake (k_1) and elimination kinetic rate constants (k_2) in earthworm *E. fetida* exposed to Ag-NP, Ag_2S-NP and $AgNO_3$ (value ± 95 % confidential interval *CI*; worm concentration based on wet weight (mg Ag Kg^{-1}); soil concentrations based on dry weight (mg Ag Kg^{-1})). Parameters with the same letters within a column are not significantly different from each other (Post hoc Tukey multiple comparison test following one way ANOVA (F (6, 191) = 236.1).

Ag form	Exposure (mg kg^{-1})	Ag species	k_1 (mg Ag kg dry soil mg Ag^{-1} kg^{-1} wet body weight day^{-1}) ± *CI*	k_2 (day^{-1}) ± *CI*	*P value of model*	BAF*
Ag-NP	9	Particulate	0.015 ± 0.010 a	0.062 ± 0.050 a	<0.001	1.5
		Total	0.061 ± 0.019 b	0.040 ± 0.013 a	<0.001	9.5
Ag_2S-NP	3.7	Particulate	0.002 ± 0.002 c	0.036 ± 0.050 a	<0.001	0.3
		Total	0.008 ± 0.002 c	0.064 ± 0.020 a	<0.001	0.8
$AgNO_3$	9.3	Particulate	0.010 ± 0.005 a	0.058 ± 0.040 a	<0.001	1.1
		Total	0.055 ± 0.007 b	0.044 ± 0.018 a	<0.001	7.8

*calculated as k_1/k_2 and corrected assuming dry body weight = 16 % wet body weight

Particulate content, sizes and elemental composition

Table 3 reports concentrations of particulate Ag as a percentage of total Ag concentration, in earthworms at different time points. Relative concentrations of particulate Ag in Ag-NPs and $AgNO_3$ exposed worms are not significantly different from each other and are lower than for Ag_2S-NPs (absolute concentrations in Supplementary Information - S4) underlining that dissolution is less relevant for Ag_2S-NPs. The occurrence of particulate Ag in the $AgNO_3$ exposure is consistent with Makama et al. [32] who measured particulate silver to account for 34 % and 4 % of total Ag in the *Lumbricus rubellus* exposed to 250 mg Ag kg^{-1} soil of Ag-NPs (PVP-coated, 50 nm) and to 15 mg Ag kg^{-1} soil of $AgNO_3$, respectively.

Table 3. Concentration of particulate Ag concentration (≥ 20 nm) (mg Ag kg^{-1} wet body weight) as percent of total content concentration (mg Ag kg^{-1} wet body weight) measured in earthworms at different time points during uptake phase and depuration phase (mean ±standard deviation).

	Uptake phase (days)				Depuration phase (days)			
% of total	7	14	21	28	35	42	49	56
Ag-NPs exposure	6.8±4.5	5.3±5.9	27.7±12.6	27.3±13.7	5.3±1.7	9.4±0.8	8.0±6.0	33.0±6.8
Ag_2S-NPs exposure	38.9±18.5	39.8±9.1	45.5±18.8	38.4±26.3	77.4±58	23.4±17.8	34.8±20.2	36.1±9.4
$AgNO_3$ exposure	3.0±1.5	5.4±4.3	8.3±6.4	22.2±7.6	6.2±3.0	3.0±1.6	6.4±1.4	17.2±8.4

Detection of nano-Ag in worms exposed to silver salt leads to the assumption that Ag^+ released from $AgNO_3$ may biogenically be transformed into particulate Ag, which may also be applicable for Ag^+ released by Ag-NPs and, to a lesser extent, by Ag_2S-NP. This is suggested by the fact that particulate Ag increases for Ag-NPs and $AgNO_3$ until 28 days exposure (Table 3) and that particulate Ag was bigger compared to the particulate Ag measured in pore water (Table 4). This size difference highlights that nano-objects in the worms were, likely, not the same particles present outside the worms and a biogenic transformation occurred. This was also visualised by SEM-EDX. Ag-nano objects were identified in worm samples exposed for 28 days to Ag-NP and $AgNO_3$ (Figure 4 and Figure 5). All detected objects were of different composition and size in comparison to the pristine Ag-NP they resulted from (in NP exposure). Sizes ranged between 75-200 nm and most of them were associated with sulphur. Ag-nano objects in worms exposed to Ag-NP and $AgNO_3$ can be associated with a variable amount of sulphur. When Ag-nano objects were detected by spICP-MS they could result in a smaller size compared to the actual size measured by microscopy techniques because spICP-MS data processing considers only silver. Ag-nano objects appear to be similar among $AgNO_3$ and Ag-

NP exposed earthworms. In the case of earthworms exposed to Ag_2S-NPs, concentrations of particulate Ag were too low for detection with SEM.

Table 4. Mean diameter sizes ± standard deviation (nm) of particulate Ag in worms exposed to Ag-NP, Ag_2S-NP and $AgNO_3$ for 28 days and in pore water extracted from soil treated for 28 days measured by spICP-MS. Please note that in case of the particulate in the worms exposed to Ag-NP and $AgNO_3$, which includes an unknown amount of S, the actual size may be underestimated by spICP-MS (see text).

	Worms Size (nm)	Pore water Size (nm)
Ag-NP	84 ± 4	37 ± 2
Ag_2S-NP	65 ± 19	27 ± 0
$AgNO_3$	81 ± 5	35 ± 3

The biogenic formation of particulate Ag has been described before as part of the detoxification system of organisms. For instance, Ag has been found to be associated with metal-rich granules [32], chloragogenous tissue and nephridia [13]. The detoxification pathway includes chloragosomes, insoluble calcium phosphate granules that immobilise the metals, and metallothionein, chelating proteins with sulphur donating ligands [50-52]. Based on above-mentioned pathways we hypothesize that metallothionein and chloragogenous tissue can actively form particulate Ag, also likely in the nano-range. This is an active process in the worm, since incubation of $AgNO_3$ with homogenised earthworm tissue and TMAH (alkaline reagent used for NPs extraction from worm tissue) did not result in the formation of Ag-particulate (Supplementary Information - S5).

Formation of nano-Ag has been reported in vivo in 1) earthworm *L. rubellus* [32] and mice lung [53], 2) biological fluids such as intestinal digestion fluid [54], and 3) fish intestinal cells [55]

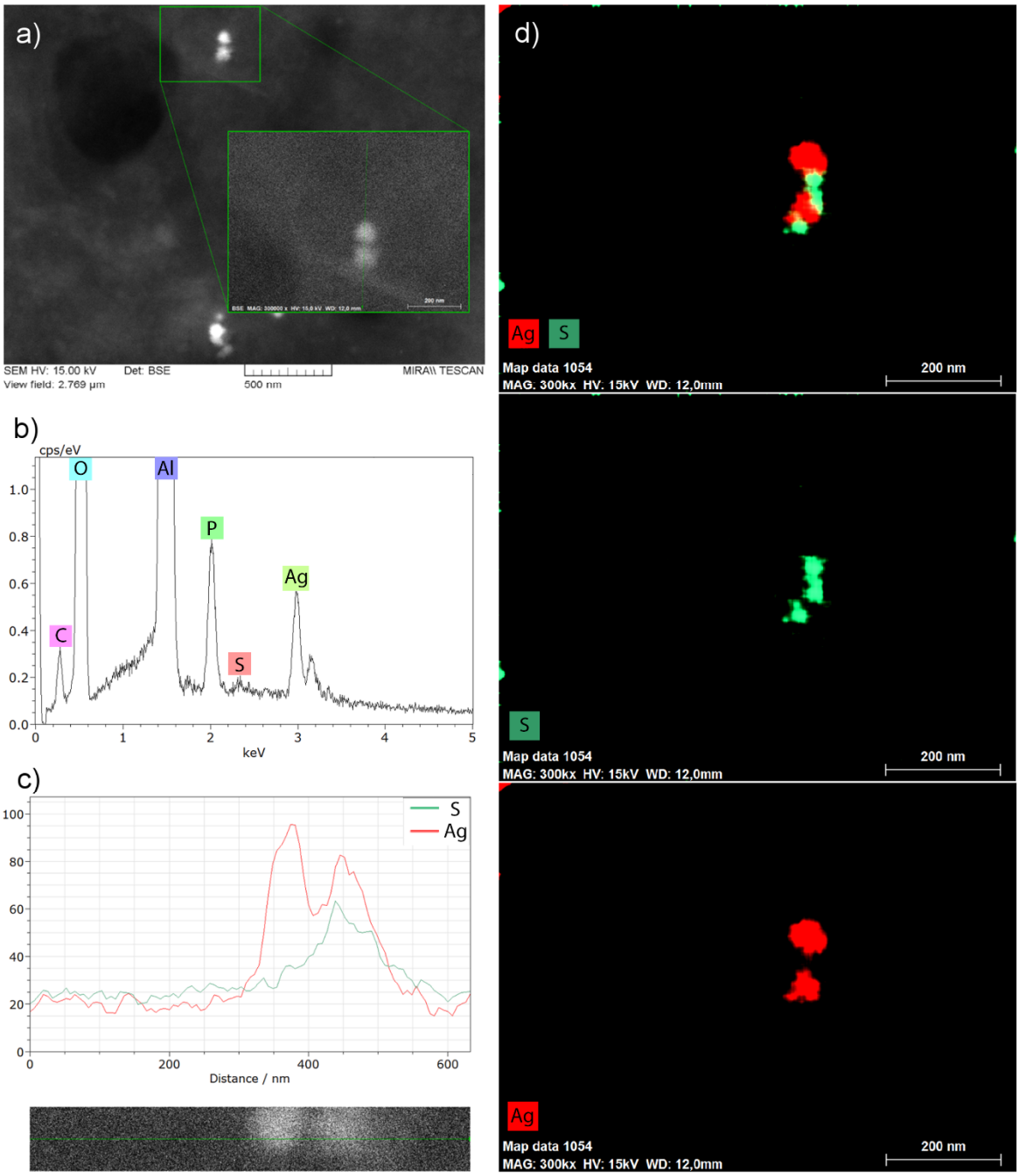

Figure 4. a) Scanning electron microscopy (SEM) picture of worm tissue exposed to Ag-NPs. Two particulate Ag objects are seen as two white dots (each of size ~100 nm); b) Energy dispersive X-ray (EDX) spectrum of the upper particle; c) EDX-line spectrum of the same particles (after clockwise rotation 90°), the left one with low S content and the right one with high S content; d) EDX-mapping of the same two Ag-nano objects, red colour indicates Ag content, green colour indicates S content.

exposed to $AgNO_3$ mainly because of precipitation of Ag^+ in particulate species such as AgCl(s) and Ag_2S(s). This may be applicable for Ag^+ released by Ag-NPs. Dissolving Ag-NPs could act as a source of ions within and outside the organism. It should be noted that in the Ag_2S-NPs stock solution used in this study not completely sulphidised Ag was present (Supplementary Information - S1), potentially resulting in some release of ions from the small amount of non-sulphidised Ag in the stock. However, it is not possible to completely exclude uptake of Ag-NPs and Ag_2S-NPs as particles, although no primary particles (Ag-NPs) could be detected in the tissue.

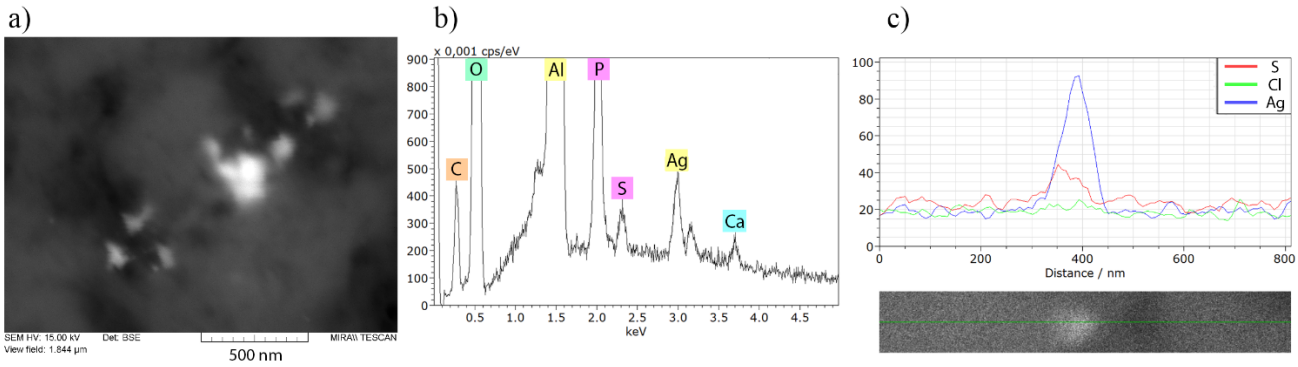

Figure 5. a) Scanning electron microscopy (SEM) picture of worms tissue exposed to $AgNO_3$. Particulate Ag objects are seen as white dots (150 nm); b) Energy dispersive X-ray (EDX) spectrum of one of the particles; c) EDX-line spectrum of the same particles reveals association to sulphur.

Conclusion

It can be concluded that uptake and elimination rate constants for worms exposed to pristine Ag-NP or $AgNO_3$ were not significantly different from each other. Uptake of (insoluble) Ag_2S-NPs was significantly lower, all indicating that ionic Ag (potentially including particles < 20 nm) was the main form taken up, irrespective of the actual form of Ag that the worms were exposed to. Interestingly, the biogenic formation of particulate Ag (~10 % of total Ag

accumulated overtime) in earthworms exposed to $AgNO_3$ led to a kinetic pattern of particulate Ag body burden similar to pristine Ag-NPs. However, it was evident that the resulting particles in the tissues were not the same as those to which worms were exposed in the Ag-NP exposure. It was demonstrated that approximately 85 % of the Ag accumulated in worms exposed to Ag-NPs and $AgNO_3$ was present in ionic form or particles with size below 20 nm (size detection limit of spICP-MS). The remarkable difference in accumulation of pristine and sulphidised form of nano-Ag, the latter reflecting the environmentally relevant form of aged Ag-particles that may accumulate in soil, indicates the fundamental need to use such relevant forms of metal NPs when performing ecotoxicological tests. The use of pristine metal NPs may lead, in the case of Ag, to an overestimation of internal uptake and associated risks. This is the first study the authors are aware of, that reports form-specific uptake kinetics of Ag in soil organisms. Further studies are needed to confirm that dissolution is an important paradigm for the assessment of uptake of (other) metal NPs in (other) soil organism and to enhance understanding about potential risks associated to delayed release of ions from metal NPs over long term periods.

Conflicts of interest

There are no conflicts to declare.

Acknowledgments

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Supplementary Information

S1 Synthesis and characterization of Ag-NP

Silver nitrate ($AgNO_3$), trisodium citrate ($Na_3C_6H_5O_7$), and tannic acid ($C_{76}H_{52}O_{46}$) were purchased from Sigma Aldrich. Briefly, 100 mL volume of aqueous solution containing sodium citrate (SC) (5 mM) and tannic acid (TA) (0.25 μM) was prepared and heated by a heating mantle in a three-neck round-bottomed flask for 15 min under vigorous stirring. After boiling had commenced, 1 mL of $AgNO_3$ (25 mM) was injected into this solution. The solution became bright yellow. Immediately after the synthesis of Ag seeds and in the same vessel, solution was diluted by extracting 19.5 mL of sample and adding 16.5 mL of MilliQ water. Then, the temperature of the solution was set to 90 °C and 500 μL of SC (25 mM), 1.5 mL of TA (2.5 mM), and 1 mL of $AgNO_3$ were sequentially injected (time delay ~ 1 min). By repeating and adjusting the amount of Ag precursor injected, different generations of Ag-NPs of progressively larger sizes were grown. Aliquots were purified by centrifugation (10000 g) in order to remove the excess of TA and further redispersed in SC 2.2 mM before sample characterization (Figure S1a, Table S1a). EDX on individual particle (Table S1b) shows that particles contain Ag (spectrum 11 within the particle) with a small layer of covering film (spectrum 12, close to the edge of the particle) which is similar in composition to the surrounding matrix (holey carbon film and additives). No sulphur could be detected.

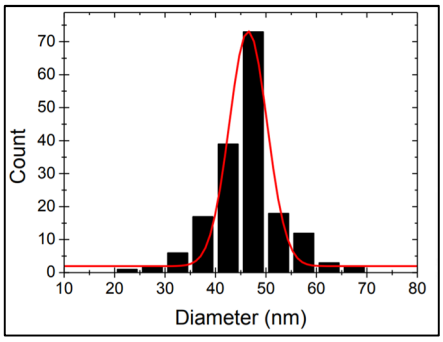
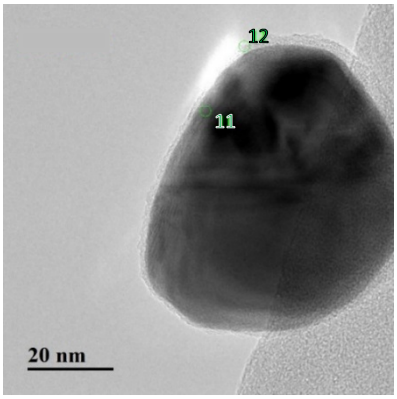


Figure S1a. Ag-NPs size distribution based on TEM analysis (particle number=318)

Table S1a. Mean diameter by intensity distribution, polydispersity Index and Z-potential measured by Dynamic Light Scattering (DLS)

	Diameter mean (nm)	Standard Deviation (nm)
Intensity	72.94	2.02
Polydispesity Index	0.137	0.024
	mV	Standard Deviation (mV)
Z-potential	-57.9	1.2

Table S1b. Particle elemental composition characterization by TEM-EDX



Spectrum Label	Spectrum 12	Spectrum 11
C	91.23	81.08
O	0.89	1.97
Si	0.22	0.35
Ag*	3.22	10.41

*Signal from the particles is relatively small due to the reduced penetration depth.

Synthesis and characterization of Ag_2S-NP

Briefly, a concentrated solution of $AgNO_3$ precursor was injected to 1 L volume of aqueous solution containing $Na_2S * 9H_2O$ and PVP 55 kDa under vigorous stirring and $[AgNO_3]$/[PVP] ratio to get the desired size. The solution became dark grey immediately and it was kept at

synthesis temperature for 15 min to ensure complete reaction of the precursors. Resultant Ag_2S nanoparticles were purified by centrifugation (1000 g) in order to remove the excess of S^{2-} and further redispersed in MilliQ water with the same PVP (1 mg/mL) before sample characterization (Figure S1b, Table S1c). EDX on individual particles (Table S1d) shows clear difference between mostly unreacted Ag (spectrum 16) and Ag_2S (spectrum 17) in the Ag/S ratio. This indicates that not all of the Ag-NPs were converted to Ag_2S. Spectrum 18 is the surrounding matrix.

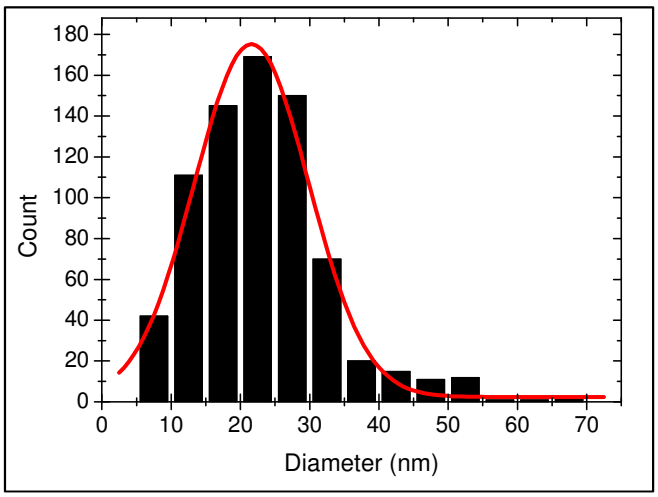
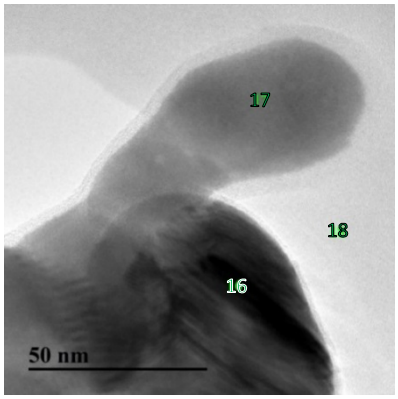


Figure S1b. Ag-NPs size distribution based on TEM analysis (particle number=759)

Table S1c. Mean diameter by intensity distribution, polydispersity Index and Z-potential measured by Dynamic Light Scattering (DLS)

	Diameter mean (nm)	Standard Deviation (nm)
Intensity	302.8	1.1
Polydispesity Index	0.85	0.03
	mV	Standard Deviation (mV)
Z-potential	-2.19	0.14

Table S1d. Particle elemental composition characterization by TEM-EDX



Spectrum Label	Spectrum 16	Spectrum 17	Spectrum 18
C	72.81	87.6	95.55
O	2.82	0	2.35
Si	0.99	0.5	0
S	0.22	2.36	0
Ag*	15.69	5.42	0
Ag/S ratio	72	2.3	n/a

*Ag signal from the particles is relatively small due to the reduced penetration depth.

S2 Soil characterization

Table S2. Characterization of natural soil

Parameter	Value	Unit
Median granular size	115	µm
Total nitrogen	1380	mg N kg^{-1}
Potassium	29	mg K kg^{-1}
pH (in water)	5.2	-
Organic matter	5.4	%
$CaCO_3$	0.2	%

S3 Statistical analysis

Table S3a. Post hoc Tukey multiple comparison of test between particulate (≥ 20nm) and total Ag concentrations of all time points in soil treated with Ag-NPs, $AgNO_3$ and Ag_2S-NPs following one way ANOVA (F (5, 18) = 19.26)). Positive confidence interval indicates that concentrations are higher in first factor, and vice versa.

	Mean Diff.	99.90 % CI of diff.	P Value
Particulate Ag-NP vs. Particulate $AgNO_3$	-0.09	-0.78 to 0.59	0.9930
Particulate Ag-NP vs. Particulate Ag_2S-NP	-0.45	-1.14 to 0.23	0.1472

Particulate $AgNO_3$ vs. Particulate Ag_2S-NP	-0.35	-1.04 to 0.33	0.3538
	Mean Diff.	99.90 % CI of diff.	P Value
Particulate Ag-NP vs. Total Ag-NP	-0.77	-1.45 to -0.08	0.0037
Particulate $AgNO_3$ vs. Total $AgNO_3$	-0.69	-1.37 to -0.0009	0.0099
Particulate Ag_2S-NP vs. Total Ag_2S	0.08	-0.61 to 0.76	0.9972
Total Ag-NP vs. Total $AgNO_3$	-0.01	-0.70 to 0.67	>0.9999
Total Ag-NP vs. Particulate Ag_2S NP	0.32	-0.37 to 1.00	0.4733
Total Ag-NP vs. Total Ag_2S	0.39	-0.29 to 1.08	0.2521

Table S3b. Post hoc Tukey multiple comparison test between concentrations of different forms of Ag in worms exposed to Ag-NPs, $AgNO_3$ or Ag_2S-NPs (concentrations at 28 days of exposure) following one way ANOVA (F (5, 17) = 194.6).

	Mean Diff.	99.90 % CI of diff.	P value
Particulate Ag-NP vs. Total Ag-NP	-6.30	-7.68 to -4.92	<0.0001
Particulate Ag-NP vs. Particulate $AgNO_3$	0.30	-1.08 to 1.68	0.9803
Particulate Ag-NP vs. Total $AgNO_3$	-6.63	-8.01 to -5.25	<0.0001
Particulate Ag-NP vs. Particulate Ag_2S-NP	2.08	0.70 to 3.46	0.0018
Particulate Ag-NP vs. Total Ag_2S-NP	1.76	0.38 to 3.14	0.0086
Total Ag-NP vs. Particulate $AgNO_3$	6.60	5.32 to 7.88	<0.0001
Total Ag-NP vs. Total $AgNO_3$	-0.33	-1.60 to 0.95	0.9599
Total Ag-NP vs. Particulate Ag_2S-NP	8.38	7.11 to 9.66	<0.0001
Total Ag-NP vs. Total Ag_2S-NP	8.06	6.78 to 9.34	<0.0001
Particulate $AgNO_3$ vs. Total $AgNO_3$	-6.92	-8.21 to -5.65	<0.0001
Particulate $AgNO_3$ vs. Particulate Ag_2S-NP	1.78	0.51 to 3.06	0.0039
Particulate $AgNO_3$ vs. Total Ag_2S-NP	1.46	0.18 to 2.74	0.0203
Total $AgNO_3$ vs. Particulate Ag_2S-NP	8.71	7.43 to 9.99	<0.0001
Total $AgNO_3$ vs. Total Ag_2S-NP	7.11	7.11 to 9.67	<0.0001
Particulate Ag_2S-NP vs. Total Ag_2S-NP	-0.32	-1.604 to 0.95	0.9611

S4 Kinetic of uptake and elimination

Table S4. Concentrations of total Ag and particulate Ag (≥ 20 nm) measured in earthworms at different time points during uptake phase and depuration phase (mg Ag kg^{-1} wet body weight; n=4; mean ± standard deviation).

		Uptake phase (days)			
Ag form		**7**	**14**	**21**	**28**
Ag-NPs	**Particulate Ag**	**0.23±0.14**	**0.37±0.50**	**2.71±2.03**	**2.26±1.02**
	Total Ag	**3.47±0.28**	**5.64±1.53**	**10.54±1.98**	**8.53±0.77**
Ag_2S-NPs	**Particulate Ag**	**0.06±0.03**	**0.12±0.03**	**0.12±0.06**	**0.08±0.03**
	Total Ag	**0.19±0.06**	**0.29±0.05**	**0.27±0.02**	**0.50±0.12**
$AgNO_3$	**Particulate Ag**	**0.10±0.06**	**0.31±0.26**	**0.78±0.58**	**1.96±0.61**
	Total Ag	**3.08±0.34**	**5.52±0.77**	**10.20±3.03**	**8.89±0.56**

		Depuration phase (days)			
Ag form		**35**	**42**	**49**	**56**
Ag-NPs	**Particulate Ag**	**0.24±0.09**	**0.43±0.09**	**0.54±0.48**	**0.95±0.17**
	Total Ag	**4.91±2.68**	**4.56±0.59**	**6.35±0.90**	**2.91±0.15**
Ag_2S-NPs	**Particulate Ag**	**0.09±0.05**	**0.04±0.02**	**0.04±0.02**	**0.06±0.02**
	Total Ag	**0.14±0.04**	**0.16±0.01**	**0.12±0.03**	**0.15±0.02**
$AgNO_3$	**Particulate Ag**	**0.23±0.17**	**0.07±0.03**	**0.41±0.13**	**0.54±0.18**
	Total Ag	**3.75±1.68**	**2.36±0.41**	**6.28±0.98**	**3.45±0.64**

S5 Potential dissolution and formation of particulate Ag during TMAH incubation

In order to assess any dissolution of nanoparticles during extraction by TMAH, three different amount of Ag-NP and Ag_2S-NPs were incubated with a solution of TMAH 20% overnight (final concentration 38 ng Ag L^{-1}, 100 ng Ag L^{-1}, 380 ng Ag L^{-1}). Analysis by spICP-MS showed a recovery of 102% and 97% for Ag-NP and Ag_2S-NP, respectively. Additionally, to assess potential spontaneous formation of nano-Ag during extraction step by TMAH, three different

amounts of $AgNO_3$ (45 µL, 90 µL, 135 µL of solution of 1 mg mL^{-1}) were added to ≈0.4 g of clean earthworms tissue and incubated following the same TMAH extraction procedure of the samples. SpICP-MS performed on these samples measured 0.04 %, 0.02 % 0.05 % nano-Ag compared to the total Ag content indicating negligible formation of particulate Ag during incubation.

Chapter 3

Particle or ion, what is the influence of dissolution on the uptake of metal nanoparticles in earthworm?

Marta Baccaro, Hans H. J. van den Berg, Nico W. van den Brink

In preparation

Abstract

A key aspect in the environmental and safety testing of metal nanoparticles is the quantification of their uptake in organisms, be it as true particle or as dissolved ions. To assess the relative importance of ionic versus particulate uptake, a study was initiated in which earthworms (*Eisenia fetida*) were exposed to Au core-Ag shell NPs (Au@Ag-NPs) and to combinations of Au-NPs, Ag-NPs, Ag and Au ions in glass jars containing natural soil for 28 days. Our hypothesis was that the Ag shell would dissolve partially or completely and that the Au core would not interact with the exposure media and would therefore behave as a tracer of the particulate uptake. Exposure concentrations were set to 1.5 mg Au kg^{-1} and 25 mg Ag Kg^{-1} for all the different forms of Au and Ag (ionic and/or particulate). Total Au and Ag concentrations were quantified in all the exposure soils, together with ethylene-diaminetetraacetic acid (EDTA) extracted metals, and in organisms by inductively coupled plasma mass spectrometry (ICP-MS). Additionally, the earthworm tissue exposed to Au@Ag-NPs was analysed by single particle inductively coupled plasma time-of-flight mass spectrometry (spICP-TOFMS) to allow the combined quantification of Au and Ag, truly part of a bimetallic particle. Analysis of earthworm tissues showed that concentrations of Ag in the earthworms were not statistically different in organisms exposed to the different forms of Ag. However, the concentration of Au in earthworms exposed to $HAuCl_4$ (ionic Au) exceeded around twenty times the Au concentrations in the worms exposed to particulate Au, which did not differ among each other. Co-exposure to ionic forms of both metals led to a different uptake pattern compared to the single metal exposure, indicating that important interactions between the ions affected their uptake from the soil. Mass measurements by spICP-TOFMS provided evidence that the uptake of the metals in their bimetallic particulate form represents approximately 5 % of the total metal amount. Size measurements by spICP-TOFMS showed that the Au core remained similar after

the uptake, while the Ag shell increased in thickness, suggesting that biotransformation processes take place at the surface of the NPs (e.g. aggregation, precipitation of Ag ions on the surface of existing particles). The study confirmed that dissolution is the main factor driving the uptake of (dissolving) metal nanoparticles in earthworms.

Introduction

The assessment of the bioaccumulation potential of metal nanoparticles (NPs) is challenged by the need to discriminate between the uptake of true NPs and their ionic counterparts that may be more available to the organisms. In case of metal NPs that may dissolve in soil, the main driver of metal NP bioavailability is attributed to dissolution processes since ions have been shown to be the form that is mostly taken up by soil organisms [1-4]. However, it was not possible to completely exclude the uptake of the metal in its particulate form. This is exemplified by studies reporting the uptake of Ag_2S-NPs in isopods [5], in earthworms [2] and in plants [6]. In the study presented in chapter 2, in which earthworms were exposed to Ag_2S-NP which are expected not to dissolve, the Ag_2S-NPs were characterised in detail and found to be only partially sulfidized, which could have led to some release of ions and ionic uptake in earthworms [2]. However, when modelling the uptake of Ag_2S-NPs by earthworms based on just the dissolved Ag concentrations in the soil (extremely low), the ionic uptake was not able to cover the entire uptake of Ag_2S-NPs, suggesting that also uptake of particulate Ag played a role [7]. Though metal NPs are known to adsorb and be retained in the soil matrix even when they do not dissolve, which reduced their bioavailability [8, 9], (part of) this fraction can still be available for organisms which ingest soil particles, like earthworms [10]. Therefore the ionic concentrations of the metal from NPs in the soil pore water may not fully explain the accumulation in the earthworm.

In order to assess the particulate uptake of (dissolving) metal NPs in earthworms in natural soils, we performed a bioaccumulation test using bimetallic NPs, Au core-Ag shell NPs (Au@Ag-NPs) and combined exposure to Au-NPs, Ag-NPs, Ag and Au ions, in all combinations. The gold core of the bimetallic nanoparticles was not expected to dissolve over

the time course of 4 week exposure, and could therefore be used as a nano-tracer of particulate uptake, while the outer shell made of Ag would interact with the exposure media and dissolve, similar to the Ag-NPs. Comparing accumulation patterns of Ag and Au from different forms provided information about how dissolution affected the uptake of the different forms by the earthworm. Additionally ethylene-diaminetetraacetic acid (EDTA) soil extractions were performed to quantify the bio-accessible (ionic) Au and Ag fractions in the soil in the different exposure scenarios [11]. Based on the results, the roles of particulate versus ionic forms in the accumulation of metal NPs into earthworms will be critically scrutinised.

Material and methods

Nanoparticle synthesis and characterization

Synthesis of 13 nm Au-NPs seeds

Briefly, 5 mL of $HAuCl_4$ solution (28.8 mmol L^{-1}) was diluted with ultrapure water up to 150 mL and heated up to 105 °C using a bath. Upon quick injection of 15 mL of sodium citrate solution (38.8 mmol L^{-1}) under reflux and stirring condition, the yellow solution became colourless within 2 minutes, then black and finally a red wine colour appeared. After 50 minutes at 105 °C, the system was cooled down to room temperature. The resulting solution was kept in the fridge without purification for future characterization and synthesis of larger Au-NPs (30 nm) and Au@Ag-NPs.

Synthesis of 30 nm Au-NPs

To a 500 mL flask containing 340 mL ultrapure water, 50 mL of 13 nm seeds Au-NPs solution, 10 mL of hydroxylamine (4 mmol L^{-1}) and 100 mL of $HAuCl_4$ solution (1.62 mmol L^{-1}) were

sequentially added under stirring. Afterwards, 15 mL of sodium citrate solution (38.8 mmol L^{-1}) was added as stabilizing agent. The resultant NPs solution was kept in the fridge without purification for future characterization and usage.

Synthesis of 60 nm Au@Ag NPs

The solution of 13 nm Au NPs was diluted 1:1 with 50 mL ultrapure water, and ascorbic acid solution (2 mmol) was added under stirring. Upon dropwise addition of 100 mL of $AgNO_3$ solution (10 mmol L^{-1}) under stirring at 50 °C, the red coloured solution became gradually brownish within 5 minutes. Polyvinylpyrrolidone (200 mg with molecular weight 40,000) was added under stirring. The system was maintained at 50°C for 10 minutes. The resulting core-shell NPs solution was kept in the fridge without purification for future characterization and usage. The mass concentration of Au core-Ag shell was determined by ICP-MS following acid digestion in *aqua regia* and shown to be 498.3±57.7 µg Ag mL^{-1} and 24.9±1.9 µg Au mL^{-1}, respectively.

Nanoparticle characterization

Characterization of all the nanoparticle solutions was performed by UV-Vis absorption spectroscopy and transmission electron microscopy (TEM). In addition the core-shell structure of Au@Ag-NPs was further confirmed by high angle annular dark-field imaging (HAADF) and element mapping by Energy Dispersive X-ray analysis (EDX).

Earthworm culture

Earthworm *Eisenia fetida* is a model organism extensively used in standardised ecotoxicological tests [12-14]. *E. fetida* were supplied by Lasebo (Nijkerkerveen, The

Netherlands) and kept in the same soil used for the experiments in an incubator at 20 ± 1 °C with 24 hours of light for approximately 2 weeks prior to the experiments and fed with horse manure from an organic farm (Bennekom, The Netherlands) with known absence of pharmaceutical use.

Soil preparation and exposure

The bioaccumulation test was based on OECD no 317 for the bioaccumulation of chemicals in terrestrial oligochaetes [13]. Earthworms were exposed in natural soil (pH 5.98 in water, organic matter content 5.4%) collected from an organic farm in The Netherlands (Proefboerderij Kooijenburg, Marwijksoord, The Netherlands), air-dried and sifted (5 mm sieve openings) before use. Glass jars with a lid were prepared with 450 g of air dried soil with additional water (20% w/w which equals approximately 47% water holding capacity). Soils were spiked with Au@Ag-NPs, Ag-NPs, Au-NPs, $AgNO_3$, $HAuCl_4$ to reach a nominal concentration of 25 mg Ag kg^{-1} and 1.5 mg Au kg^{-1} for all treatments (for actual concentrations see Figure 5). The ratio between nominal Ag/Au concentrations spiked in the soils were as measured in the Au@Ag-NPs dispersion. Soil, water and metal solutions were homogeneously mixed by an automatic mixer for 3 minutes and jars were filled. After 24 hours, 8 adult *E. fetida* earthworms with an average weight of 478.31 ± 44 mg (n=360) per earthworm were randomly placed into every experimental unit (5 jar per treatments).

Sampling

After 28 days, jars were separately emptied. Aliquots of soil were sampled and stored at −20 °C. Worms were collected, carefully rinsed with deionized water, dried in a double layer of

tissue paper and placed in glass Petri dishes to depurate in the incubator at 20 °C for 24 hours. After depuration, worms were rinsed again with deionized water and dried. Worms from each jar were pooled and snap frozen in liquid nitrogen and stored at -80 °C for further analysis.

EDTA extraction of soil

A 1 g of soil was mixed with 10 mL of disodium EDTA 0.05 M at pH 7.0 and shaken overhead overnight (~18 hours). The solution was filtered through preconditioned 0.45 µm filters (soaked overnight in 0.1 M $CuNO_3$ (99.9%, Sigma Aldrich)) and directly acid digested for Ag and Au quantification.

Extraction and analysis of total Ag and Au

The extraction of total Ag and Au from soil, worm tissues and EDTA soil extracts was performed by microwave assisted (MARS 6, microwave system, CEM Corporation, USA) acid digestion in *aqua regia* (1:3 nitric acid–hydrochloric acid). Aliquots of samples were weighed (~0.5 g of wet soil or worm tissue; 2.5 mL of soil EDTA extract) and placed in Teflon vessels with 6 mL of HCl 37% (Merck, Germany) and 2 mL of HNO_3 69% (Merck, Germany). A two steps temperature programme was used (ramp to 160 °C in 10 min, 30 min hold, ramp to 200 °C in 10 min and 30 min hold). Samples were analysed for total Au and Ag, using ICP-MS Nexion 350D (Perkin-Elmer Inc., Waltham, MA). The metals quantified were Ag (*m/z* 107 and 109), Au (*m/z* 197) and Rh (*m/z* 103) as internal standard. The calibration curve was prepared from standards of Ag^+ and Au^+(standard stock solution 1000 mg L^{-1} Ag, Merck, Germany) and standard of Rh^{3+} (standard stock solution 1000 mg L^{-1} Ag, Merck, Germany). The limit of detection (LOD) of silver (m/z 107) and for gold was calculated as the mean of digested blank + 3 standard deviations blank and found to be 0.5 µg L^{-1} for all metals.

Extraction and particulate analysis of Au@Ag-NPs

Au@Ag-NPs were extracted from the earthworm tissue by incubation of ~425±22 mg (n=5) of finely ground worm tissue in 8 mL of alkaline solution (tetramethylammonium hydroxide (TMAH) 20 %) overnight [15]. The samples were diluted 1000x prior to analysis by single particle Inductively Coupled Plasma Time of Flight Mass Spectrometry (spICP-TOFMS, icpTOF 2R, TOFWERK AG, Switzerland). This instrument records a broad range of the atomic mass spectrum (7-250 m/z) at 46 μs intervals, permitting the *quasi*-simultaneous detection of multiple elements on a particle-by-particle basis. The principles of spICP-MS are described elsewhere [16, 17]. Briefly, a known particle size standard is used to determine the transport efficiency of the nebulized sample to the plasma, which is then used to calculate a mass flux calibration slope. This calibration curve slope is then used to relate the ion signal intensity to mass, which is subsequently converted into diameter assuming a geometry and a density (see formulas in paragraph S5). The dissolved metal curves used to relate the signal to the mass were prepared in trace metal grade nitric acid. Transport efficiency was calculated by the use of well-characterized 100 nm gold nanoparticles (BBI solutions) resulting in a transport efficiency of 6.43%. The dwell time was set at 3 ms and data on mass, volume and size were selected in order to only process the intensity signals when Au and at least one Ag isotope (Ag 107 and Ag 109) were detected. Particle events were detected using a modified method adopted from Pace et al. [18]. Here particles events are described as those signal intensities which exceed a threshold set by the iterative application of a $\mu+3\sigma$ cut-off (where μ is the average of all intensities and σ is the standard deviation).

Results

Nanoparticle characterization

TEM showed that Ag-NPs and Au-NPs were spherical shaped particles with size equal to 50.0±7.4 nm (minor axis± SD, N=371) and 33.1±5.8 nm (minor axis± SD, N=1110), respectively (Figure 1a-b). *Quasi*-spherical Au@Ag-NPs were achieved *via* a seeded approach by using 13.1±2.6 nm (minor axis ± SD, N=463) Au NPs as seeds (Figure 2). The size increases to 54.1±16.7 nm (minor axis± SD, N=298) after coating with a Ag layer (Figure 1c). Size distributions are reported in Figure S1, supplemental Information.

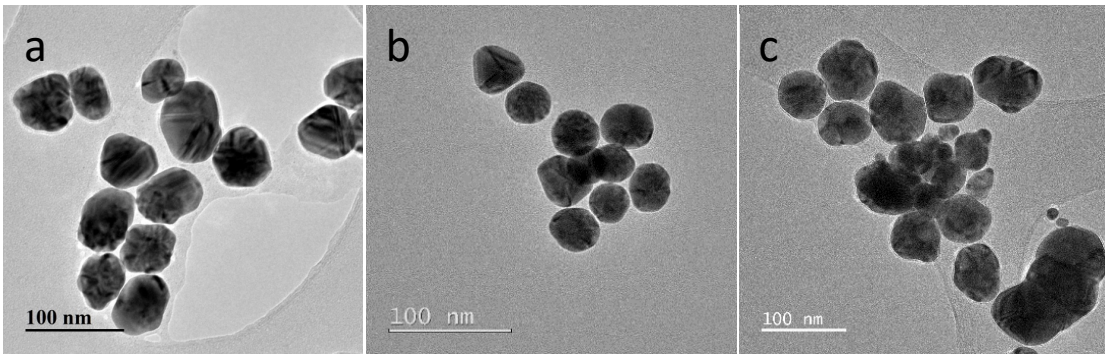


Figure 1. TEM images of a) Ag-NPs, b) Au-NPs, c) Au@Ag-NPs

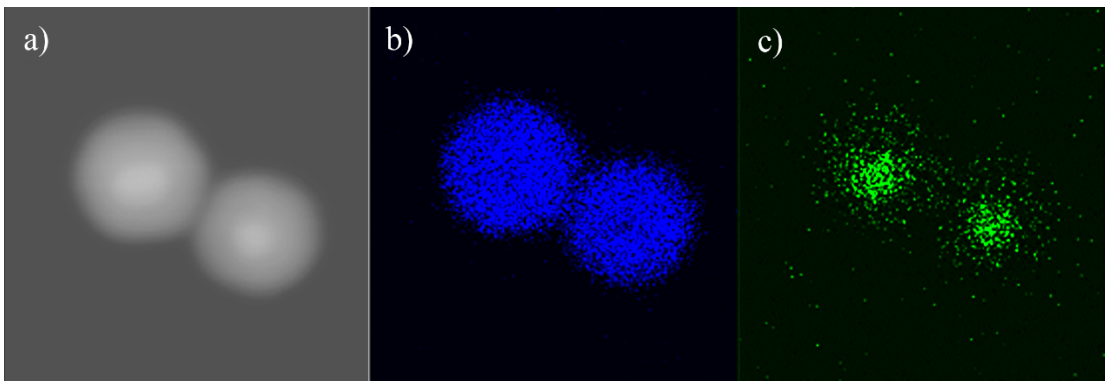


Figure 2. a) HAADF images of Au@Ag-NPs and EDS mapping of b) silver element and c) gold element

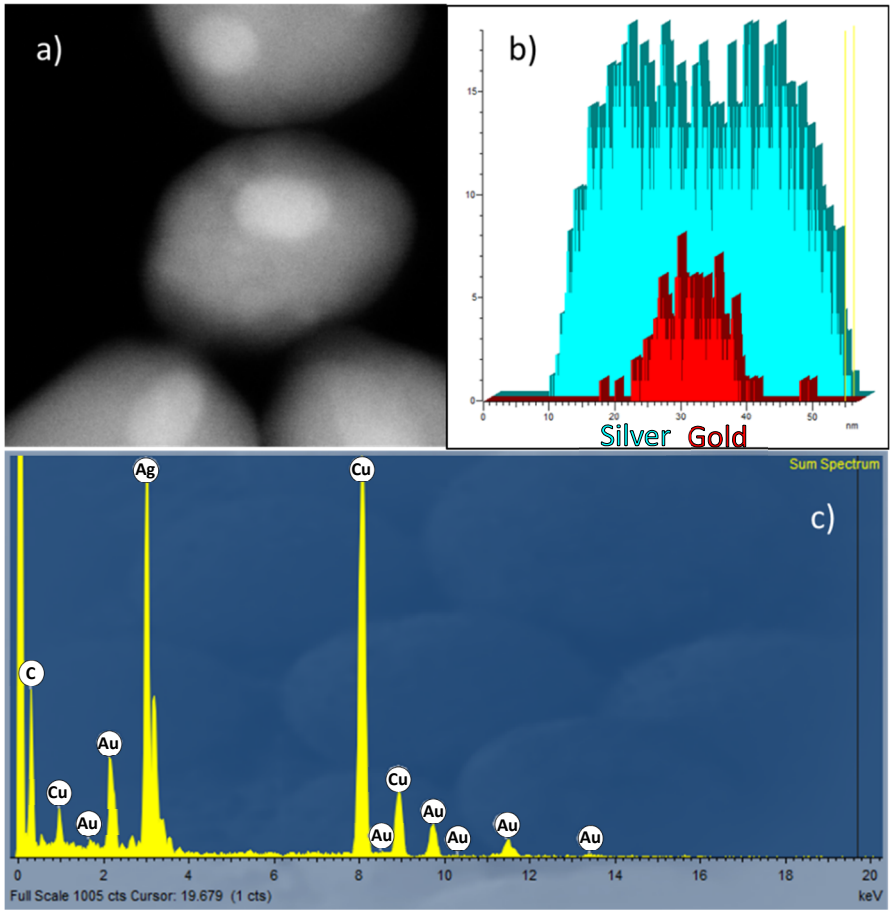


Figure 3. a) HAADF of one individual particle, b) EDX line spectra (light blue indicates the Ag element, the red colour indicates the Au element) and c) EDX spectrum of the same particle.

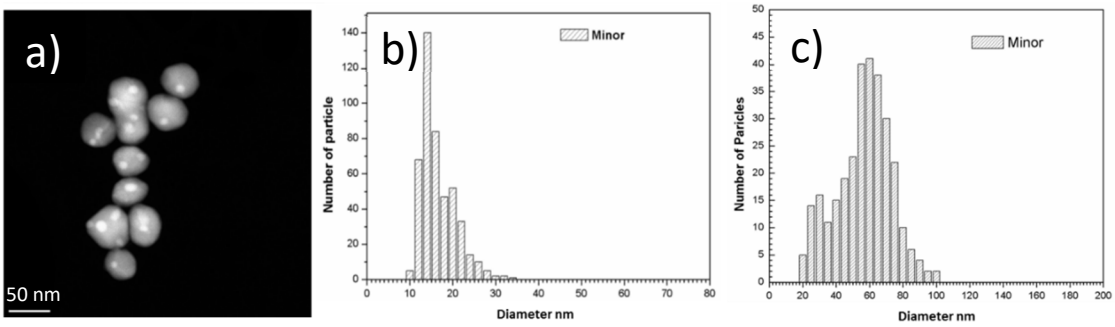


Figure 4. a) HAADF image of Au@Ag-NPs where double/multiple cores are visible as white dots within the particles, b) size distribution (minor axes± SD, 13.1±2.6, N=463) of Au core and c) of Au@Ag-NPs as Au-Ag structure (minor axis± SD, 54.1±16.7, N=298).

Indicated by HAADF image (Figure 4), some particles consist of more than one Au cores, which appeared to be brighter in contrast than the Ag shell. The equivalent size of the Au core detected by spICP-TOFMS was larger than the size of Au seed NPs, ~30 nm *vs* ~13 nm, which is likely attributed to the fact that some core-shell NPs consist of multi Au core (Figure 3). After coating with an Ag layer, a significant shift on absorbance, from 525 nm to 450 nm, was observed (supplementary information - Figure S2). An increase in hydrodynamic size was also observed, which is in consistence with TEM and UV-vis results. After removing particulate pieces in the Au@Ag-NPs stock solution by ultrafiltration only negligible ionic form of Ag or Au was detected in the ultra-filtrate by ICP-MS (supplemental information - paragraph S4).

Exposure characterization

Actual concentrations of Ag and Au in soil showed average recoveries of 88±8% and 107±16% of the nominal concentrations for Ag and Au, respectively (Figure 5). EDTA extracted concentrations of Ag and Au were very variable (Figure 6). Results of statistical analysis (supplemental information -Table S3) showed .

Bioaccumulation in the earthworms

The concentrations of Ag and Au in earthworms exposed to the different treatments is presented in Figure 7. None Ag internal concentration was statistically different from any other Ag internal concentration. The single ionic Au exposure led to an Au uptake which was always statistically different from all the others Au exposures (Table S1- supplemental information). Uptake of ionic Au in combined exposure with Ag significantly varied according to the form of Ag, in particular earthworms accumulated 2.07 ± 0.77 mg Au kg^{-1} wet weight and 7.69 ± 1.73 when exposed to Au^{+} Ag^{+} and Au^{+} Ag-NPs, respectively. The organisms exposed to

Au@Ag-NPs were also analysed by spICP-TOFMS, which allowed for multi-element detection of nanoparticles [19]. Results of concentrations of bimetallic NPs in the worms showed that 0.02±0.01 mg Au kg^{-1} wet weight and 0.59±0.43 mg Ag kg^{-1} wet weight were present as part of bimetallic nanoparticles. Table 1 reports the mass concentrations and thickness of the Ag shell and diameter of the Au core of the bimetallic nanoparticles detected in the stock solution and in the earthworms. In the paragraph S5 (supplementary information), we reported the formulas used for the calculation of the volume of the shell and particle mass concentrations.

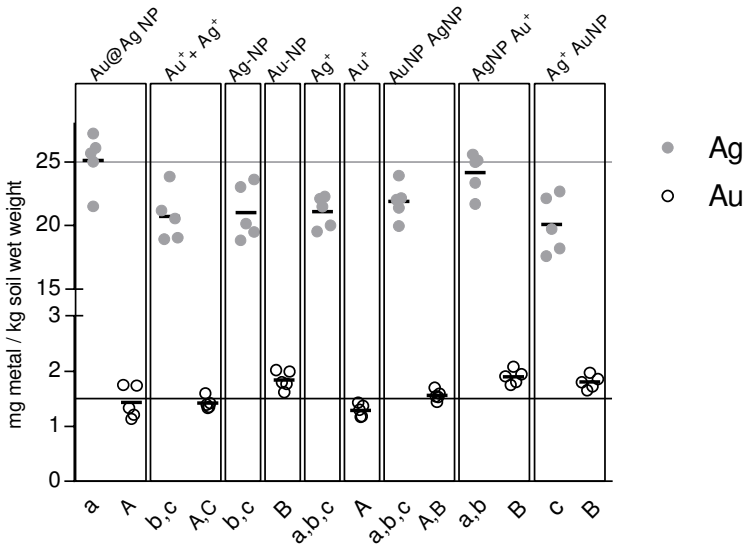


Figure 5. Total Ag and Au exposure concentrations (n=5) in spiked soil measured by ICP-MS following acid digestion (mg kg^{-1} wet weight). Solid lines represent the intended nominal concentration for Ag (grey line) and Au (black line). Solid marks indicate average concentrations for the treatment. Average values with the same letters are not significantly different (*post hoc* Tukey multiple comparison test following one-way ANOVA; capital letters for Au, normal letters for Ag).

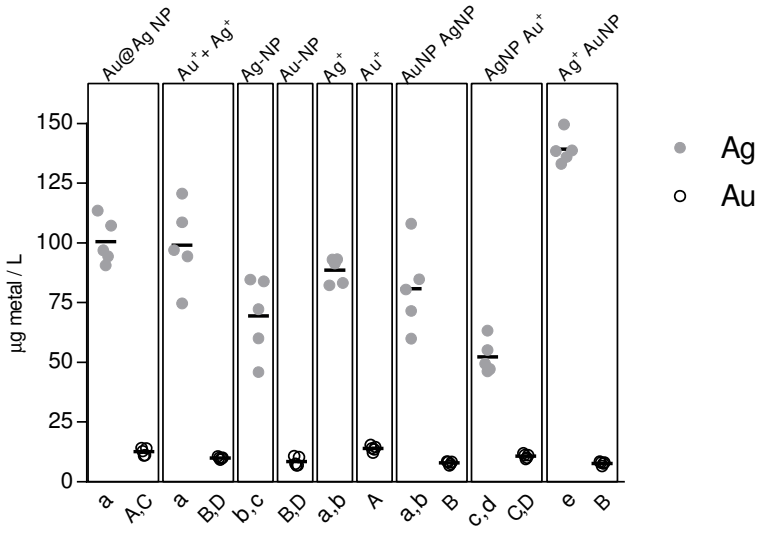


Figure 6. Total Ag and Au concentrations (n=5) in EDTA soil extracts measured by ICP-MS following acid digestion. Average values with the same letters are not significantly different (*post hoc* Tukey multiple comparison test following one-way ANOVA; capital letters for Au, normal letters for Ag).

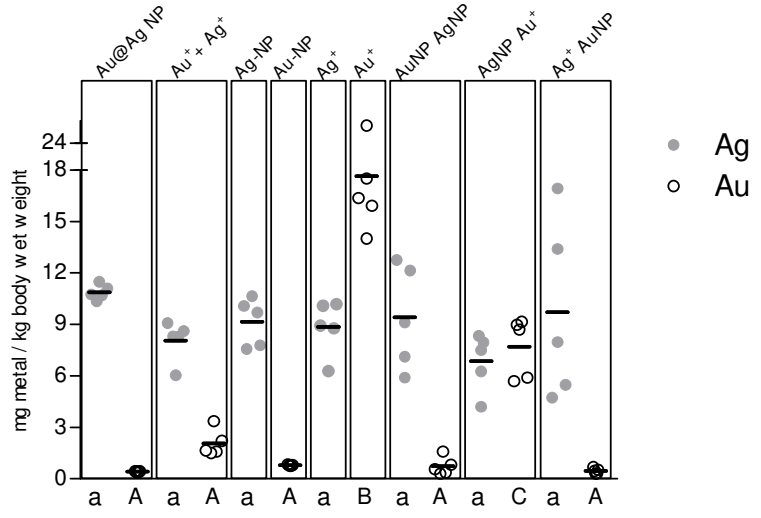


Figure 7. Total Ag and Au concentrations in earthworm tissue after 28 days exposure measured by ICP-MS following acid digestion. Average values with the same letters are not significantly different (*post hoc* Tukey multiple comparison test following one-way ANOVA; capital letters for Au, normal letters for Ag).

Table 1. Concentrations (as bimetallic particles) and thickness of the Ag shell and diameter of the Au core of the bimetallic nanoparticles detected in the stock solution and in earthworm tissue samples by spICP-TOFMS (average ± standard deviation).

	Thickness shell Ag (nm)	Diameter core Au (nm)	Mass Ag (µg mL^{-1})	Mass Au (µg mL^{-1})
Stock solution (n=3)	21.7 ± 1.0	30.9 ± 0.2	289.6 ± 2.8	26.5 ± 0.3
Average earthworms (n=5)	48.1 ± 3.9	31.8 ± 1.2	0.59 ± 0.43	0.02 ±0.01

Discussion

Regardless the form in which Ag was added to the soil, Ag uptake in earthworms was not significantly different amongst Ag treatments. This supports the hypothesis that Ag from the bimetallic nanoparticles (Au@Ag-NPs) dissolved and that the released Ag ions were taken up by the worms in a similar way compared to ionic Ag from $AgNO_3$. The same process has been discussed by a kinetic model in chapter 2 [2].

The comparison amongst the concentrations of Au in earthworms exposed to all the combined treatments highlights statistically significant differences. Although the concentrations in earthworms exposed to particulate Au (Au@Ag-NPs and Au-NPs) did not show statistically differences and were < 1 mg Au kg^{-1}, when the exposure included only ionic Au, the concentration in the worms reached 17.6±4.0 mg Au kg^{-1}. To the authors knowledge the only studies that assessed the bioavailability and uptake of Au and Au-NPs in earthworms are the works of Unrine et al. [20] and Bourdineaud et al. [21] who also reported that *E. fetida* exposed to $HAuCl_4$ and Au-NPs accumulated both ionic Au and nano Au, but that the ionic form was taken up to a larger extent. However, in the current study when earthworms were exposed to

Au^+ in the presence of Ag (Au^+ vs Ag-NP Au-NP, Au^+ vs Ag-NP Au^+, Au^+ vs Ag^+ Au-NP) their Au internal concentrations were significantly lower than when earthworms were only exposed to Au^+ suggesting a potential interaction between the two element in the soil and/or in the process of uptake into the worms (Figure 7). The nucleation of bimetallic Ag-Au nanocrystals from the interactions of Ag^+ and Au^+ with fulvic and humic acids has been reported [22, 23]. In the present study it is hypothesised that the complexation of Ag and Au ions with soil components may have decreased the amount of free and available metal ions to earthworms. Au concentrations in worms exposed to combined Au^+Ag^+ in the soils were significantly lower than in the ones exposed to Au^+ and Ag-NPs, likely due to that fact that the Ag ions in the Ag^+ exposures directly interacted with the Au ions, while in the Au^+ Ag-NP exposures the Ag first needed to dissolve before it could interact with the Au ions. This left a time window of relatively efficient Au accumulation by the earthworms at the start of the experimental time frame. The effect of the complexation on bioavailability (lower uptake of Ag when co-exposed with Au) could not be detected in the case of Ag (no significant differences) likely due to the excess of free Ag^+ compared to Au^+ (25 mg Ag kg^{-1} vs 1.5 mg Au kg^{-1}). The explanation for the different Au uptake between earthworms in the single and in the co-exposure with Ag can also lay in the competition among cations at the uptake site, due to its higher affinity for Au than the affinity for Ag. It is more likely that both complexation in the soil and cation competition played a role in the resulting different uptake of Ag and Au [24].

The quantification of Au@Ag-NPs as bimetallic nanoparticles highlighted that 5.2% and 4.0% of Ag and Au (based on total quantifications) taken up in the earthworms were present as bimetallic nanoparticles indicating an uptake as particulate. However the detection of particles with sizes below 20 nm can be challenging due to a relative high background of dissolved silver ions. This could have resulted in an underestimation of the bimetallic particles concentrations,

although the recovery of Au in the stock solution quantified by spICP-TOFMS is equal to 106 % (24.9±1.9 µg mL^{-1} by ICP-MS vs 26.5 ± 0.3 by spICP-TOFMS). From the size distribution of the Au core particles in the stock it can be calculated that the particles with size lower than 20 nm represents around 75% (TEM size distribution, Figure 4b). In the worst case in which just 25% of the Au-NPs were detected, this would lead to an estimated true gold particulate uptake of 0.08 mg Au kg^{-1} instead of 0.02 mg Au kg^{-1}. By calculating the Ag needed to have a shell of 22 nm around a 13 nm gold core (median value of the size distribution), the Ag belonging to a bimetallic nanoparticles would be 3.19 mg Ag kg^{-1} which represent 29% of the total Ag taken up in the earthworm. Nevertheless, as already mentioned above, the mass Au recovery by spICP-TOFMS was calculated to be 106% (Table 1) and for this reason we propose that it is more likely that the detected Au@Ag-NPs had multiple Au cores and spICP-TOFMS detected these as a single Au core of ~30 nm (Figure 4).

The comparison between the sizes of the bimetallic nanoparticles within the earthworms and in the stock solution detected by the same analytical techniques spICP-TOFMS (Table 1), suggested that the shell underwent some transformations because the Au core size in both cases (before and after uptake) remained unvaried as ~30 nm while the mass of Ag increased suggesting that the thickness of the Ag shell increased ~26.4 nm. The fact that the Au core size did not change after uptake indicates that Au did not interact with the surrounding media in the soil or tissues. The increasing shell thickness suggests that aggregation or precipitation processes may have occurred at the surface of the shell instead. The quantification of the exact increase in thickness is not possible due to a too broad size distribution of the initial Au@Ag-NPs stock solution (Figure 4c). In Merrefield et al. [25], Au@Ag-NPs were also used to study transformations of Ag-NPs in complex media and size was monitored in hard water with the addition of fulvic acid. At similar concentrations to the concentrations measured after EDTA

extraction in the present study (> 1 µg L^{-1}) they reported an increase in the Ag shell thickness but not in the Au core size. In the present study the authors considered two possible scenarios to explain the increase in the Ag shell thickness. The organic matter could form a corona at the NPs surface and Ag bound afterwards [26], causing a surface growth in the soil solution; and/or the Ag dissolved in the soil solution and the resulting nearly naked Au cores were taken up. In this case, the Ag precipitation or adsorption on the surface of the NPs occurred biogenically, within the organism. The second proposed scenario is most likely to take place since the results showed that ions were the form of metal mostly taken and therefore dissolution was the predominant process in the soil solution. The formation of the biocorona has been reported *in vivo* and *in vitro* [27-30], and soft and hard coronas have been reported to trap Ag ions and to form Ag_2S on the surface of Ag-NPs [31]. Ag ions could originate from the nanoparticle itself or be located in the earthworm environment. If this mechanism takes place, spICP-TOFMS would detect the particle as a bimetallic particle with a larger Ag shell compared to the original one. We propose that 5% of the detected bimetallic nanoparticles in the earthworms underwent similar processes and that the Ag shell resulted to be thicker (~+26 nm). We exclude any effect caused by the method used to extract the NPs from the earthworm tissue. Dong et al. [32] showed that the sizes of Ag-NPs extracted by alkaline solution from the tissues were nearly identical to those in H_2O, suggesting that the Au-NP core was also stable in alkaline extracted tissue and retained its original size (core diameter) [15, 33, 34].

Since analyses of metals extracted by *aqua regia* is not representative of the bioavailable fraction, we extract the metals from the soil of each experimental jar by the chelating agent EDTA which has been used as chemical extractant to obtain information on the phytoavailable metal fraction in soils [35]. EDTA is known to be able to displace carbonate-bound metals and also metals in organometallic complexes which may represent the available fraction to soil-

feeding organisms [36, 37]. The concentrations of Ag and Au in the EDTA extractions showed no direct relationships with earthworm concentrations in the different treatments. Pearson r coefficients were equal to 0.22 (n=35, R^2=0.04, p>0.05) and 0.64 (n=35, R^2=0.30, p<0.001) for Ag and Au, respectively (Figure 8). Au concentrations in worms show a significant correlation with Au concentrations in the EDTA soil extracts. However, the higher Au uptake in the treatment with Au^+ and Ag-NPs Au^+ did not match higher Au concentration in the EDTA soil extracts of this treatments than the others. Therefore, we conclude that concentrations of Ag and Au in the EDTA extractions are not a good proxy for their availability for earthworms.

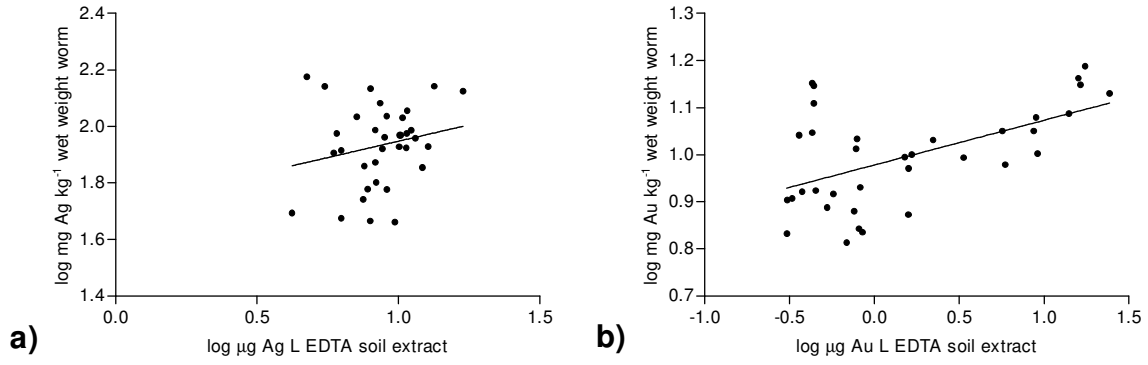


Figure 8. Plot of log transformed Ag (a) and Au (b) concentrations in the earthworms as a function of log transformed Ag and Au concentrations in the EDTA soil extracts.

Conclusion

The quantification of Ag and Au in earthworms exposed to bimetallic NPs and different combination of nano- and ionic form of Au and Ag allowed to confirm that dissolution is the driving factor for the uptake of metal NPs in earthworms. The accumulation of particulate metals from Au@Ag-NPs represented only ~5% of the total amount of each metal within the earthworm. Still, transformation of the bimetallic NPs occurred and was highlighted by the

measure of unvaried Au core and an increase of the Ag shell thickness likely due to biogenic precipitation or adsorption of Ag on the surface of the NPs in the earthworm. No statistical differences were found among the accumulated concentrations of Ag in any case, regardless the Ag form, while in the case of Au the exposure to ionic Au only led to an Au uptake which was statistically higher from all the others where Au was present. The co-exposure to the two metals in different forms brought to different accumulation patterns compared to the single metal exposure set ups substantiating the importance of testing toxicity of chemical mixtures. Metal concentrations in the EDTA soil extract (considered the bio-accessible fraction) correlated with the concentrations in the earthworms only in the case of Au.

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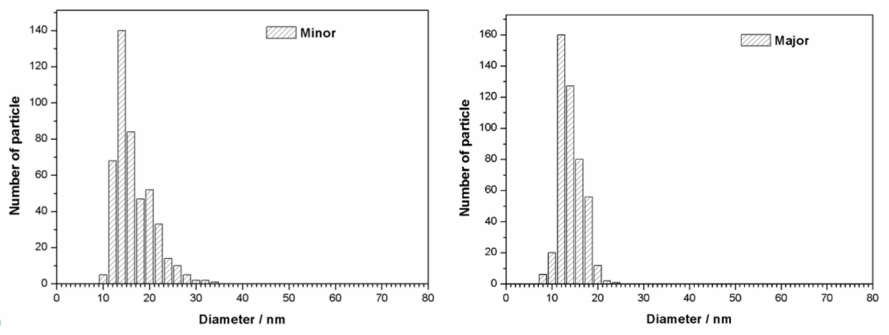
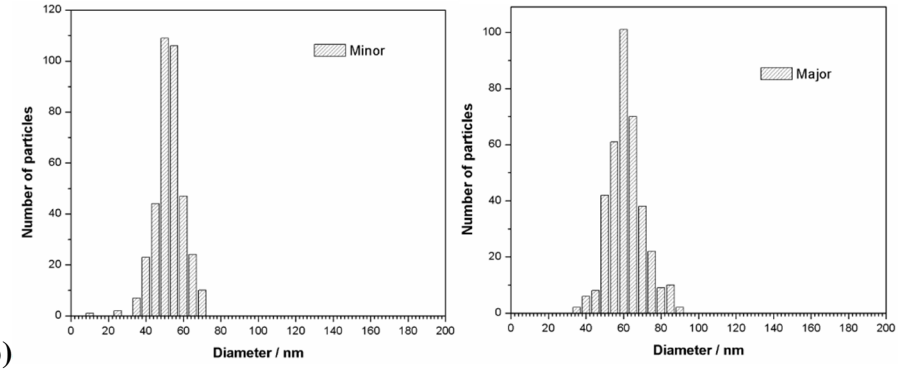
Supplementary information

S1 Figures. Size distribution based on minor (left) and major (right) axes of a) Au-NPs, b) Ag-NPs, c) Au@Ag@NPs.



a)



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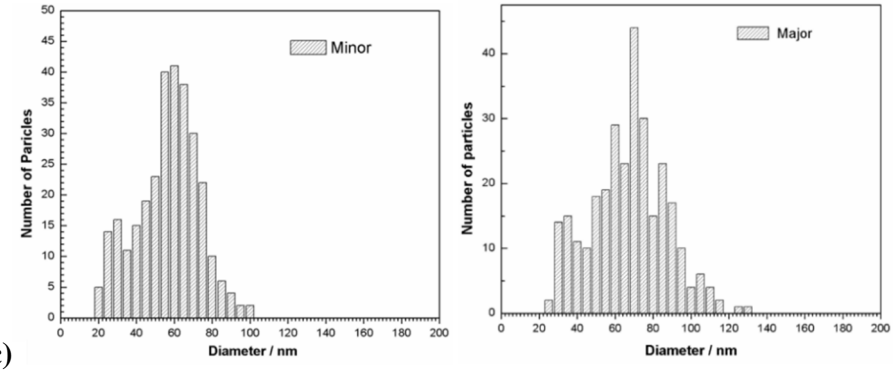
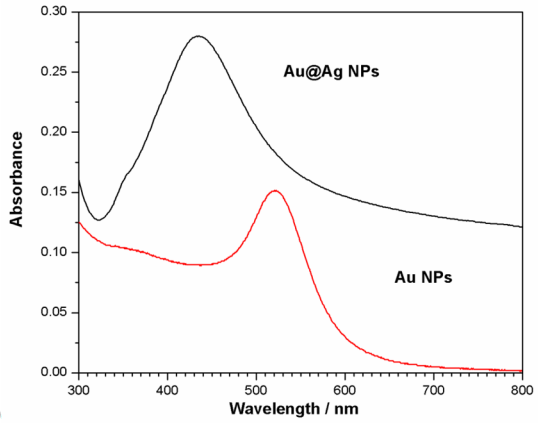
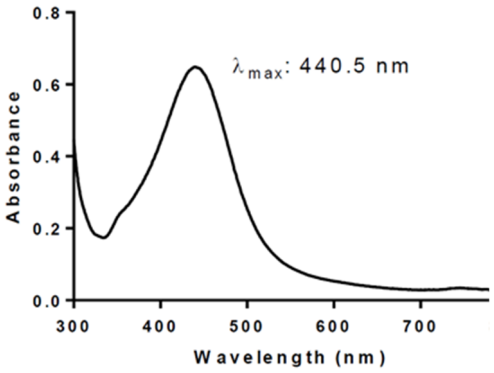


c)

S2 Figures. UV-VIS absorption of Au@Ag NPs and Au-NPs seeds (a) and AgNPs (b).



a)



b)

S3 Tables

Post hoc multiple comparison test following one way ANOVA amongst Ag concentrations in EDTA soil extracts from all the treatments with Ag and amongst Au concentrations in EDTA soil extracts from all the treatments with Au. Positive confidence interval indicates that concentrations are higher in first factor, and vice versa.

	95% CI of diff.	P Value	
Ag in Ag@Au-NP vs Ag in Ag^+ Au^+	-0.13 to 0.15	>0.9999	
Ag in Ag@Au-NP vs Ag-NP	0.03 to 0.31	0.0088	**
Ag in Ag@Au-NP vs Ag^+	-0.08 to 0.19	0.8754	
Ag in Ag@Au-NP vs Ag in Ag-NP Au-NP	-0.04 to 0.24	0.2768	
Ag in Ag@Au-NP vs Ag in Ag-NP Au^+	0.15 to 0.42	<0.0001	****
Ag in Ag@Au-NP vs Ag in Ag^+ Au-NP	-0.28 to -0.004	0.0417	*
Ag in Ag^+ Au^+ vs Ag-NP	0.02 to 0.30	0.0158	*
Ag in Ag^+ Au^+ vs Ag^+	-0.09 to 0.18	0.9506	
Ag in Ag^+ Au^+ vs Ag in Ag-NP Au-NP	-0.05 to 0.23	0.3972	
Ag in Ag^+ Au^+ vs Ag in Ag-NP Au^+	0.13 to 0.41	<0.0001	****
Ag in Ag^+ Au^+ vs Ag in Ag^+ Au-NP	-0.30 to -0.01	0.0240	*
Ag-NP vs Ag^+	-0.26 to 0.02	0.1449	
Ag-NP vs Ag^+ in Ag-NP Au-NP	-0.21 to 0.07	0.6877	
Ag-NP vs Ag in Ag-NP Au^+	-0.02 to 0.25	0.1581	
Ag-NP vs Ag in Ag^+ Au-NP	-0.45 to -0.17	<0.0001	****
Ag^+ vs Ag in Ag-NP Au-NP	-0.09 to 0.19	0.9310	
Ag^+ vs Ag in Ag-NP Au^+	0.09 to 0.37	0.0002	***
Ag^+ vs Ag in Ag^+ Au-NP	-0.33 to -0.06	0.0019	**
Ag in Ag-NP Au-NP vs Ag in Ag-NP Au^+	0.05 to 0.32	0.0039	**
Ag in Ag-NP Au-NP vs Ag in Ag^+ Au-NP	-0.38 to -0.10	0.0001	***
Ag in Ag-NP Au^+ vs Ag in Ag^+ Au-NP	-0.57 to -0.29	<0.0001	****
Au in Ag@Au-NP vs Au in Ag^+ Au^+	0.30 to 5.02	0.0192	*
Au in Ag@Au-NP vs Au-NP	1.78 to 6.49	0.0001	***
Au in Ag@Au-NP vs Au^+	-3.67 to 1.04	0.5754	
Au in Ag@Au-NP vs Au in Ag-NP Au-NP	2.40 to 7.11	<0.0001	****
Au in Ag@Au-NP vs Au in Ag-NP Au^+	-0.53 to 4.18	0.2134	

Au in Ag@Au-NP vs Au in Ag^+Au-NP	2.53 to 7.25	<0.0001	****
Au in Ag^+ Au^+ vs Au-NP	-0.89 to 3.83	0.4483	
Au in Ag^+ Au^+ vs Au^+	-6.34 to -1.62	0.0002	***
Au in Ag^+ Au^+ vs Au in AgNP AuNP	-0.26 to 4.45	0.1075	
Au in Ag^+ Au^+ vs Au in AgNP Au^+	-3.19 to 1.52	0.9152	
Au in Ag^+ Au^+ vs Au in Ag^+ AuNP	-0.13 to 4.58	0.0734	
Au-NP vs Au^+	-7.81 to -3.09	<0.0001	****
Au-NP vs Au in Ag-NP Au-NP	-1.74 to 2.98	0.9789	
Au-NP vs Au in Ag-NP Au^+	-4.66 to 0.05	0.0581	
Au-NP vs Au in Ag^+ Au-NP	-1.60 to 3.11	0.9456	
Au^+ vs Au in Ag-NP Au-NP	3.71 to 8.43	<0.0001	****
Au^+ vs Au in Ag-NP Au^+	0.79 to 5.50	0.0038	**
Au^+ vs Au in Ag^+ Au-NP	3.85 to 8.56	<0.0001	****
Au in Ag-NP Au-NP vs Au in Ag-NP Au^+	-5.28 to -0.57	0.0079	**
Au in Ag-NP Au-NP vs Au in Ag^+ Au-NP	-2.22 to 2.49	>0.9999	
Au in Ag-NP Au^+ vs Au in Ag^+ Au-NP	0.71 to 5.42	0.0050	**

Post hoc multiple comparison test following one way ANOVA amongst Ag concentrations in earthworms from all the treatments with Ag and amongst Au concentrations in earthworms from all the treatments with Au. Positive confidence interval indicates that concentrations are higher in first factor, and vice versa.

	95% CI of diff.	P Value	
Ag in Ag@Au-NP vs Ag in Ag^+ Au^+	-2.31 to 7.93	0.5970	
Ag in Ag@Au-NP vs Ag-NP	-3.41 to 6.84	0.9339	
Ag in Ag@Au-NP vs Ag^+	-3.10 to 7.14	0.8680	
Ag in Ag@Au-NP vs Ag^+ in Ag-NP Au-NP	-3.66 to 6.59	0.9683	
Ag in Ag@Au-NP vs Ag in Ag-NP Au^+	-1.10 to 9.14	0.2017	
Ag in Ag@Au-NP vs Ag in Ag^+Au-NP	-3.96 to 6.29	0.9899	
Ag in Ag^+Au^+ vs Ag-NP	-6.22 to 4.03	0.9929	
Ag in Ag^+Au^+ vs Ag^+	-5.91 to 4.33	0.9988	
Ag in Ag^+Au^+ vs Ag^+ in Ag-NP Au-NP	-6.47 to 3.78	0.9793	
Ag in Ag^+Au^+ vs Ag in Ag-NP Au^+	-3.91 to 6.33	0.9879	
Ag in Ag^+Au^+ vs Ag in Ag^+ Au-NP	-6.77 to 3.48	0.9461	
Ag-NP vs Ag^+	-4.82 to 5.43	>0.9999	
Ag-NP vs Ag^+ in Ag-NP Au-NP	-5.37 to 4.87	>0.9999	
Ag-NP vs Ag in Ag-NP Au^+	-2.82 to 7.43	0.7833	
Ag-NP vs Ag in Ag^+Au-NP	-5.672 to 4.578	0.9999	
Ag^+ vs Ag^+ in Ag-NP Au-NP	-5.679 to 4.57	0.9998	
Ag^+ vs Ag in Ag-NP Au^+	-3.125 to 7.125	0.8731	
Ag^+ vs Ag in Ag^+ Au-NP	-5.976 to 4.274	0.9982	
Ag^+ in Ag-NP Au-NP vs Ag in Ag-NP Au^+	-2.57 to 7.68	0.6947	
Ag^+ in Ag-NP Au-NP vs Ag in Ag^+ Au-NP	-5.42 to 4.83	>0.9999	
Ag in Ag-NP Au+ vs Ag in Ag+ Au-NP	-7.98 to 2.27	0.5811	
Au in Ag@Au-NP vs Au in Ag^+ Au^+	-5.02 to 1.72	0.7135	
Au in Ag@Au-NP vs Au-NP	-3.75 to 2.99	0.9998	
Au in Ag@Au-NP vs Au^+	-20.59 to -13.84	<0.0001	****
Au in Ag@Au-NP vs Au in Ag-NP Au-NP	-3.69 to 3.06	>0.9999	
Au in Ag@Au-NP vs Au in Ag-NP Au^+	-10.64 to -3.89	<0.0001	****
Au in Ag@Au-NP vs Au in Ag^+Au-NP	-3.41 to 3.33	>0.9999	
Au in Ag^+ Au^+ vs Au-NP	-2.10 to 4.64	0.8904	
Au in Ag^+ Au^+ vs Au^+	-18.94 to -12.20	<0.0001	****
Au in Ag^+ Au^+ vs Au in AgNP AuNP	-2.04 to 4.71	0.8658	

Au in Ag^+ Au^+ vs Au in AgNP Au^+	-8.99 to -2.25	0.0002	***
Au in Ag^+ Au^+ vs Au in Ag^+ AuNP	-1.76 to 4.98	0.7355	
Au-NP vs Au^+	-20.21 to -13.47	<0.0001	****
Au-NP vs Au in Ag-NP Au-NP	-3.31 to 3.44	>0.9999	
Au-NP vs Au in Ag-NP Au^+	-10.26 to -3.52	<0.0001	****
Au-NP vs Au in Ag^+ Au-NP	-3.03 to 3.71	>0.9999	
Au^+ vs Au in Ag-NP Au-NP	13.53 to 20.28	<0.0001	****
Au^+ vs Au in Ag-NP Au^+	6.58 to 13.32	<0.0001	****
Au^+ vs Au in Ag^+ Au-NP	13.80 to 20.55	<0.0001	****
Au in Ag-NP Au-NP vs Au in Ag-NP Au^+	-10.33 to -3.58	<0.0001	****
Au in Ag-NP Au-NP vs Au in Ag^+ Au-NP	-3.10 to 3.65	>0.9999	
Au in Ag-NP Au^+ vs Au in Ag^+ Au-NP	3.85 to 10.6	<0.0001	****

S4 Paragraph

Ultrafiltration (Millipore Amicon Bioseparations Stirred Cell – 400 mL) was performed in order to quantify the presence of ions in the Au@Ag-NPs stock solution. The membrane applied was a Millipore ultrafiltration membranes with a filter (diameter 90 mm) made of regenerated cellulose with pores size 1000 NMWL (or nominal molecular weight limit) corresponding to a pore size of around 1 nm. NPs where diluted in deionised water (DW) with ratio 1:13.3 for a total volume of 100 mL. The solution was transferred to the ultrafiltration cell along with the magnetic stirrer bar, and the cell was appropriately closed by locking to lid to avoid any gas leakage when connected to the nitrogen gas. The nitrogen gas was released into the cell at a pressure of 1.2 bars; this caused the ions to be push through the membrane and the constant stirring of the solution prevent the agglomeration of the NPs. After collecting 50 mL of the ion solution, the filtration was stopped, and 50 mL of DW was added into the cell, bring back it volume content to 100 mL and the NPs ultra-filtered again. This process was repeated four times and a total of five vials of 50 mL ionic solution was collected. The solutions were then acidified with 2% nitric acid (HNO_3, Sigma Aldrich) and kept at room temperature for ICP-MS analysis.

Au ions resulted to be 0.3% of the total Au concentration and Ag ions <0.001 % of the total Ag concentration in the stock solution.

Paragraph S5

Formulas for the calculation of the shell thickness of core shell and the mass concentrations of Au@Au-NPs

$$Radius\ of\ particle\ r = \frac{Diameter}{2}$$

$$Volume\ of\ particle\ V_p = \frac{4}{3}\,\pi\, r^3$$

$$Volume\ of\ the\ shell\ V_s = \frac{4}{3}\,\pi\,(R^3 - r^3)$$

where r is the radius of the Au core, R is the radius of the bimetallic particle.

$$Particle\ mass\ concentration = particle\ concentration * \rho * V$$

where ρ is the density

$$Particle\ concentration = \left(\frac{number\ o\ particles}{transport\ efficiency}\right) / \left(\frac{flow\ rate}{reading\ time}\right)$$

Chapter 4

Is a short-term toxicokinetic study able to predict the uptake of metal NPs in earthworms after nine months?

Marta Baccaro, Hans H. J. van den Berg, Nico W. van den Brink

In preparation

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 toxicokinetics 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 experiment previously conducted under similar conditions for 4 weeks (chapter 2). 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, internalized Ag was five times lower than for the other treatments after nine months. Ag concentrations in soil pore water did not change over time, pH decreased and electrical conductivity increased in all treatments. Metallothioneins in exposed earthworms were not statistically different from levels in untreated earthworms. Finally, the short-term kinetic rate constants predicted the bioaccumulation in earthworms exposed to Ag-NP and $AgNO_3$ after nine months, although for Ag_2S-NPs the bioaccumulation was somewhat under-predicted. Although, the rate of accumulation of Ag_2S-NPs is lower than that of Ag-NPs or $AgNO_3$ and thus potentially of lower concern, 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.

Introduction

Highlighting any difference 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 [1-3]. 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 [4]. Their behaviour in the soil is mainly affected by dissolution and redox reactions [5]. In chapter 2, we indeed demonstrated that in earthworms exposed to Ag-NP and $AgNO_3$ for 28 days, ~85% (average of both treatments) of the Ag accumulated in the earthworms was present as ions and accumulation patterns did not statistically differ between ionic Ag and pristine Ag-NPs. The bioaccumulation of Ag from Ag_2S-NPs was significantly lower, likely because of their low dissolvability. Although it appeared more and more clear that the availability of ions, released from metal NPs could explain most of the uptake [6], still some studies consistently reported particulate uptake [7, 8]. In soil, besides dissolution also other processes such as heteroaggregation and adsorption of NPs to soil particles and dissolved organic matter occur [9]. Since such speciation processes can strongly affect bioavailability, they should be studied, using dynamic approaches [10, 11]. However, due to difficulties to pinpoint standardized tests that assess effects of such 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 exposure time frames. To address this we performed a long-term exposure study under similar conditions used for the short term experiment in chapter 2.

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 modelled their uptake over time, deriving uptake rate constants for each Ag form, which can be compared to the rate constants derived in the earlier short-term study (chapter 2). Soil pore water was extracted to track changes in the more bioavailable metal fraction of the soil (<0.45 µm) [12], although it is known that NPs do not reach a steady state between the different phases in the soil [13]. Additionally, metallothionein (MT) levels in the earthworms were quantified at different time points assuming that synthesis of MT would occur during a prolonged time only when NPs would release ions since the induction of the synthesis of MT has been widely reported for dissolved metals [14, 15]. Finally, the comparison between the short-term (28 days) and long-term (nine months) toxicokinetic model allowed to conclude whether results of the short term toxicokinetic study can be applied to predict outcomes of longer term exposure scenarios, which are often more environmentally relevant.

Material and Methods

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 hours light, the earthworms were selected based on their weight, 380 ± 90 mg (average ± standard deviation, n=336). Before the start of the experiment, earthworms were depurated in petri dish with moist filter paper for 24 hours. Natural soil was collected from Proefboerderij Kooijenburg, Marwijksoord, The Netherlands, air dried and sieved (5 mm sieve openings).

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. The characterization of the nanomaterials used here are reported in chapter 2. In the control soil only water was added. Soil, water and Ag were homogeneously mixed by an automatic mixer for 3 minutes. After spiking the soil, jars were closed with a lid and kept in the incubator at 20°C for 24 hours, 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 every 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 allowed evaporation of water from the soil. Every ~3 days, jars were weighed and the loss of water was replenished by addition of Milli Q water. Sampling time points were set after 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 analysed. 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 hours. After depuration, earthworms were weighed, snap-frozen in liquid nitrogen and chopped for subsequent analysis.

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 [16]. The suspension was shaken at room temperature (20°C ± 1°C) for 1 hour after which it was allowed to settle overnight before the measurement (Handy Lab 680FK, SI Analytics, Germany).

Extraction of soil pore water for Ag analysis

Soil pore water was extracted by adaptation of the method reported in Waalewijn-Kool et al. [17]. 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 hours 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 minutes (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.

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 [18, 19]. 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. 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}.

Metallothionein analysis

Semi-quantification of MT was performed according to the protocol of Viarengo et al. [20] 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 minutes 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 minutes 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 [21]. 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 [22, 23].

Modelling 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 [24]. In the present study and in chapter 2, 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. [25]. The model is described by the equation (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). The elimination rate constants k_2 were assumed not to be different from the ones of the short-term experiment (chapter 2) because the capacity of excretion is mainly related on the physiology of the earthworm and therefore was assumed to be the same. Standard error and 95% confidential intervals of all parameters were calculated. Kinetic rate constants of the different treatments were compared.

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 of the short-term experiments were used to calculate the Ag concentrations in earthworm after 270 days of exposure. Uptake kinetic rate constants k_1 from short- and long-term toxicokinetic studies were compared, while elimination kinetic rate constants k_2 were kept identical.

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 the uptake kinetic rate constants k_1 amongst treatments and between short- and long-term exposure, a t test was used. All the analyses were performed by GraphPad Prism 7.0.

Results

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 (Figure 1a, Tables S1 – supplementary information). 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 Figure 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 – supplementary information).

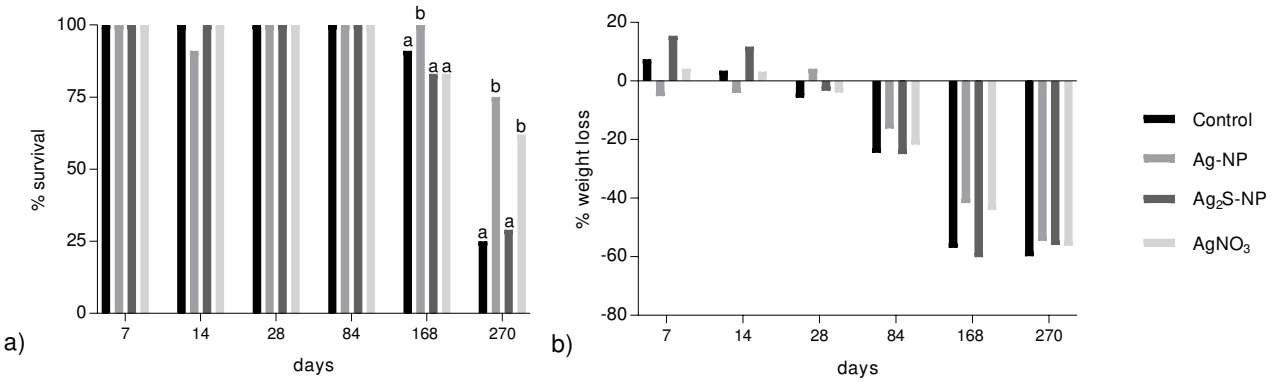


Figure 1. Percentage of survival (a) and weight loss (b) of the survivals compared to time 0 (n=12 for all treatments and time point except for day 270 for which n=24)

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 kg^{-1} dry weight for soils spiked with Ag-NP, Ag_2S-NP and $AgNO_3$, respectively (mean ± standard deviation). Internalized Ag concentrations in the depurated earthworms are shown in Figure 2 (for details see Table S2 – supplementary information). Concentrations of Ag in earthworms exposed to Ag-NP and $AgNO_3$ did not show any statistical difference at any time point, 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 all the time points (Tables S4 – supplementary information). When testing statistical 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 S5 – supplementary information).

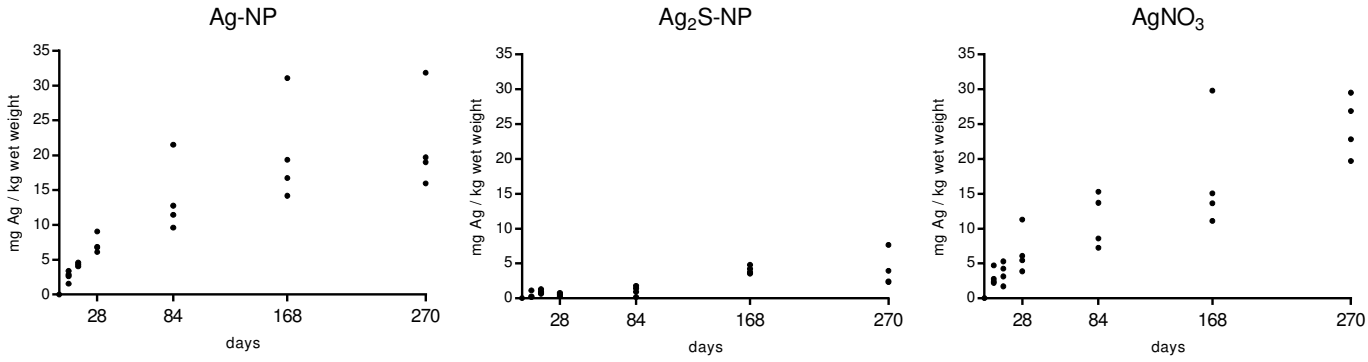


Figure 2. Time-dependent accumulation of Ag in earthworm *E. fetida* exposed to 15 mg Ag kg^{-1} dry weight soil of Ag-NPs, Ag_2S-NPs and $AgNO_3$ in natural soil.

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 (Figure 3). 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, indicating that the decrease in pH was highest in control soil, $AgNO_3$ and Ag-NP spiked soils.

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 µS cm^{-1} 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 S6 and S7 – supplementary information.

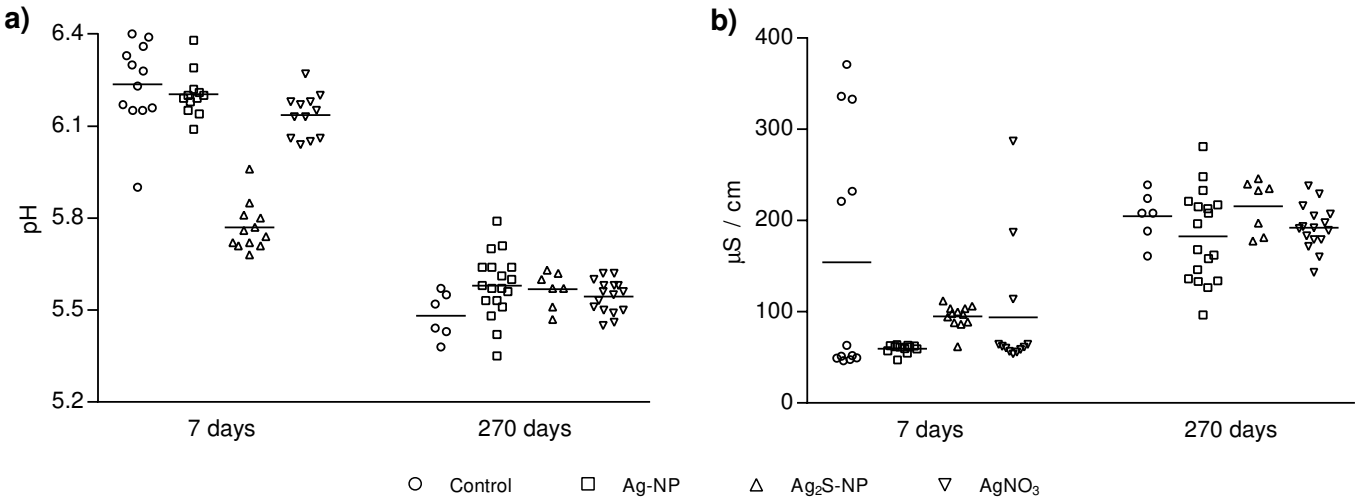


Figure 3. a) pH and b) electrical conductivity (µS cm^{-1}) values of control soils and soils (n=12) spiked with 15 mg Ag kg^{-1} of Ag-NP, Ag_2S-NP and $AgNO_3$ and incubated for 7 and 270 days.

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 Figure 4 (statistical analysis in Tables S8 and S9 – supplementary information).

Metallothionein quantification

In Figure 5 the concentrations of metallothionein in the earthworms unexposed and exposed to Ag-NP, Ag_2S-NP, $AgNO_3$ and cadmium are reported. MT concentrations did not differ significantly from the control with the exception of the MT concentration in the earthworms exposed to $AgNO_3$ for 6 months. Earthworms exposed to cadmium (positive control) showed higher MT levels than unexposed earthworms except for the control at day 84 (Tables S10 – supplementary information).

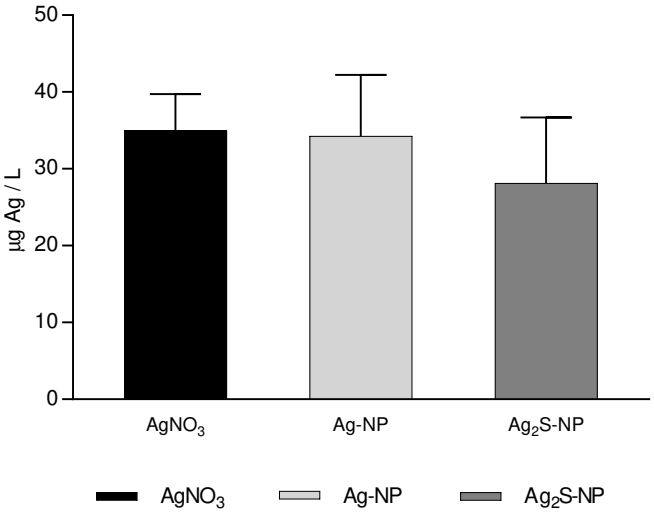


Figure 4. Average Ag concentrations in soil pore water extracted in soil aliquots of all the treatments (mean ± standard error, n=18).

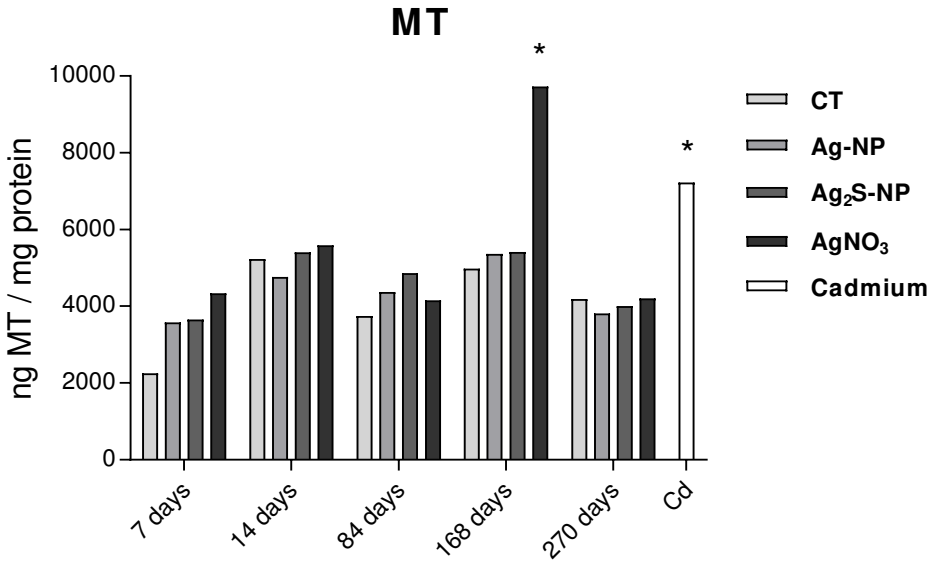


Figure 5. Metallothionein concentrations in earthworms (ng MT mg^{-1} protein; average value, n=2) unexposed and exposed to Ag-NP, Ag_2S-NP, $AgNO_3$ in all the time points. Cadmium is the positive control. Asterisks represents statistically significant difference with the control.

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) [26] and nine months (long-term). Elimination kinetic rate constants of the short-term study were used in the model of the long-term study. 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. As reported in chapter 2, the elimination kinetic rate constants did not statistically differ from each other for all the treatments. Comparison between the uptake kinetic rate constants (k_1) of the long-term model and the ones of the short-term model did not highlight any statistical difference.

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 ± 95% confidential interval. Elimination kinetic rate constants k_2 of the short-term study were used in the long-term study. Parameters with the same lowercase letter are not statistically different within the same exposure time. Parameters with the same capital letter are not statistically different between the long-term and short-term exposure.

	k_1 (mg Ag kg dry soil mg Ag^{-1} kg^{-1} wet body weight day^{-1}) ± SE	**k_2 (day^{-1}) ± SE**
Long-term exposure		
Ag_2S-NP	0.010 ± 0.002 a, A	0.064 ± 0.020 c, C
Ag-NP	0.052 ± 0.004 b, A	0.040 ± 0.013 c, C
$AgNO_3$	0.057 ± 0.006 b, A	0.044 ± 0.018 c, C
Short-term exposure		
Ag_2S-NP	0.008 ± 0.002 a, A	0.064 ± 0.020 c, C
Ag-NP	0.061 ± 0.019 b, A	0.040 ± 0.013 c, C
$AgNO_3$	0.055 ± 0.007 b, A	0.044 ± 0.018 c, C

Results of the statistical analysis are reported in Tables S11 – supplementary information. Confidential intervals for the regression lines were calculated for both models (long- and short-term exposure) and results are plotted in Figure 6.

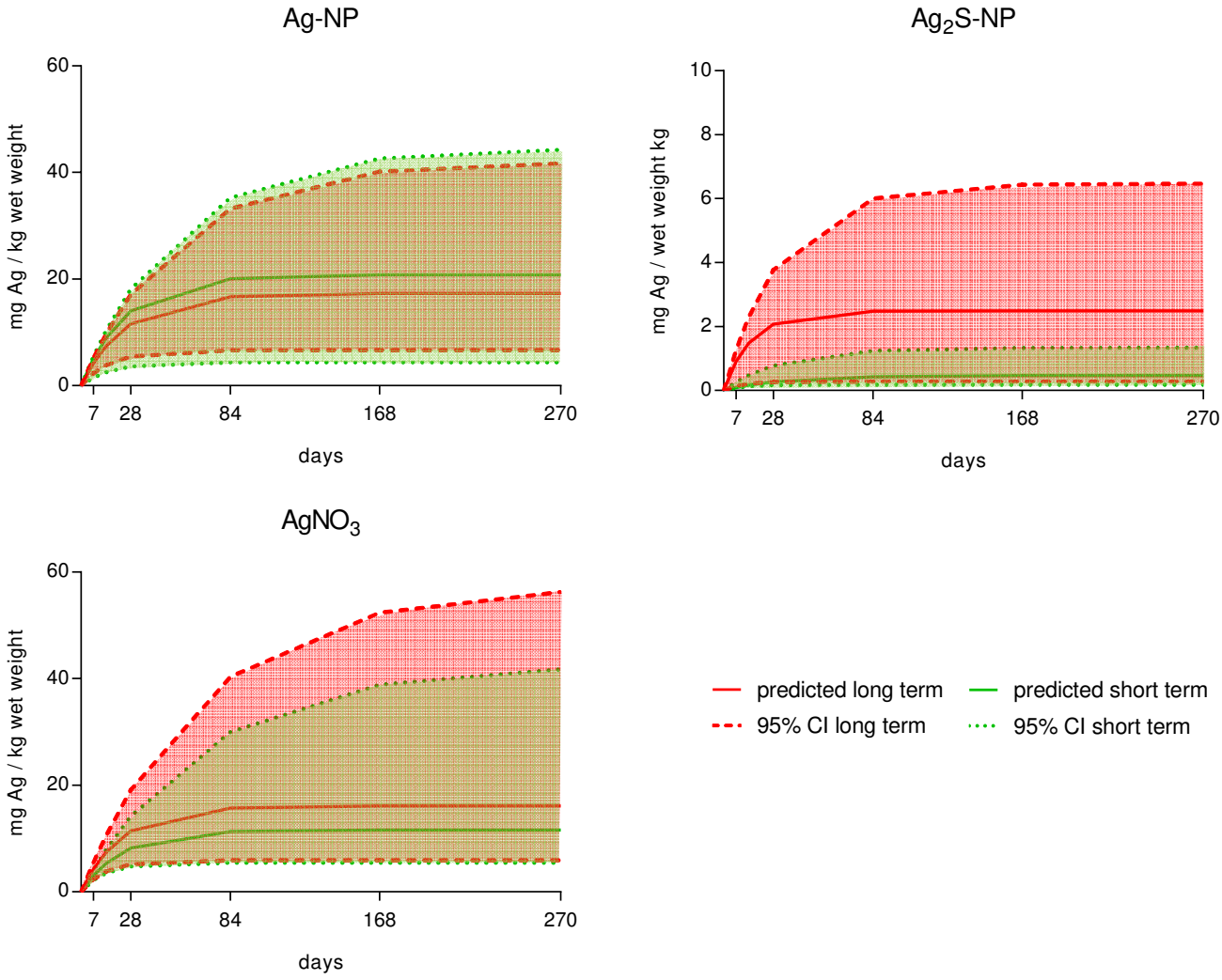


Figure 6. Comparison between the model calculated with the kinetic constants derived from the experimental data of the present study (red lines) and the prediction based using the kinetic constants from the model of a short-term bioaccumulation test (green lines) [26]. Note the different Y axis in the figure for the Ag_2S-NPs.

Discussion

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 [27]. 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 which was not 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 selected earthworms 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. Food was limited to 1.5 g of oven dry horse manure every two months in order to avoid increase of the amount of organic matter in the soil, potentially affecting the bioavailability due to the interaction between the increased organic matter and the NPs [28]. Addition of high amount of organic matter may also alter 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 [29]. 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. [30] 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. However, another study [31] showed that

earthworm *L. rubellus* raised pH in soil leachates likely because of conversion of calcium oxalate into calcium carbonate [32].

Bioavailability of Ag and accumulation in earthworms

A decrease in pH could lead to a tendency toward dissolution of Ag-NPs over time [33]. Sekine et al. [34] 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 this thesis, a similar scenario was reflected in the statistically significant increase of electrical conductivity in all soils of the current study where Ag was present for 270 days, which suggests an increased amount of ions and electrolytes, also in the sulfidized Ag-NP samples. Indeed, Sekine et al. [34] 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 Ag ions a steady state was reached within the different phases of the soil [35], 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. [36] tested the dissolution as function of pH and dissolved oxygen in sand columns in 48 hours. Results showed that dissolution decreased over time because of

oxidation of the surface of NPs. Molleman and Hiemstra [37] 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. [38] 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 hours.

The surface of Ag_2S-NPs is aged, covered (for the majority) by sulphur, 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 [35]. Indeed, the stability of Ag_2S-NPs has been recently reconsidered because studies showed that Ag_2S-NPs can dissolve in the aquatic environments [39, 40]. 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 [37, 41].

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-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 bioaccumulation study with exposure during 28 days (chapter 2), 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 S12 – supplementary information). When assuming that most uptake is ionic (chapter 2 and 3), this would suggest that the Ag_2S-NPs released more ions under conditions with additions of water and are more unstable under these conditions [42], hence may be reactive to changes in the environmental conditions such as rain fall over a long time.

MT-induction

Metallothionein (MT) measurement did not show any difference between exposed and control earthworms, and also did not vary over time. It has been reported that MT induction can be related to sources of stress other than uptake of metals [43, 44] and it is possible that isolation and weight loss 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. [45] 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 [46] 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 [47, 48], it is possible that if any MT induction occurred due to the presence of the metal, it took place within the first days (1 to 4 days) of exposure, which may have been missed in the current study.

Comparison between toxicokinetic rate constants

Comparison between the uptake kinetic rate constants amongst 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 a comparable value 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. Comparison between the uptake kinetic rate constants k_1 of long- and short-term exposure experiments did not highlight significant differences (Table 1). When tracing the confidentiality intervals of long- and short-term models in the graphs (Figure 6), it appeared clearer how the short term models predicts experimental data of accumulation after exposure to the different Ag forms over nine months. While the short term model was found to predict the bioaccumulation in the longer exposure for the pristine form and the $AgNO_3$ 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, as discussed in the previous section. 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 [25].

Conclusion

Accumulation of Ag in Ag-NP and $AgNO_3$ exposed earthworms did not statistically differ after nine months exposure. In Ag_2S-NP exposed earthworms the internalized concentrations were five times lower compared to the other treatments. The Ag concentrations in pore water did not reflect the uptake pattern and metallothionein concentrations in the exposed earthworms were not different from the control group. The uptake kinetic rate constants derived from a short-term exposure model of a previous study 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. The bioaccumulation of Ag_2S-NPs in earthworms cannot be explained by the concentrations of Ag measured at a specific time in pore water. Therefore, a better description of the dissolution dynamics of Ag_2S-NP in soil is necessary to improve their toxicokinetic predictions in earthworms. 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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Supplementary information

S1 Tables. Fisher test between survival data of all treatments after 168 days. Exact p values are reported when are higher than 0.0001.

	Ag-NP	$AgNO_3$	Ag_2S-NP	CT
Ag-NP	/	<0.0001	<0.0001	0.0032
$AgNO_3$	<0.0001	/	>0.9999	0.1400
Ag_2S-NP	<0.0001	>0.9999	/	0.1400
CT	0.0032	0.1400	0.1400	/

Fisher test between survival data of all treatments after 270 days. Exact p values are reported when are higher than 0.0001.

	Ag-NP	$AgNO_3$	Ag_2S-NP	CT
Ag-NP	/	0.0673	<0.0001	<0.0001
$AgNO_3$	0.0673	/	<0.0001	<0.0001
Ag_2S-NP	<0.0001	<0.0001	/	0.6330
CT	<0.0001	<0.0001	0.6330	/

Table S2. Concentrations of Ag measured in earthworms at different time points (mg Ag kg^{-1} wet body weight; n=4; mean ± standard deviation).

	7 days	14 days	28 days	84 days	168 days	270 days
Ag-NP	2.62±0.77	4.31±0.23	7.19±1.29	13.83±5.29	20.34±7.46	21.63±6.99
Ag_2S-NP	0.45±0.45	0.92±0.27	0.55±0.19	1.07±0.71	4.07±0.56	4.09±2.49
$AgNO_3$	3.03±1.15	3.60±1.54	6.67±3.23	11.21±3.89	17.40±8.42	24.73±4.33

S3 Tables. Turkey multiple comparison test following one way ANOVA amongst weight loss of all the treatment at the different time point.

7 days exposure

Tukey's multiple comparisons test	Mean Diff.	99.00% CI of diff.	Significant?	Adjusted P Value
Control vs. Ag-NP	12.67	-9.92 to 35.25	No	0.2640
Control vs. Ag_2S-NP	-7.87	-30.46 to 14.71	No	0.6609
Control vs. $AgNO_3$	3.40	-19.18 to 25.99	No	0.9593
Ag-NP vs. Ag_2S-NP	-20.54	-43.12 to 2.05	No	0.0221
Ag-NP vs. $AgNO_3$	-9.26	-31.85 to 13.32	No	0.5346
Ag_2S-NP vs. $AgNO_3$	11.27	-11.31 to 33.86	No	0.3635

14 days exposure

Tukey's multiple comparisons test	Mean Diff.	99.00% CI of diff.	Significant?	Adjusted P Value
Control vs. Ag-NP	7.59	-14.38 to 29.57	No	0.6658
Control vs. Ag_2S-NP	-8.20	-29.7 to 13.29	No	0.5919
Control vs. $AgNO_3$	0.40	-21.09 to 21.89	No	>0.9999
Ag-NP vs. Ag_2S-NP	-15.8	-37.77 to 6.176	No	0.0971
Ag-NP vs. $AgNO_3$	-7.20	-29.17 to 14.78	No	0.7020
Ag_2S-NP vs. $AgNO_3$	8.62	-12.89 to 30.09	No	0.5539

28 days exposure

Tukey's multiple comparisons test	Mean Diff.	99.00% CI of diff.	Significant?	Adjusted P Value
Control vs. Ag-NP	-9.90	-29.73 to 9.94	No	0.3636
Control vs. Ag_2S-NP	-2.37	-22.2 to 17.47	No	0.9790
Control vs. $AgNO_3$	-1.68	-21.51 to 18.15	No	0.9923
Ag-NP vs. Ag_2S-NP	7.53	-12.3 to 27.37	No	0.5969
Ag-NP vs. $AgNO_3$	8.22	-11.62 to 28.05	No	0.5261
Ag_2S-NP vs. $AgNO_3$	0.69	-19.15 to 20.52	No	0.9995

84 days exposure

Tukey's multiple comparisons test	Mean Diff.	99.00% CI of diff.	Significant?	Adjusted P Value
Control vs. Ag-NP	-8.35	-25.68 to 8.972	No	0.3940
Control vs. Ag_2S-NP	0.47	-16.86 to 17.79	No	0.9997
Control vs. $AgNO_3$	-2.80	-20.12 to 14.53	No	0.9506
Ag-NP vs. Ag_2S-NP	8.82	-8.50 to 26.14	No	0.3461
Ag-NP vs. $AgNO_3$	5.55	-11.77 to 22.88	No	0.7163
Ag_2S-NP vs. $AgNO_3$	-3.26	-20.59 to 14.06	No	0.9245

168 days exposure

Tukey's multiple comparisons test	Mean Diff.	99.00% CI of diff.	Significant?	Adjusted P Value
Control vs. Ag-NP	-15.32	-36.8 to 6.15	No	0.0998
Control vs. Ag_2S-NP	3.18	-18.76 to 25.12	No	0.9627
Control vs. $AgNO_3$	-13.1	-35.58 to 9.38	No	0.2300
Ag-NP vs. Ag_2S-NP	18.51	-2.97 to 39.98	No	0.0325
Ag-NP vs. $AgNO_3$	2.22	-19.81 to 24.25	No	0.9869
Ag_2S-NP vs. $AgNO_3$	-16.29	-38.77 to 6.19	No	0.0924

270 days exposure

Tukey's multiple comparisons test	Mean Diff.	99.00% CI of diff.	Significant?	Adjusted P Value
Control vs. Ag-NP	-5.15	-29.44 to 19.14	No	0.8966
Control vs. Ag_2S-NP	-3.78	-32.64 to 25.08	No	0.9726
Control vs. $AgNO_3$	-3.56	-28.39 to 21.28	No	0.9647
Ag-NP vs. Ag_2S-NP	1.37	-21.56 to 24.31	No	0.9972
Ag-NP vs. $AgNO_3$	1.59	-16.01 to 19.19	No	0.9906
Ag_2S-NP vs. $AgNO_3$	0.22	-23.29 to 23.73	No	>0.9999

S4 Tables. Tukey multiple comparison test following ANOVA test amongst treatments between Ag concentration in earthworms at the different time points

7 days exposure

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
AgNP vs. $AgNO_3$	-0.41	-2.08 to 1.25	No	0.7746
AgNP vs. Ag_2S-NP	2.17	0.50 to 3.84	Yes	0.0136
$AgNO_3$ vs. Ag_2S-NP	2.58	0.92 to 4.25	Yes	0.0049

14 days exposure

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
AgNP vs. $AgNO_3$	0.71	-1.08 to 2.51	No	0.5334
AgNP vs. Ag_2S-NP	3.39	1.59 to 5.19	Yes	0.0013
$AgNO_3$ vs. Ag_2S-NP	2.67	0.88 to 4.47	Yes	0.0063

28 days exposure

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
AgNP vs. $AgNO_3$	0.52	-3.45 to 4.49	No	0.9294
AgNP vs. Ag_2S-NP	6.65	2.68 to 10.62	Yes	0.0030
$AgNO_3$ vs. Ag_2S-NP	6.13	2.16 to 10.1	Yes	0.0050

84 days exposure

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
AgNP vs. AgNO3	2.62	-4.91 to 10.14	No	0.6117
AgNP vs. Ag2S-NP	12.76	5.24 to 20.29	Yes	0.0027
AgNO3 vs. Ag2S-NP	10.15	2.62 to 17.67	Yes	0.0112

168 days exposure

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
AgNP vs. $AgNO_3$	-4.39	-14.63 to 5.85	No	0.4840
AgNP vs. Ag_2S-NP	16.24	6.00 to 26.48	Yes	0.0042
$AgNO_3$ vs. Ag_2S-NP	20.63	10.39 to 30.87	Yes	0.0008

270 days exposure

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
AgNP vs. $AgNO_3$	4.23	-8.26 to 16.72	No	0.6268
AgNP vs. Ag_2S-NP	17.56	5.07 to 30.04	Yes	0.0088
$AgNO_3$ vs. Ag_2S-NP	13.33	0.84 to 25.81	Yes	0.0373

S5 Tables. Turkey multiple comparison test following ANOVA test amongst Ag concentration in earthworms at the different time points for the same treatment.

Ag_2S-NP

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
7 days vs. 14 days	-0.47	-2.96 to 2.01	No	0.9892
7 days vs. 28 days	-0.099	-2.59 to 2.39	No	>0.9999
7 days vs. 84 days	-0.62	-3.11 to 1.87	No	0.9649
7 days vs. 168 days	-3.62	-6.11 to -1.14	Yes	0.0024
7 days vs. 270 days	-3.65	-6.14 to -1.16	Yes	0.0023
14 days vs. 28 days	0.375	-2.11 to 2.86	No	0.9963
14 days vs. 84 days	-0.15	-2.64 to 2.34	No	>0.9999
14 days vs. 168 days	-3.15	-5.64 to -0.66	Yes	0.0088
14 days vs. 270 days	-3.17	-5.66 to -0.68	Yes	0.0083
28 days vs. 84 days	-0.52	-3.01 to 1.97	No	0.9834
28 days vs. 168 days	-3.53	-6.01 to -1.04	Yes	0.0032
28 days vs. 270 days	-3.55	-6.04 to -1.06	Yes	0.0030
84 days vs. 168 days	-3.00	-5.49 to -0.51	Yes	0.0130
84 days vs. 270 days	-3.02	-5.51 to -0.54	Yes	0.0123
184 days vs. 270 days	-0.02	-2.51 to 2.47	No	>0.9999

Ag-NP

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
7 days vs. 14 days	-1.69	-12.34 to 8.96	No	0.9953
7 days vs. 28 days	-4.57	-15.23 to 6.08	No	0.7463
7 days vs. 84 days	-11.21	-21.87 to -0.56	Yes	0.0358
7 days vs. 168 days	-17.72	-28.37 to -7.06	Yes	0.0006
7 days vs. 270 days	-19.01	-29.66 to -8.36	Yes	0.0003
14 days vs. 28 days	-2.88	-13.54 to 7.77	No	0.9512
14 days vs. 84 days	-9.52	-20.18 to 1.13	No	0.0956
14 days vs. 168 days	-16.03	-26.68 to -5.37	Yes	0.0018
14 days vs. 270 days	-17.32	-27.97 to -6.67	Yes	0.0008
28 days vs. 84 days	-6.64	-17.29 to 4.01	No	0.3898
28 days vs. 168 days	-13.14	-23.8 to -2.49	Yes	0.0109
28 days vs. 270 days	-14.44	-25.09 to -3.79	Yes	0.0048
84 days vs. 168 days	-6.50	-17.16 to 4.15	No	0.4114
84 days vs. 270 days	-7.80	-18.45 to 2.86	No	0.2344
184 days vs. 270 days	-1.29	-11.95 to 9.36	No	0.9987

$AgNO_3$

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
7 days vs. 14 days	-0.98	-10.95 to 8.99	No	0.9995
7 days vs. 28 days	-4.05	-14.03 to 5.91	No	0.7853
7 days vs. 84 days	-8.60	-18.57 to 1.38	No	0.1153
7 days vs. 168 days	-14.78	-24.75 to -4.81	Yes	0.0020
7 days vs. 270 days	-22.11	-32.08 to -12.14	Yes	<0.0001
14 days vs. 28 days	-3.08	-13.05 to 6.89	No	0.9182
14 days vs. 84 days	-7.62	-17.59 to 2.35	No	0.1984
14 days vs. 168 days	-13.8	-23.77 to -3.83	Yes	0.0040
14 days vs. 270 days	-21.13	-31.1 to -11.16	Yes	<0.0001
28 days vs. 84 days	-4.54	-14.51 to 5.43	No	0.6996
28 days vs. 168 days	-10.73	-20.7 to -0.753	Yes	0.0309
28 days vs. 270 days	-18.05	-28.02 to -8.08	Yes	0.0002
84 days vs. 168 days	-6.18	-16.16 to 3.79	No	0.3949
84 days vs. 270 days	-13.51	-23.48 to -3.54	Yes	0.0048
184 days vs. 270 days	-7.33	-17.3 to 2.64	No	0.2308

S6 Tables. Turkey multiple comparison test following ANOVA test amongst pH measurements between soils of all treatments for all time points.

Comparison amongst treatments after 7 days

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
Control vs. Ag-NP	0.032	-0.07 to 0.14	No	0.8478
Control vs. Ag_2S-NP	0.47	0.36 to 0.57	Yes	<0.0001
Control vs. $AgNO_3$	0.1	-0.004 to 0.20	No	0.0633
Ag-NP vs. Ag_2S-NP	0.43	0.33 to 0.54	Yes	<0.0001
Ag-NP vs. $AgNO_3$	0.068	-0.04 to 0.17	No	0.3084
Ag_2S-NP vs. $AgNO_3$	-0.37	-0.47 to -0.27	Yes	<0.0001

Comparison amongst treatments after 270 days

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
Control vs. Ag-NP	-0.10	-0.20 to 0.004	No	0.0625
Control vs. Ag_2S-NP	-0.08	-0.20 to 0.034	No	0.2400
Control vs. $AgNO_3$	-0.06	-0.16 to 0.042	No	0.3926
Ag-NP vs. Ag_2S-NP	0.01	-0.08 to 0.11	No	0.9859
Ag-NP vs. $AgNO_3$	0.04	-0.04 to 0.11	No	0.5601
Ag_2S-NP vs. $AgNO_3$	0.02	-0.07 to 0.12	No	0.9121

Comparison between pH at day 7 and day 270 in the same treatment

Unpaired t test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
Control day 7 vs. Control day 270	-0.75 ± 0.062	-0.88 to -0.62	yes	<0.0001
Ag-NP day 7 vs. Ag-NP day 270	-0.62 ± 0.03	-0.69 to -0.55	yes	<0.0001
Ag_2S-NP day 7 vs. Ag_2S-NP day 270	-0.20 ± 0.03	-0.27 to -0.13	yes	<0.0001
$AgNO_3$ day 7 vs. $AgNO_3$ day 270	-0.59 ± 0.02	-0.64 to -0.54	yes	<0.0001

S7 Tables. Turkey multiple comparison test following ANOVA test amongst electrical conductivity (EC) measurements between soils of all treatments for all time points.

Comparison amongst treatments after 7 days

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
Control vs. Ag-NP	94.75	11.56 to 177.9	Yes	0.0199
Control vs. Ag_2S-NP	59.35	-23.84 to 142.5	No	0.2408
Control vs. $AgNO_3$	60.57	-22.62 to 143.8	No	0.2250
Ag-NP vs. Ag_2S-NP	-35.4	-118.6 to 47.79	No	0.6694
Ag-NP vs. $AgNO_3$	-34.18	-117.4 to 49	No	0.6931
Ag_2S-NP vs. $AgNO_3$	1.22	-81.97 to 84.4	No	>0.9999

Comparison amongst treatments after 270 days

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
Control vs. Ag-NP	21.87	-25.07 to 68.81	No	0.6020
Control vs. Ag_2S-NP	-10.91	-66.31 to 44.48	No	0.9522
Control vs. $AgNO_3$	12.64	-35.02 to 60.31	No	0.8930
Ag-NP vs. Ag_2S-NP	-32.79	-77.14 to 11.57	No	0.2131
Ag-NP vs. $AgNO_3$	-9.23	-43.44 to 24.98	No	0.8882
Ag_2S-NP vs. $AgNO_3$	23.56	-21.56 to 68.68	No	0.5091

Comparison between EC at day 7 and day 270 in the same treatment

Unpaired t test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
Control day 7 vs. Control day 270	50.55 ± 56.01	-68.19 to 169.3	No	0.3802
Ag-NP day 7 vs. Ag-NP day 270	123.4 ± 14.49	93.74 to 153.1	yes	<0.0001
Ag_2S-NP day 7 vs. Ag_2S-NP day 270	120.8 ± 9.66	100.4 to 141.2	yes	<0.0001
$AgNO_3$ day 7 vs. $AgNO_3$ day 270	98.47 ± 19.21	58.98 to 138	yes	<0.0001

S8 Tables. Bonferroni multiple comparison test following ANOVA test amongst Ag concentrations in soil pore water of all the treatments at the different time points

7 days

Bonferroni's multiple comparisons test	Mean Diff.	99.90% CI of diff.	Significant?	Adjusted P Value
Ag-NP vs. $AgNO_3$	5.15	-26.11 to 36.41	No	0.8209
Ag-NP vs. Ag_2S-NP	22.85	-8.41 to 54.11	No	0.0052
$AgNO_3$ vs. Ag_2S-NP	17.71	-13.55 to 48.96	No	0.0182

14 days

Bonferroni's multiple comparisons test	Mean Diff.	99.90% CI of diff.	Significant?	Adjusted P Value
Ag-NP vs. $AgNO_3$	-14.33	-43.93 to 15.28	No	0.0367
Ag-NP vs. Ag_2S-NP	1.64	-27.97 to 31.24	No	>0.9999
$AgNO_3$ vs. Ag_2S-NP	15.96	-13.64 to 45.57	No	0.0228

28 days

Bonferroni's multiple comparisons test	Mean Diff.	99.90% CI of diff.	Significant?	Adjusted P Value
Ag-NP vs. $AgNO_3$	-30.19	-149.3 to 88.93	No	0.3397
Ag-NP vs. Ag_2S-NP	14.24	-104.9 to 133.4	No	>0.9999
$AgNO_3$ vs. Ag_2S-NP	44.43	-74.69 to 163.5	No	0.1028

84 days

Bonferroni's multiple comparisons test	Mean Diff.	99.90% CI of diff.	Significant?	Adjusted P Value
Ag-NP vs. $AgNO_3$	36.82	-57.55 to 131.2	No	0.0871
Ag-NP vs. Ag_2S-NP	42.05	-52.31 to 136.4	No	0.0518
$AgNO_3$ vs. Ag_2S-NP	5.232	-89.13 to 99.59	No	>0.9999

168 days

Bonferroni's multiple comparisons test	Mean Diff.	99.90% CI of diff.	Significant?	Adjusted P Value
Ag-NP vs. $AgNO_3$	-1.05	-165.4 to 163.2	No	>0.9999
Ag-NP vs. Ag_2S-NP	-42.77	-207.1 to 121.5	No	0.3168
$AgNO_3$ vs. Ag_2S-NP	-41.71	-206 to 122.6	No	0.3381

270 days

Bonferroni's multiple comparisons test	Mean Diff.	99.90% CI of diff.	Significant?	Adjusted P Value
Ag-NP vs. $AgNO_3$	-0.835	-24.68 to 23.01	No	>0.9999
Ag-NP vs. Ag_2S-NP	-1.243	-25.09 to 22.61	No	>0.9999
$AgNO_3$ vs. Ag_2S-NP	-0.408	-24.26 to 23.44	No	>0.9999

S9 Tables. Bonferroni's multiple comparison test following ANOVA test amongst Ag concentration in soil pore water at the different time points for the same treatment

Ag_2S-NP

Bonferroni's multiple comparisons test	Mean Diff.	99.90% CI of diff.	Significant?	Adjusted P Value
7 vs. 14	12.62	-82.87 to 108.1	No	>0.9999
7 vs. 28	12.85	-82.64 to 108.3	No	>0.9999
7 vs. 84	-1.08	-96.57 to 94.41	No	>0.9999
7 vs. 168	-44.18	-139.7 to 51.31	No	0.2615
7 vs. 270	-4.21	-99.7 to 91.28	No	>0.9999
14 vs. 28	0.23	-95.26 to 95.72	No	>0.9999
14 vs. 84	-13.7	-109.2 to 81.79	No	>0.9999
14 vs. 168	-56.8	-152.3 to 38.69	No	0.0609
14 vs. 270	-16.83	-112.3 to 78.66	No	>0.9999
28 vs. 84	-13.93	-109.4 to 81.56	No	>0.9999
28 vs. 168	-57.03	-152.5 to 38.46	No	0.0593
28 vs. 270	-17.06	-112.6 to 78.43	No	>0.9999
84 vs. 168	-43.1	-138.6 to 52.39	No	0.2963
84 vs. 270	-3.13	-98.63 to 92.35	No	>0.9999
168 vs. 270	39.97	-55.52 to 135.5	No	0.4247

Ag-NP

Bonferroni's multiple comparisons test	Mean Diff.	99.90% CI of diff.	Significant?	Adjusted P Value
7 vs. 14	33.83	-23.27 to 90.94	No	0.0624
7 vs. 28	21.46	-35.65 to 78.56	No	0.6749
7 vs. 84	-20.27	-77.38 to 36.83	No	0.8422
7 vs. 168	21.44	-35.67 to 78.54	No	0.6777
7 vs. 270	19.88	-37.22 to 76.99	No	0.9053
14 vs. 28	-12.38	-69.48 to 44.73	No	>0.9999
14 vs. 84	-54.11	-111.2 to 3.00	No	0.0016
14 vs. 168	-12.4	-69.5 to 44.71	No	>0.9999
14 vs. 270	-13.95	-71.06 to 43.15	No	>0.9999
28 vs. 84	-41.73	-98.84 to 15.37	No	0.0141
28 vs. 168	-0.02	-57.13 to 57.08	No	>0.9999
28 vs. 270	-1.58	-58.68 to 55.53	No	>0.9999
84 vs. 168	41.71	-15.4 to 98.81	No	0.0142
84 vs. 270	40.15	-16.95 to 97.26	No	0.0189
168 vs. 270	-1.55	-58.66 to 55.55	No	>0.9999
84 vs. 270	40.15	7.941 to 72.37	Yes	0.0124
168 vs. 270	-1.55	-33.77 to 30.66	No	>0.9999

$AgNO_3$

Bonferroni's multiple comparisons test	Mean Diff.	99.90% CI of diff.	Significant?	Adjusted P Value
7 vs. 14	14.36	-56.5 to 85.21	No	>0.9999
7 vs. 28	-13.88	-84.73 to 56.98	No	>0.9999
7 vs. 84	11.39	-59.46 to 82.25	No	>0.9999
7 vs. 168	15.23	-55.62 to 86.09	No	>0.9999
7 vs. 270	13.9	-56.96 to 84.75	No	>0.9999
14 vs. 28	-28.23	-99.09 to 42.62	No	0.5284
14 vs. 84	-2.96	-73.82 to 67.89	No	>0.9999
14 vs. 168	0.88	-69.98 to 71.73	No	>0.9999
14 vs. 270	-0.46	-71.31 to 70.39	No	>0.9999
28 vs. 84	25.27	-45.59 to 96.12	No	0.8277
28 vs. 168	29.11	-41.74 to 99.96	No	0.4619
28 vs. 270	27.77	-43.08 to 98.63	No	0.5669
84 vs. 168	3.841	-67.01 to 74.7	No	>0.9999
84 vs. 270	2.50	-68.35 to 73.36	No	>0.9999
168 vs. 270	-1.34	-72.19 to 69.52	No	>0.9999

S10 Tables. Dunnett multiple comparisons test following ANOVA test amongst metallothionein concentrations for all the treatments at each time point.

7 days

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
CT vs. Ag-NP	-2831	-6863 to 1202	No	0.1558
CT vs. Ag_2S-NP	-1404	-5437 to 2628	No	0.6040
CT vs. $AgNO_3$	-2087	-6120 to 1946	No	0.3250

14 days

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
CT vs. Ag-NP	470.7	-1164 to 2106	No	0.7252
CT vs. Ag_2S-NP	-181.6	-1816 to 1453	No	0.9827
CT vs. $AgNO_3$	-361.1	-1996 to 1274	No	0.8531

84 days

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
CT vs. Ag-NP	-873.7	-4907 to 3160	No	0.8605
CT vs. Ag_2S-NP	-1371	-5405 to 2663	No	0.6206
CT vs. $AgNO_3$	-663.8	-4698 to 3370	No	0.9365

168 days

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
CT vs. Ag-NP	-395.8	-4041 to 3250	No	0.9840
CT vs. Ag_2S-NP	-443.2	-4089 to 3202	No	0.9763
CT vs. Ag^+	-4749	-8394 to -1103	Yes	0.0181

270 days

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
CT vs. Ag-NP	379.9	-4591 to 5351	No	0.9957
CT vs. Ag_2S-NP	186.5	-4784 to 5157	No	0.9998
CT vs. Ag^+	-17.5	-4988 to 4953	No	0.9999

T test between metallothionein concentrations in earthworms untreated and earthworms exposed to cadmium

	7 days p value	14 days p value	84 days p value	168 days p value	270 days p value
Control vs Cadmium	0.0004	0.0119	0.1555	0.0272	0.0073

Tables S11. Comparison between kinetic rate constants derived from the different treatments of the long-term exposure (n=24) and between long- and short-term exposure (n=32).

Ag-NP long term vs $AgNO_3$ long term

	Ag-NP		$AgNO_3$		Differences between parameters				
	Mean	SE	Mean	SE	Difference between means	Mean Squared Error	t-value	total degrees of freedom	p-value
k1	0.052	0.004	0.057	0.006	-0.004	0.007	-0.625	46	0.535
k2	0.040	0.013	0.044	0.018	-0.004	0.022	-0.180	46	0.0858

Ag-NP long term vs Ag_2S-NP long term

	Ag-NP		Ag_2S-NP		Differences between parameters				
	Mean	SE	Mean	SE	Difference between means	Mean Squared Error	t-value	total degrees of freedom	p-value
k1	0.052	0.004	0.010	0.002	0.042	0.005	9.166	46	<0.00001
k2	0.040	0.013	0.064	0.020	-0.024	0.024	-1.006	46	0.319

$AgNO_3$ long term vs Ag_2S-NP long term

	$AgNO_3$		Ag_2S-NP		Differences between parameters				
	Mean	SE	Mean	SE	Difference between means	Mean Squared Error	t-value	total degrees of freedom	p-value
k1	0.057	0.006	0.010	0.002	0.047	0.006	7.915	46	<0.00001
k2	0.044	0.018	0.064	0.020	-0.020	0.027	-0.743	46	0.461

Ag_2S-NP short term vs Ag_2S-NP long term

	Short term		Long term		Differences between parameters				
	Mean	SE	Mean	SE	Difference between means	Mean Squared Error	t-value	total degrees of freedom	p-value
k1	0.008	0.002	0.010	0.002	-0.002	0.002	-0.792	54	0.4318

Ag-NP short term vs Ag-NP long term

	Short term		Long term		Differences between parameters				
	Mean	SE	Mean	SE	Difference between means	Mean Squared Error	t-value	total degrees of freedom	p-value
k1	0.061	0.019	0.052	0.004	0.009	0.019	0.444	54	0.658

$AgNO_3$ short term vs $AgNO_3$ long term

	Short term		Long term		Differences between parameters				
	Mean	SE	Mean	SE	Difference between means	Mean Squared Error	t-value	total degrees of freedom	p-value
k1	0.055	0.007	0.057	0.006	-0.001	0.009	-0.199	54	0.8438

Table S12. T-test between Ag concentrations in earthworms exposed to Ag-NP, Ag_2S-NP and $AgNO_3$ with (present work) and without (chapter 2) addition of water for 28 days.

	P value
Ag-NP short term vs Ag-NP long term	0.2097
$AgNO_3$ short term vs $AgNO_3$ long term	0.3387
Ag_2S-NP short term vs Ag_2S-NP long term	0.0122

Chapter 5

Bioturbation of Ag_2S-NPs in soil columns by earthworms

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Abstract

Sewage sludge contains Ag_2S-NPs causing NP exposure of soil fauna when sludge is applied as soil amendment. Earthworm bioturbation is an important process affecting many soil functions. Bioturbation may be affected by the presence of Ag_2S-NPs, but the earthworm activity itself may also influence the displacement of these NPs that otherwise show little transport in the soil. The aim of this study was to determine effects of Ag_2S-NPs on earthworm bioturbation and effect of this bioturbation on the vertical distribution of Ag_2S-NPs. Columns (12 cm) of a sandy loamy soil with and without *Lumbricus rubellus* were prepared with and without 10 mg Ag kg^{-1}, applied as Ag_2S-NPs in the top 2 cm of the soil, while artificial rainwater was applied at ~1.2 mm day^{-1}. The soil columns were sampled at three depths weekly for 28 days and leachate was collected from the bottom. Total Ag measurements showed more displacement of Ag to deeper soil layers in the columns with earthworms. The application of rain only did not significantly affect Ag transport in the soil. No Ag was detected in column leachates. X-ray tomography showed that changes in macro porosity and pore size distribution as a result of bioturbation were not different between columns with and without Ag_2S-NPs. Earthworm activity was therefore not affected by Ag_2S-NPs at the used exposure concentration. Ag concentrations along the columns and the earthworm density allowed the calculation of the bioturbation rate. The remarkable effect on the Ag transport in the soil shows that earthworm burrowing activity is a relevant process that must be taken into account when studying the fate of nanoparticles in soils.

Introduction

Earthworms mix soils by their burrowing activity. This is fundamental for the soil formation and its functioning. Ingestion and egestion of soil and construction of burrows impact the structure and chemistry of soil, its water holding capacity and drainage, aeration, as well as the distribution and fate of essential elements and organic matter [1, 2]. The activity of earthworms can lead to a complete mixing of the soil over a few years [3] and this process can displace strongly adsorbed contaminants or nutrients [4, 5]. Apart from moving soil, earthworms create burrows, which may represent preferential routes for the transport of rain water including dissolved nutrients or contaminants [6]. In turn, burrowing activity of earthworms can be affected by exposure to contaminants, as shown for imidacloprid [7] and carbaryl [8]. In this way, contaminants present in e.g. sludge from waste water treatment plants (WWTPs) may affect the behaviour of earthworms. Because of the wide use of Ag-NPs in consumer goods, WWTP-sludge can contain Ag_2S-NPs, which are the main product of the chemical transformation of manufactured Ag-NPs captured by biosolids in WWTPs [9, 10]. The low dissolvability of Ag_2S-NPs may lead to relatively low bioavailability of Ag for soil organisms (chapter 2) [11] and plants [12], suggesting lower toxicity compared to pristine Ag-NPs or ionic Ag [13, 14]. However, earthworm behavioural alterations may not be directly linked to the uptake of chemicals but to e.g. sensing and detection of the Ag [15, 16]. For instance, avoidance of Ag-NPs by different earthworm species has been observed [15-18] and it was found to be a sensitive endpoint, not directly related to dissolution of Ag-NPs and not related to Ag uptake and body burden.

Column transport experiments with repacked soils have shown that NPs generally are relatively immobile having transport distances of only a few centimetres under saturated flow conditions [19]. Interaction of NPs with air-water interfaces reduces their mobility even more

in non-saturated soils [20]. Greater mobility of Ag-NPs was observed in sand columns than in sandy loam soil columns where the retention of Ag-NPs was higher than 90% [21]. With no or little transport, NPs would accumulate in the upper soil layers only, but column experiments do not account for biologically mediated NP transport by e.g. earthworms, plants. To better understand the fate of NPs in the soil, there is a need to assess how earthworms affect their transport in the top soil. In this work, for the first time, we therefore quantitatively compared transport distances of Ag_2S-NPs related to percolating water or to bioturbation. For this, a series of experiments was conducted using Ag_2S-NP as a model for aged forms of Ag-NPs, using a field-relevant earthworm species, *Lumbricus rubellus* and including artificial rain. The experiments were performed in a series of microcosms in which we assessed the influence of the burrowing activity of earthworms on the vertical transport of Ag_2S-NPs. A bioturbation rate was calculated, useful to predict the influence of the earthworms in distributing metal-based NPs. Furthermore, we quantified the uptake of Ag_2S-NPs in the earthworms and the potential effect of the presence of Ag_2S-NPs in the top soil on the burrowing activity.

Materials and methods

NPs and soil characterisation

Uncoated Ag_2S-NPs were tailor-made synthesised and characterized by Applied Nanoparticles (Barcelona, Spain) and Oxford Materials Characterization Service (University of Oxford, UK). Particles had diameter 28.0±9.0 nm (mean±standard deviation), measured by transmission electron microscopy (TEM) (number of particles=1620, number of images=30), ζ-potential was -22.1±0.6 mV in water (200 µg Ag_2S-NP ml^{-1}, conductivity 0.158±0.001 mS

cm^{-1}, pH 8.52). In Paragraph S1 in Supplementary information, TEM images and STEM/EDX (scanning transmission electron microscope/energy dispersive X-ray) analyses provide the elemental composition of the single particle (Ag/S ratio higher than two). A natural sandy-loam soil (pH 5.98, organic matter content 2.71 %, CEC 8 mmol/100 g) collected from an uncontaminated location in The Netherlands (Proefboerderij Kooijenburg, Marwijksoord) was air-dried and sifted (5 mm sieve openings) before use. Additional soil characterization parameters are reported in Tables S1 and S2 – supplementary information.

Earthworms

Earthworms (*Lumbricus rubellus*) were obtained from a non-polluted field site near Nijkerkerveen in the Netherlands and maintained for acclimatisation in experimental natural soil at 15±1 °C with 24 hours light for 2 weeks until use. A bed of dried alder leaves (*Alnus glutinosa*) from an uncontaminated site in the Netherlands (Vossemeerdijk, Dronten) was placed on top of the soil allowing natural feeding behaviour. Before the start of the experiment, adult clitellated earthworms were selected, based on their weight and allowed to void gut contents on wet filter paper for 48 hours. The final average weight per earthworm was 0.82±0.08 g (mean ± standard deviation; n=320).

Soil column preparation and exposure

Experiments were conducted in polyvinyl chloride (PVC) columns (n=64, diameter 7.5 cm, length 15 cm) with a top-cap with a hole (diameter 5 mm) for aeration. The bottom consisted of a mesh (diameter 150 µm openings) which allowed water to leach out but kept the soil in place. The columns were filled with 450 g of air-dried soil up to a depth of 12 cm. Initial moisture content was set at 17.5% w/w (~40 % of water holding capacity, WHC) for all

columns. Homogenisation of soil and water was ensured by the use of an automatic mixer. On top of each column, a 75 g soil (air dried weight, equal to ~1.8 cm) without or with 10 mg Ag kg^{-1} dry weight soil as Ag_2S-NPs was added. After 24 hours adult depurated *L. rubellus* (n=5) were randomly introduced on top of every experimental unit. This resembles an approximate density of ~2500 individuals/m^2. Although such a density is five times the highest field density reported in literature [22] a relatively high density was chosen to allow for detectability of the mixing processes. After the worms entered the soil, 3 g of dry alder leaves were distributed on the soil surface. Soil columns were carefully placed in the incubator (15±1 °C) to avoid soil structure disturbance. Artificial rain water (ARW) was prepared (0.01 mM NaCl, 0.0053 mM $(NH_4)_2SO_4$, 0.0059 mM $NaNO_3$ and 0.0039 mM $CaCl_2$ in demineralised water), at pH 5.1. Five days a week, 7.5 mL of ARW (~1.2 mm day^{-1}) was added to the surface of 50% of the columns by slowly dripping the volume with the use of a pipette avoiding the edges of the columns. The amount of ARW was calculated based on the average precipitation in the Netherlands. Four different experimental treatments (n=16 per treatment) were carried out simultaneously in a factorial design: i) with/without worms, ii) with/without artificial rain.

Sampling

The experiments ran for 28 days, each week four replicates per treatment were randomly selected. Three different layers of soil, denoted as top, middle and bottom were sampled at 0-2, 6-8, 10-12 cm depth. Soil in between these layers was discarded due to difficulties in sampling distinct layers of soil in the column with accuracy. Soil was sampled by pushing out the exact amount of soil from the bottom until the designated depth using a graduated solid cylinder. The soil samples were weighed and stored in sealed polyethylene bags at -20°C for further chemical analysis. Earthworms were sampled as they were found and their vertical

position within the column was recorded. After depuration on moist filter paper for 48 hours in the dark at 15±1 °C, earthworms were washed, pad dried, weighed, killed in liquid nitrogen and freeze dried for 46 hours.

X-ray tomography and image analysis

In addition to the destructive collection of samples, changes in soil macro porosity were quantified by X-ray tomography over time. Additional soil columns were prepared for this purpose, i.e. 3 replicates with earthworms and with Ag_2S-NPs in the top layer, 3 replicates with earthworms and without Ag_2S-NPs, 3 replicates without earthworms and without Ag_2S-NPs. Rain was not applied to keep the density difference (between soil and air) as high as possible, essential to obtain a high quality x-ray signal. The scans were done weekly over 28 days (including time 0) using a GE Phoenix v|tome|x m tomographer (General Electric, Wunstorf, Germany). The system contains two X-ray sources. A 240 kV micro focus tube with tungsten target was employed. X-rays were produced with a voltage of 180 kV and a current of 150 µA. A 0.2 mm Cu filter was used to avoid beam hardening. The images were recorded by a GE DXR detector array with 2024 × 2024 pixels (pixel size 200µm). The detector was located 815 mm from the X-ray source. The columns were placed at a distance of 272.04 mm from the X-ray source allowing a spatial resolution of 66.67 µm. A full scan consisted of 1500 projections over 360°. The first image was skipped. The saved projection is the average of 3 images where every image was obtained over 250 ms exposure time. GE reconstruction software (Wunstorf, Germany) was used to calculate the 3D structure via back projection. The analysis of the 3D images using Avizo imaging software (version 9.2.0) allowed the creation of colour maps of the pore size.

Soil pore water extraction and leachate collection

Because centrifugal extraction did not yield enough soil pore water, soil pore water was extracted by saturating 20 g of wet soil sampled from the different depths, from columns treated for 28 days. After 24 hours of equilibration, water was centrifuged through glass wool at 2000 *g* for 35 min (Hermle Z400K, Germany). The collected water was filtered through a 0.45 µm cellulose acetate syringe filter (Chromafil, Macherey-Nagel, Germany). Glass wool and filters were conditioned by soaking them in a solution 0.1 M of $CuNO_3$ (99.9%, Sigma Aldrich) overnight before use, in order to avoid adsorption of Ag on the surface of the glass fibres and filters [23]. Water leachate of the columns was accumulated in a Petri dish at the bottom of the columns after 12, 19 and 21 days of exposure and stored in a -20°C freezer until chemical analysis.

Chemical analysis

Total Ag concentrations in soil, dry worm tissues, soil pore water and water leachates were measured using a Nexion 350D ICP-MS (Perkin-Elmer Inc., Waltham, MA) following microwave-assisted acid digestion in *aqua regia* (1:3 Nitric Acid- Hydrochloric Acid) using a MARS 5 microwave (CEM corporation, USA). An aliquot of each sample was weighed (~0.5 g of wet soil or worms) and placed in Teflon vessels with 6 mL of HCl 37 % (Merck, Darmstadt) and 2 mL of HNO_3 69 % (Merck, Darmstadt). A smaller volume of acids (3 mL HCl and 1 mL HNO_3) was used for the digestion of pore water and leachates (1 mL per sample). The calibration curve was prepared by diluting a 1000 mg L^{-1} Ag standard stock solution (Merck, Darmstadt) in acid matching matrix. Rhodium was used as an internal standard. The limit of detection (LOD) of silver (*m*/*z* 107) was 0.12 ug L^{-1} (expressed as the average of the Ag concentration in blank samples (n=10) plus three standard deviations)

whereas the limit of quantification (LOQ) was 0.14 μg L^{-1} (expressed as average of the Ag concentration in blank samples (n=10) plus ten standard deviations). The moisture content of soil samples where ARW was applied was determined by drying moist soil in the oven at 110±5 °C for 20 hours or until weight was constant. In the samples without the addition of ARW, the moisture content was assumed to be constant at 17.5 % dry weight soil, based on weekly weighing of the columns.

Quality control

For every batch of samples, analytical quality was assured by using blanks and an external standard of Ag obtaining an average recovery of 93±6%. Spiking tests of Ag_2S-NPs and Ag^+ (from $AgNO_3$) with the experimental soil showed an average recovery of 70±5% and 84±6%, respectively.

Calculation of the Ag dispersion rate due to earthworm bioturbation

The resulting Ag concentrations in the different soil layers, with and without earthworms and Ag_2S-NPs, were fitted by a bioturbation model. The model works in one dimension by dividing the soil into a number of layers L, each with a depth d_l (m) and Ag concentration $[Ag]_l$ (mg kg^{-1}). Ag concentrations are assumed constant within each layer and were calculated each (user-defined) model time step δt (s) by assuming that a certain depth of soil (and thus amount of Ag) was instantaneously mixed between any two neighbouring layers within this time step. A soil turnover rate $v_{l:l+1}$ (m s^{-1}) is defined such that the depth of soil that is mixed between layers l and $l+1$ each time step is given by $v_{l:l+1}\delta t$. The average depth that earthworms burrow to, h (i.e. the diffusion path length), can be used to relate the soil turnover

rate to the biodiffusion coefficient (m^2 s^{-1}) $D_{l:l+1}$ as $D_{l:l+1} = v_{l:l+1}h$ [24]. The so-called bioturbation rate k_{bioturb} (s^{-1}) is given by

$$k_{\text{bioturb},l:l+1} = \frac{v_{l:l+1}}{d_l} \tag{1}$$

and the Ag concentration of a given layer l at time $t + 1$ is calculated as

$$\begin{aligned}[\text{Ag}]_{l,t+1} = [\text{Ag}]_{l,t} + k_{\text{bioturb},l:l+1,t}\delta t\left([\text{Ag}]_{l+1,t} - [\text{Ag}]_{l,t}\right) \\ + k_{\text{bioturb},l-1:l,t}\delta t\left([\text{Ag}]_{l-1,t} - [\text{Ag}]_{l,t}\right)\end{aligned} \tag{2}$$

In Equation 2, the second term on the right-hand side represents Ag mixing from the layer below, whilst the third term represents Ag mixing from the layer above. Note that the bioturbation rate is also dependent on time as it is likely to be a function of time-dependent parameters such as the density of earthworms in a given soil layer.

In the following, we make the assumption that the soil turnover rate (and thus bioturbation rate) is directly proportional to the density of earthworms in a given layer w_l (m^{-3}) [24] such that

$$v_{l:l+1} = \beta w_l \,, \tag{3}$$

where β (m^4 s^{-1}) is a bioturbation fitting parameter. The soil profile is defined as having 6 layers of equal depth (2 cm) such that model layers 1 (the top-most layer), 4 and 6 correspond, respectively, to the top, middle and bottom soil layers in the experimental setup. A worm density of 9431 individuals/m^3 (based on 5 worms being added to a column with soil volume

of 530 cm^3) was used which corresponds to ~2500 individuals/m^2 assuming earthworms mainly populate the first 20 cm of the soil profile. The model was run with a daily time step. Model parameterisation provided a value for the bioturbation fitting parameter β by application of the Levenberg-Marquardt algorithm.

Results

Earthworm bioaccumulation

The actual Ag concentration of the contaminated soil, mimicking sludge, was measured to be 6.62±0.43 mg Ag kg^{-1} soil dry weight (average ± standard deviation, n=3) and the Ag background in clean soil was 0.03±0.01 mg Ag kg^{-1} soil dry weight (average ± standard deviation, n=6).

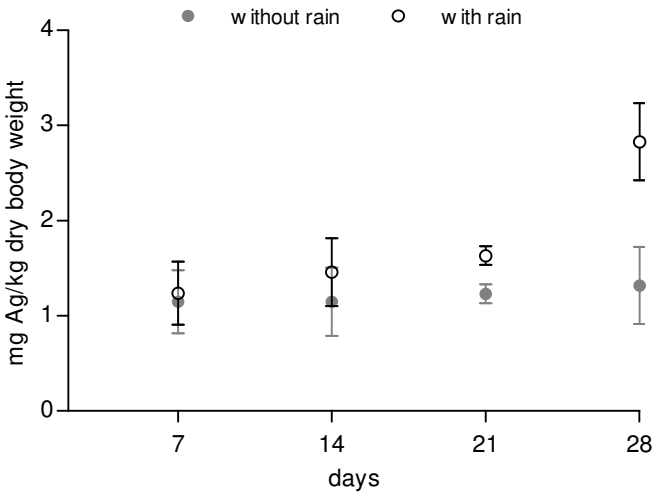


Figure 1. Time dependent concentrations (mg Ag kg^{-1} body weight, mean ± standard deviation, n=4) of total Ag in earthworms (*Lumbricus rubellus*) exposed to Ag_2S-NPs in the top 2 cm of soil columns with (O) and without (●) application of artificial rain.

After 28 days, earthworms accumulated significantly different Ag concentrations, up to 1.36±0.04 and 2.01±0.87 mg Ag kg^{-1} dry body weight in the experiments without and with ARW, respectively. The concentrations of Ag in the earthworms in the absence of rain did not change significantly over time (Figure 1, Table S3 – supplementary information).

In contrast, the Ag concentrations in the earthworms increased significantly over time when ARW was applied (Figure 1, Table S3 – supplementary information), resulting in a significant interaction between two factors, time and treatment (Table S4 – supplementary information).

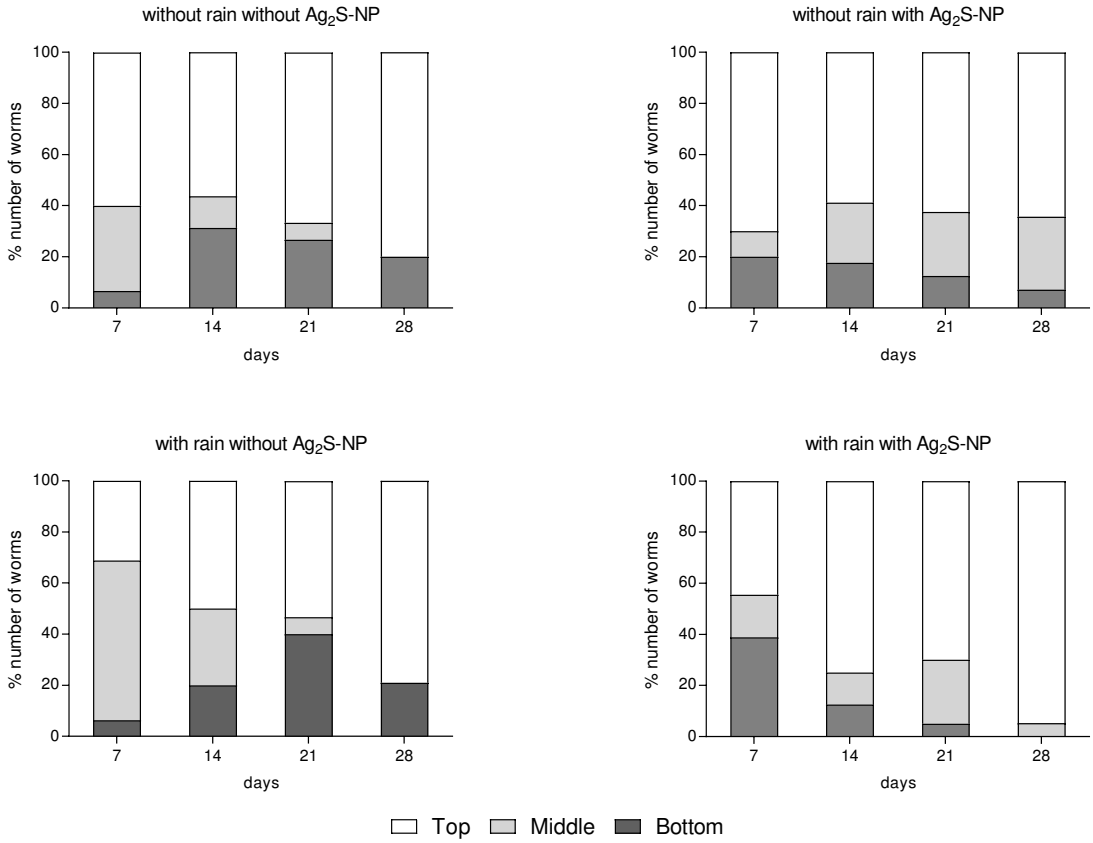


Figure 2. Depth distribution of earthworms in Kooijenburg soil with or without Ag_2S-NPs and with and without the application of rain at different time points. Columns were sampled at the three different depths (4 cm height).

The vertical distribution of the earthworms within the columns was recorded during sampling. The overall recovery of earthworms was 87% and 90% in the treatment without and with

application of ARW, respectively. Three 4 cm layers (top, middle and bottom) were considered. Earthworms were found throughout the soil columns although they seemed to prefer the top layer (Figure 2). Ag_2S-NPs did not affect the vertical distribution of the earthworms whereas the addition of ARW significantly increased the average number of earthworms in the top layer (Table S5 – supplementary information).

Burrowing behaviour

The effect of the presence of Ag_2S-NPs on the burrowing behaviour of earthworms was assessed by comparing the change of the macro porosity of the soil between the treatments with Ag_2S-NPs and introduction of earthworms. Effects on the macro porosity were calculated by changes in the absolute macro porosity [25] and in the distribution of pore sizes [26]. Figure 3 shows the size distribution of the pores (mm) after 28 days. The largest pores, diameter between 3.8 and 7.5 mm, represented approximately 16.3% of all pores in columns with both Ag_2S-NPs and worms, 10.8% in columns without Ag_2S-NP but with worms, and 0.8% in columns without Ag_2S-NPs and without worms. Pore size distributions of soil in columns with earthworms did not differ significantly between columns with Ag_2S-NPs and without Ag_2S-NPs at 28 days (Table S6 – supplementary information). Also, the change of absolute porosity with time was not significant between columns with and without Ag_2S-NPs in the presence of the worms (Tables S7A – supplementary information). Porosity and pore distribution were always significantly different from the columns without earthworms (Tables S7B and S7C – supplementary information). Changes of porosity between layers at day 7 and day 28 were compared amongst treatments showing no significant difference between the columns with and without Ag_2S-NPs (Figure 4, Table S8 – supplementary information). Figure 5 shows longitudinal profiles of three columns of the different treatments at day 28. The images

illustrate the presence of pores and their size is indicated by the colour scale. While control treatments without worms contained only small pores, both treatments including earthworms presented pores with sizes between 2 mm and 6 mm after 28 days. The profile and cross section maps of the other time points are shown in supplementary information (paragraph S9).

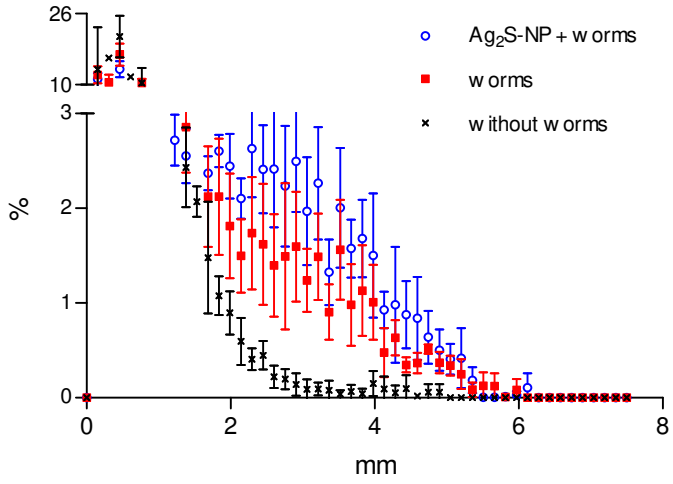


Figure 3. Pore size distributions of Kooijenburg soil in columns with Ag_2S-NPs and earthworms (*Lumbricus rubellus*), without Ag_2S-NPs and with earthworms and without earthworms or Ag_2S-NP after 28 days.

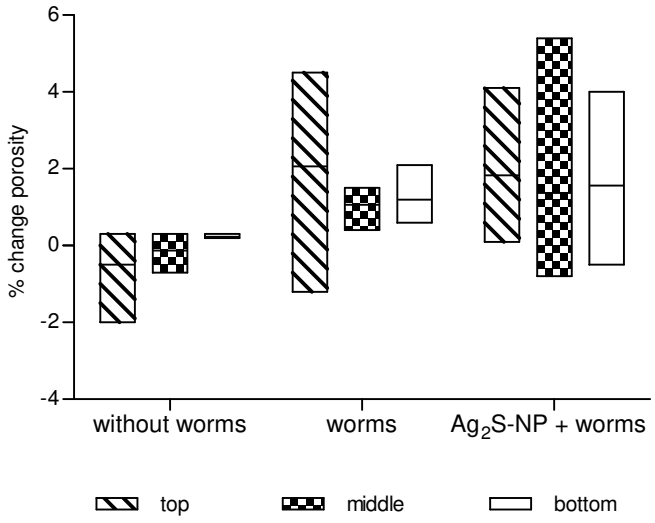
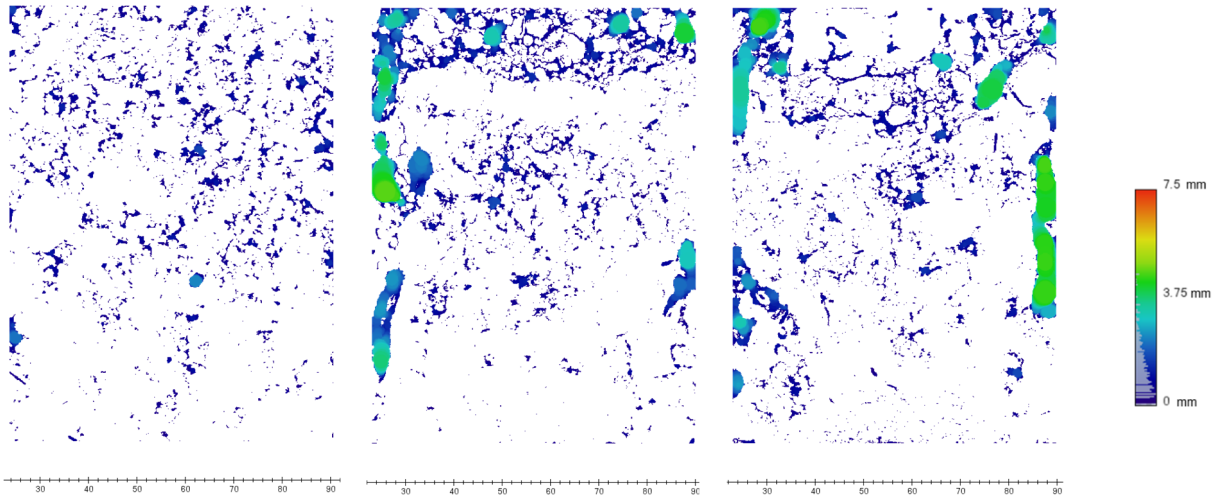


Figure 4. Change of porosity at three depths (top, middle, bottom) of Kooijenburg soil in columns with Ag_2S-NPs and earthworms (*Lumbricus rubellus*), without Ag_2S-NP and with earthworms and without earthworms between day 7 and 28.



without worms worms Ag_2S-NPs + worms

Figure 5. Colour maps of the pore size distribution in longitudinal profile of the Kooijenburg soil columns at the end of the incubation (28 days) with Ag_2S-NPs and worms, with and without earthworms.

Vertical transport of Ag in soil

Quantification of total Ag concentrations at the three depths in the soil columns allowed to calculate the time-dependent change in depth profiles of Ag_2S-NPs. Figure 6a illustrates the results of the experiments without the application of ARW. In the columns with earthworms, the Ag concentrations in middle and bottom layers was significantly higher than the background concentration in control soil after 7 days of incubation and increased with time (Table S10 – supplementary information).

In columns without worms, Ag concentrations in deeper soil layers were not different from background values in control soils indicating a limited vertical transport of Ag. Significant differences between treatments (with and without earthworms) were found for all the time points as Ag concentrations in middle and bottom layers increased with time (Tables S10 and S11 – supplementary information). Also with application of ARW, the activity of the

earthworms led to a time dependent vertical transport of Ag (Figure 6b) which did not occur in columns without the organisms (Table S10 – supplementary information). Differences between these treatments was significant after only 7 days. The ARW application played no significant effect in the vertical transport of Ag_2S-NPs in both cases with and without earthworms except at 21 days in the presence of earthworms (Tables S11 and S12 – supplementary information).

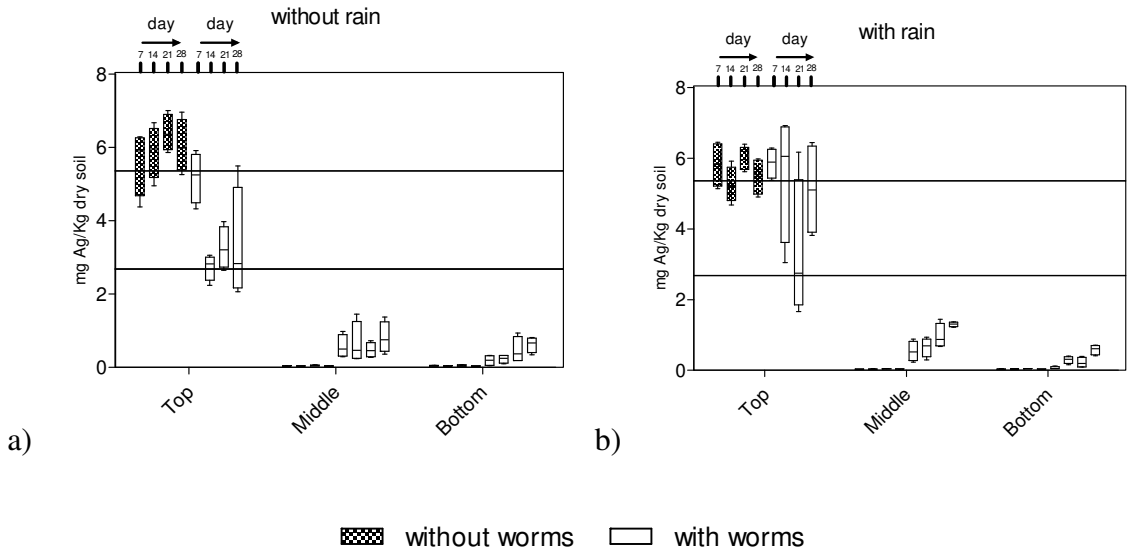


Figure 6. a) Ag concentrations at three depths of columns with Kooijenburg soil, with a top layer spiked with Ag_2S-NPs, with and without earthworms for the treatments without artificial rain water overtime, b) Ag concentrations at three depths of soil in columns with and without earthworms for the treatments with artificial rain water over time.

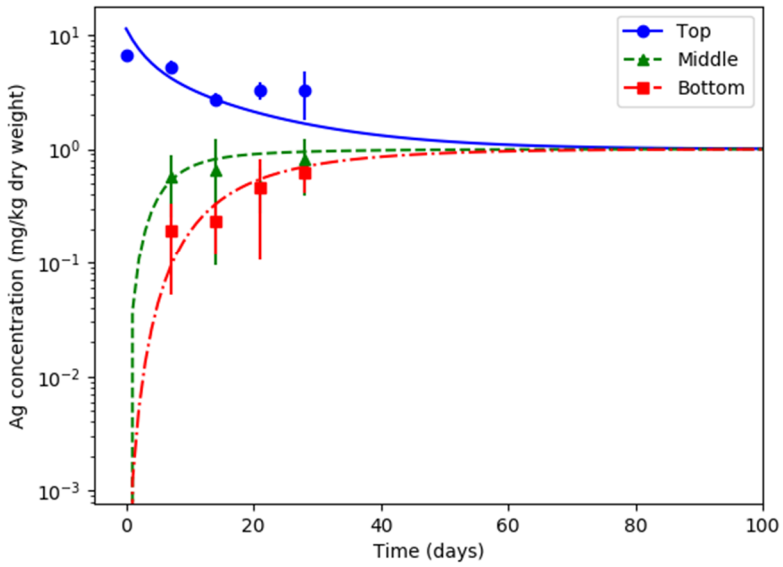
Soil pore water and leachates

Concentrations of Ag in soil pore water extracted from soil at three depths in the columns after 28 days were only quantifiable in the top soil of the columns with ARW but without earthworms (36.7±2.1 µg Ag L^{-1}, mean ± standard deviation, n=4).

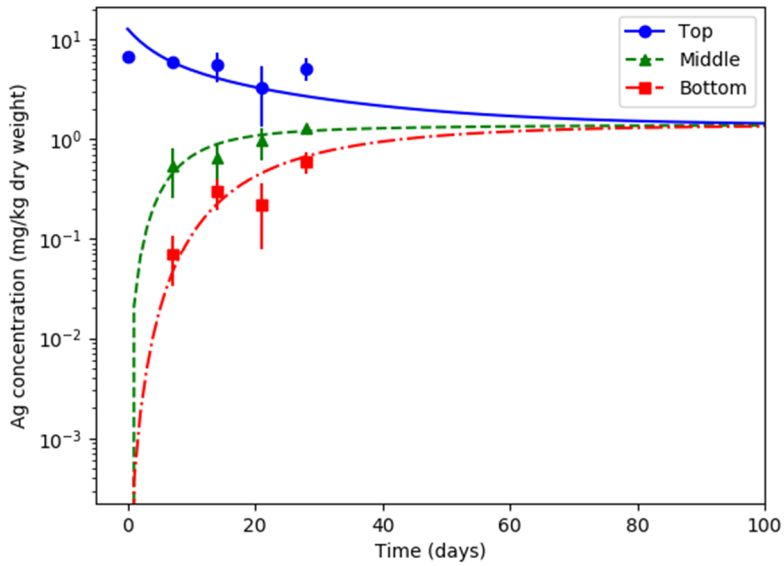
It was possible to collect volumes of percolated water at the bottom of all the columns after 12, 19 and 21 days. However, Ag concentrations in the leachates were below the limit of quantification in all the samples suggesting that transport of Ag_2S-NPs via percolating water through the soil is negligible relative to the displacement caused by earthworm bioturbation.

Bioturbation rate

The fits of the bioturbation model to the resultant Ag concentrations, with worms and Ag_2S-NPs, with and without ARW are shown in Figure 7. The log of concentrations was taken before fitting to provide better sensitivity to the lower concentrations in the deeper soil layers. The fit resulted in a bioturbation fitting parameters of $\beta = 4.80 \times 10^{-12} \pm 0.99^{-12}$ m^4 s^{-1} and $\beta = 3.56 \times 10^{-12} \pm 0.65^{-12}$ m^4 s^{-1} (value ± 95% confidence interval) for the treatments without and with rain, respectively. The corresponding soil turnover rate of $v = 0.39 \pm 0.04$ cm day^{-1} (Equation 3) for the treatments without rain yielded a bioturbation rate of $k_{\text{bioturb}} = 2.3 \times 10^{-6} \pm 0.26 \times 10^{-6}$ s^{-1}, while $v = 0.29 \pm 0.02$ cm day^{-1} resulted in $k_{\text{bioturb}} = 1.68 \times 10^{-6} \pm 0.14 \times 10^{-6}$ s^{-1} were calculated for the treatments with the application of rain (value ± 95% confidence interval). The model indicated that complete mixing – defined as concentrations in separate layers being within 0.01 mg kg^{-1} of each other – could (hypothetically) be reached after approximately 100 days in stable conditions and after 150 days when rain was applied.



a)



b)

Figure 7. Development over time of experimental Ag concentrations at three different depths in Kooijenburg soil in columns with Ag_2S-NPs spiked layer on top, for the treatments with earthworms (*L. rubellus*) and Ag_2S-NPs without (a) and with artificial rainwater (b), fitted by the bioturbation model. Concentrations are log-transformed to provide better sensitivity to lower concentrations in the deeper soil layers.

Discussion

Although only the top layer of the soil columns was treated, earthworms did accumulate Ag from Ag_2S-NPs. The uptake of Ag from this specific form of Ag-NPs was already studied in our previous work using the same soil [11] where *E. fetida* exposed to 3.7±1.1 mg Ag kg^{-1} accumulated up to 0.50±0.12 mg Ag kg^{-1} wet body weight after 28 days. This equates to ~3.1 mg Ag kg^{-1} dry body weight, assuming dry body weight = 16% wet body weight [27]. In that study the Ag_2S-NPs were homogeneously mixed with the soil and exposure concentration was about half of that in the current study. When using the modelling parameters from that study (uptake rate constant k_1 = 0.008 mg Ag kg dry soil mg Ag^{-1} kg^{-1} wet body weight day^{-1} and elimination rate constant k_2 = 0.064 day^{-1}) and applying the concentrations detected in the different soil layers, assuming that the earthworms spent on average approximately 60-75% in top soil depending on the application of ARW (derived from the depth distribution of earthworms within the columns, Figure 2) the modelled concentration in the worms at day 28 in the treatment without ARW is approximately 1.69 ± 0.19 mg Ag kg^{-1} dry weight. For the earthworms in the treatment with ARW the results tend to be slightly higher due to the fact that worms in this treatment occur somewhat more in the upper layer. The modelled concentrations vary a bit, which is depending on the timing of their occurrence in the different layers (averages and standard deviations based on 50 runs). The modelled concentrations are similar to the measured concentrations (Figure 1, 28 days), which would indicate that the uptake of Ag in the worms follows the kinetic rate constants as derived by chapter 2 [11], while differences between treatments are associated with differences in behaviour of the worms.

The differences between the treatments with and without ARW may be associated with the higher moisture content in the soil columns where rain was applied daily. Despite the open

bottom allowing the drainage of water, a moisture content of 50.5±4.8 % WHC, higher than the initial one (~40% WHC), was recorded at the bottom of the soil columns. Indeed, the data (Figure 2) suggest that worms preferred the top layer of the columns, which was drier than the bottom (-4% WHC from the moisture content of the bottom). Detailed data on moisture content at the three depths of soil columns of the treatment with the application of ARW are reported in the supplementary information (Figure S13 – supplementary information). Comparison between absolute macro porosity and size distributions also suggested that the earthworms did not avoid the contaminated soil as they altered the macro porosity of soil columns to a similar extent regardless of the presence of Ag_2S-NP at environmentally relevant concentrations (Figure 3, Figure 4 and Table S8). Earthworms had a large impact on the redistribution of the Ag_2S-NPs, moving approximately 9% of the Ag from top to bottom layer in 28 days. Other studies reported that earthworms are responsible of mobilisation of contaminants and that the involved mechanisms can be complex and metal-species-soil specific [28]. Earthworms can transport and increase the availability of metals [29], likely including metal NPs, by their feeding activity, i.e. by ingestion of soil and production of casts elsewhere with chemical, biological and physical properties differing from the surrounding soil [30, 31]. Additionally, earthworm burrows change soil structure and properties which in turn can affect the water flow through the soil. This and the increased aeration of the soil may increase the mobilisation of soluble contaminants [32]. In the present study, an average amount of daily rain (1.2 mm day^{-1}) did not significantly affect the transport of Ag_2S-NPs in unsaturated soil conditions, likely because of their low dissolvability and their rapid attachment to soil surfaces and/or air/water interfaces [33]. However, the use of sandy loam soil may have influenced the results as this kind of soil does not tend to form preferential flow paths. Whether the amount and intensity of the rainfall are critical is debated. Makselon et al. [34] reported an enhanced Ag-NPs transport when rain events were more frequent and more

intense and ascribed this phenomenon to high pore water flow velocities and/or the mobilisation of Ag-NP-soil colloids associations. However, Löv et al. reported very little effect of very high rain intensities on colloid mobilisation with in intact cores [35]. In absence of worms, rainfall resulted in increased pore water Ag concentrations, potentially related to the higher dissolution of the Ag_2S-NPs or increased detachment of the NPs from the soil following a decrease in ionic strength. In the presence of worms, this increase in soil pore water was not obvious, possibly due to increased vertical transport, diluting the relatively low soil pore water concentrations below LOD. Nevertheless, these results indicate a complex interaction between soil pore water kinetics and earthworm activity in affecting the environmental fate of metal NPs.

The present study also shows that bio-mediated transport of Ag_2S-NPs may exceed physical chemical transport in soils. Bioturbation therefore has to be considered when discussing NP bioavailability because a higher mixing rate implies a lower local NP concentration in the different strata.

In order to predict the bioturbation rate of Ag_2S-NPs due to earthworm activity, the experimental data related to the treatment without rain were fitted using the previously described bioturbation model, yielding a bioturbation rate of $k_{\mathrm{bioturb}} = 2.3 \times 10^{-6} \pm 0.26 \times 10^{-6}$ s^{-1} across the soil column for the experiment with controlled conditions and $k_{\mathrm{bioturb}} = 1.68 \times 10^{-6} \pm 0.14 \times 10^{-6}$ s^{-1} for the experiment with the rainfall. Complete mixing of the soil column due to bioturbation was predicted to occur within 100-150 days. Treating this dispersion rate as directly proportional to earthworm density resulted in a significant fit of the experimental data (Figure 7).

Apart from quantifying the rate at which bioturbation proceeds, validating the model against experimental data is of relevance for predictive models of nanomaterial fate, on which bioturbation may have a large impact. The difficultly in sourcing data for such models makes

the simple linear relationship between bioturbation rate and earthworm density, presented here, highly attractive. Indeed, spatially resolved earthworm density data for the EU already exist [22], and the dependence of earthworm density on land-use and land-management has been quantified [36]. Nevertheless, the linear relationship between bioturbation rate and earthworm density may have limitations. Earthworm burrowing activity likely reaches an upper limit at higher densities, when earthworms may affect each other's mobility. Additionally, the model does not consider the potential changes of burrowing activity due to the presence of other earthworm species in field conditions [37]. The extrapolation of our columns data may also lead to some overestimation due to the high earthworm density and to the fact that worms can enter diapause and/or quiescence under specific environmental conditions and be less active [38, 39]. However, in the realistic case in which Ag_2S-NPs are present in biosolids, the higher organic matter content of the sludge could lead to a higher availability of nutrients and to a higher density of earthworms. High organic matter is also shown to decrease the transport of Ag-NPs due to rain along soil columns, resulting in lower Ag concentration in the effluent water [40].

Finally, the degree of impact of earthworm bioturbation on the transport of Ag already seen in this short-term study requires including such process when studying and quantifying the fate of metal NPs in the soil compartment. The incorporation of the biological mixing into the framework of a physical transport model is expected to be even more important to reproduce long term redistribution as shown by Jarvis and his group concerning ^{137}Cs [41].

Conclusions

The present study provides evidence that earthworm bioturbation plays an important role in the vertical transport of Ag_2S-NPs in soil. Rainfall did not lead to displacement of Ag_2S-NPs

indicating that in the case of hardly dissolvable metal NPs and unsaturated soil conditions, bio-mediated transport overcomes physical chemical transport. Earthworm bioturbation was quantified by assessing the changes of the macro porosity in the soil columns. Results indicated that earthworms burrowing activity was not affected by the presence of Ag_2S-NPs at the experimental concentrations.

Whilst the relatively short term of the experiment and the high density of earthworms, we proposed a linear relationship between bioturbation rate and the abundance of earthworms that is applicable to future bioturbation studies.

In overall the present study has demonstrated the importance of taking into account the bioturbation (animal burrowing and floralturbation) while studying the fate of NPs in the soil.

Acknowledgements

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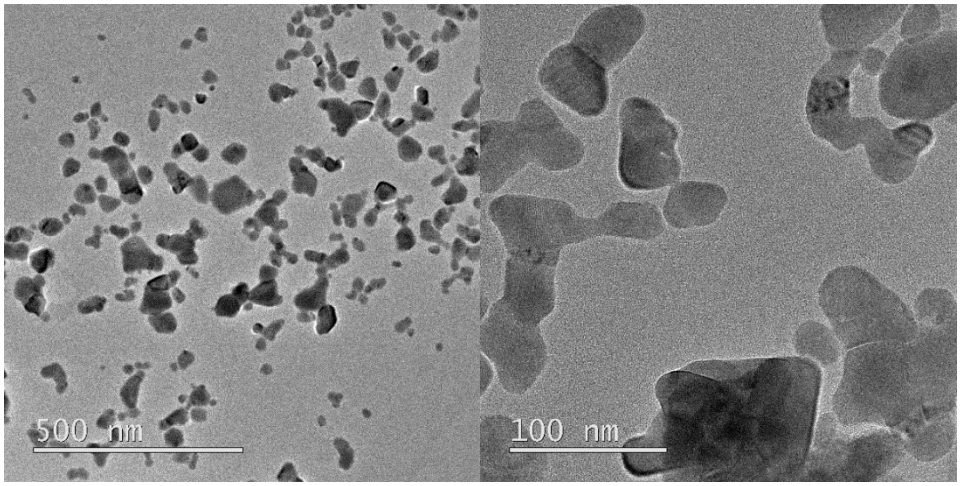
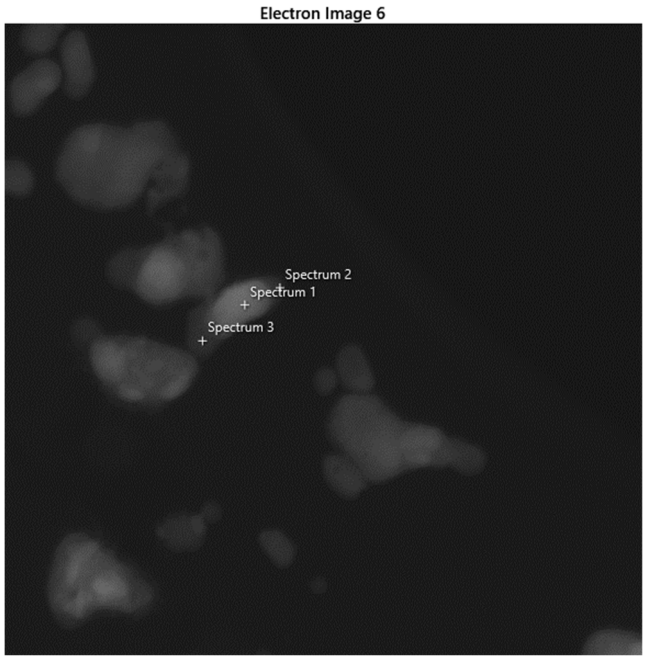
Supplementary Information

Paragraph S1

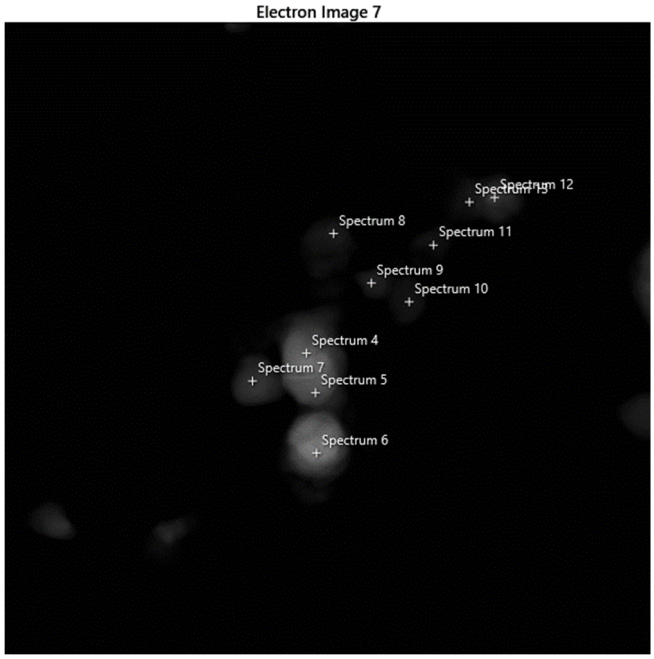
Bright field TEM pictures of the Ag_2S-NPs in stock solution.

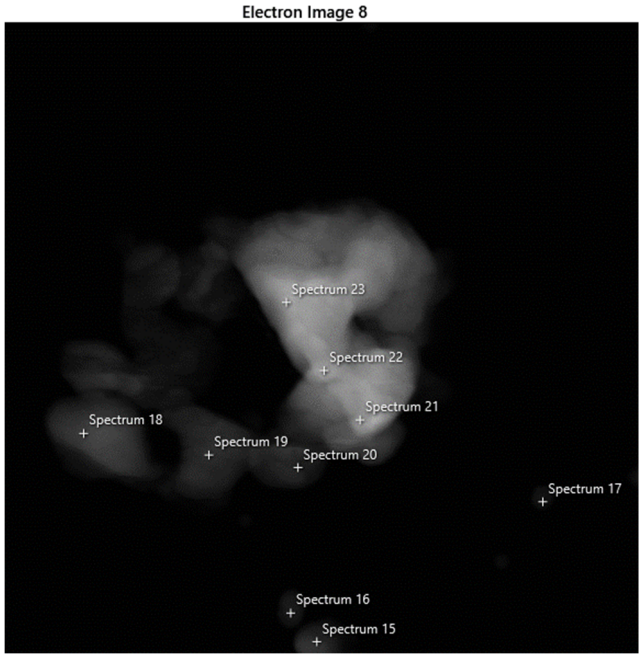


STEM/EDX (scanning transmission electron microscope/energy dispersive X-ray) pictures of the Ag_2S-NPs in stock solution and relative Ag and S atomic %.



Spectrum Label	S	Ag	Ag/S ratio
Spectrum 1	0	4.52	no sulphur signal, Ag only
Spectrum 2	0.47	2.72	5.8
Spectrum 3	0.81	4.33	5.3





Spectrum Label	S	Ag	Ag/S ratio
Spectrum 4	9.81	28.54	2.9
Spectrum 5	12.09	16.69	1.4
Spectrum 6	3.34	30.11	9
Spectrum 7	9.63	22.66	2.4
Spectrum 8	6.79	24.53	3.6
Spectrum 9	5.54	14.38	2.6
Spectrum 10	5.65	14.92	2.6
Spectrum 11	6.61	13.28	2
Spectrum 12	6.24	18.07	2.9
Spectrum 13	3.71	9.56	2.6
Spectrum 15	5.57	19.79	3.6
Spectrum 16	7.89	18.14	2.3
Spectrum 17	1.84	17.14	9.3
Spectrum 18	9.17	18.66	2
Spectrum 19	4.92	14.44	2.9
Spectrum 20	5.22	17.42	3.3
Spectrum 21	6.27	18.13	2.9
Spectrum 22	2.79	21.21	7.6
Spectrum 23	0.83	22.73	27.4

Table S1. Exchangeable base concentrations in the Kooijenburg soil used for the earthworm bioturbation experiment with Ag_2S-NPs.

Ammonium acetate extractable concentrations	
Mg 285.2	9.1 mg kg^{-1}
Ca 317.93	97.3 mg kg^{-1}
Mn 257.61	1.4 mg kg^{-1}
Na 589.6	2.2 mg kg^{-1}
K 766.5	16.0 mg kg^{-1}

Table S2. Phosphorus, manganese, aluminium and iron concentrations in the Kooijenburg soil used for the earthworm bioturbation experiment with Ag_2S-NPs.

Ammonium oxalate extraction	
P 213.6	777 mg kg^{-1}
Mn 257.6	142 mg kg^{-1}
Fe 259.9	3049 mg kg^{-1}
Al 308.2	3005 mg kg^{-1}

Table S3

Post hoc Tukey multiple comparison test between total Ag concentrations in earthworms exposed to Ag_2S-NPs in Kooijenburg soil treatments without rain and with rain at different time points following one way ANOVA (F (5, 18) = 19.26)). Positive confidence interval indicates that concentrations are higher in first factor, and vice versa.

Treatment without artificial rain			
	Mean Diff.	95% CI of diff	P value
7 days vs 14 days	0.0002433	-0.7164 to 0.7169	>0.9999
7 days vs 21 days	-0.08338	-0.7469 to 0.5801	0.9796
7 days vs 28 days	-0.1711	-0.8878 to 0.5456	0.8827
14 days vs 21 days	-0.08362	-0.8003 to 0.6331	0.9835
14 days vs 28 days	-0.1714	-0.9375 to 0.5948	0.9008
21 days vs 28 days	-0.08774	-0.8044 to 0.6289	0.9811

Treatment with artificial rain			
	Mean Diff.	95% CI of diff	P value
7 days vs 14 days	-0.2201	-1.3160 to 0.8760	0.913
7 days vs 21 days	-0.3943	-1.4900 to 0.7018	0.7145
7 days vs 28 days	-1.591	-2.6870 to -0.4950	0.0048
14 days vs 21 days	-0.1742	-1.2700 to 0.9218	0.9638
14 days vs 28 days	-1.371	-2.4670 to -0.2749	0.0136
21 days vs 28 days	-1.197	-2.2930 to -0.1007	0.0311

Table S4

Two way ANOVA test between total Ag concentrations in earthworms exposed to Ag_2S-NPs in Kooijenburg soil in treatments without rain and with rain at different time points together.

Source of variation	F	P value
Treatment	12.91 (1, 22)	0.0016
Time	5.77 (3, 22)	0.0046
Interaction	3.74 (3, 22)	0.0261

Table S5

Three way ANOVA between percentage of the position of earthworms at three depths in Kooijenburg soil columns with and without application of artificial rain and the presence and absence of Ag_2S-NPs over time.

Source of variation	df	Mean square	F	P value
Layer * Ag_2S-NPs * time	6	0.840	0.103	0.995
Layer * Ag_2S-NPs * rain	2	4.771	0.638	0.534
Layer * rain * time	6	20.951	5.466	0.001

Table S6

Post hoc Tukey multiple comparison test between pore size distributions (expressed as number of pixels) in Kooijenburg soil in columns with Ag_2S-NPs and earthworms, with earthworms and without earthworms following one way ANOVA (F (2, 49) = 38.15). Positive confidence intervals indicate that concentrations are higher in first factor, and vice versa.

	Mean Diff.	95% CI of diff	P value
Ag_2S-NP + worm vs worm	25447	-250429 to 301323	0.9741
Ag_2S-NP + worm vs control	372289	96413 to 648165	0.0048
worm vs control	346842	70966 to 622718	0.0095

Table S7A

Multiple regression analysis of changes in porosity of Kooijenburg soil between treatments with earthworms and with earthworms in presence of Ag_2S-NPs over time (Adjusted R^2= 0.26, F(2, 30)=10.54 p value<0.01).

	Coefficients	Standard Error	P value
Intercept	1.039	0.035	<0.001
Time	0.008	0.002	<0.001
Treatment	-0.039	0.035	0.283

Table S7B

Multiple regression analysis of changes in porosity of Kooijenburg soil between treatments without earthworms and with earthworms over time (Adjusted R^2= 0.71, F(2, 30)=14.08, p value<0.01).

	Coefficients	Standard Error	P value
Intercept	0.975	0.026	<0.001
Time	0.004	0.001	0.002
Treatment	0.111	0.026	<0.001

Table S7C

Multiple regression analysis of changes in porosity of Kooijenburg soil between treatments without earthworms and with earthworms and Ag_2S-NP over time (Adjusted R^2= 0.26, F(2, 30)=6.05, p value<0.01).

	Coefficients	Standard Error	P value
Intercept	0.975	0.033	<0.001
Time	0.004	0.002	0.011
Treatment	0.036	0.017	0.039

Table S8

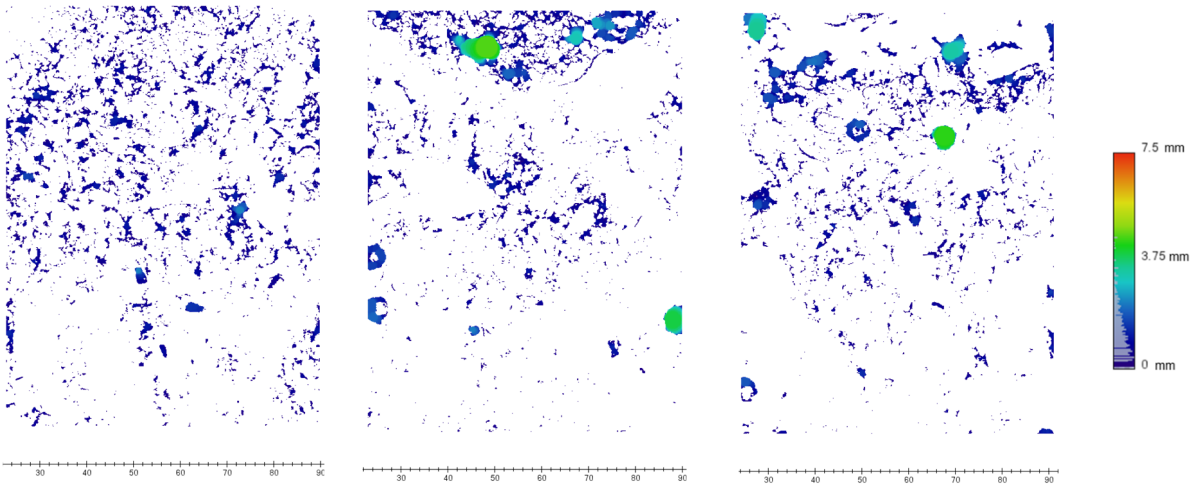
Two way ANOVA between changes of porosity of Kooijenburg soil (between day 7 and day 28) at three depths amongst treatments with and without Ag_2S-NPs and with and without earthworms.

	Interaction		layers		treatment	
	F	p value	F	p value	F	p value
Worm vs control	0.58	0.5744	0.09	0.9175	5.81	0.0329
Ag_2S-NP + worm vs control	0.11	0.8970	0.03	0.9658	3.40	0.0900
Worm vs Ag_2S-NP + worm	0.03	0.9694	0.17	0.8453	0.02	0.8954

Paragraph S9

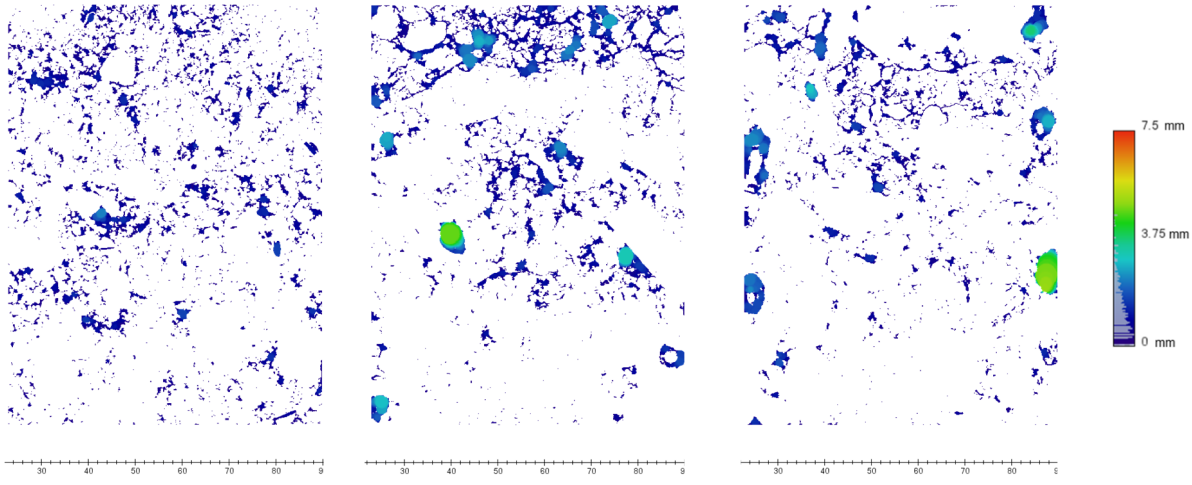
Colour maps of the pore size distribution in longitudinal profile of one soil column of the three treatments (with and without earthworms, with earthworms and Ag_2S-NPs) at day 7, 14 and 21.

Time 7 day



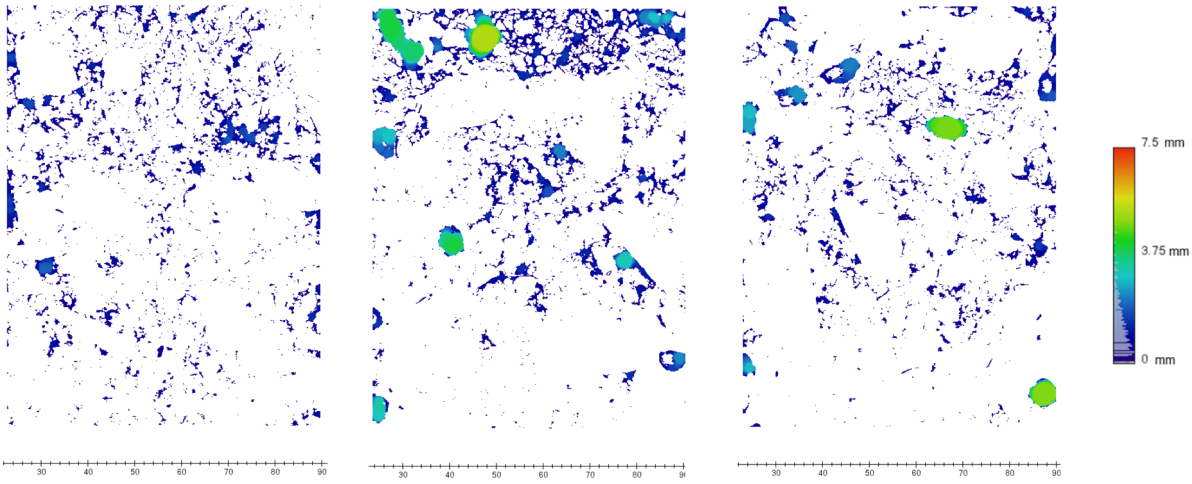
without worms | worms | Ag_2S-NPs + worms

Time 14 days



without worms | worms | Ag_2S-NPs + worms

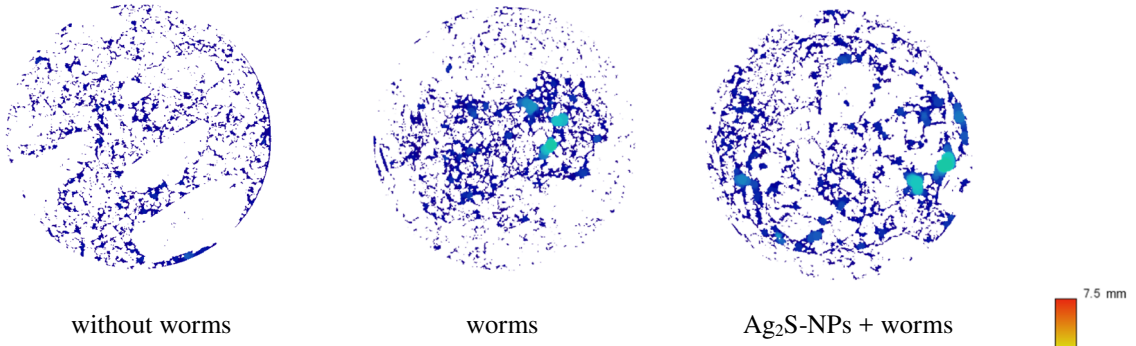
Time 21 day



without worms worms Ag_2S-NPs + worms

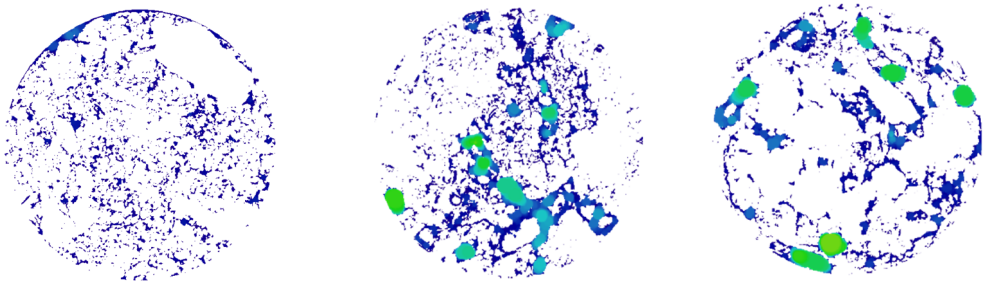
Colour maps of the pore size distribution in cross sections of one soil column of the three treatments (with and without earthworms, with earthworms and Ag_2S-NPs) at days 0, 7, 14, 21 and 28.

Time 0 day



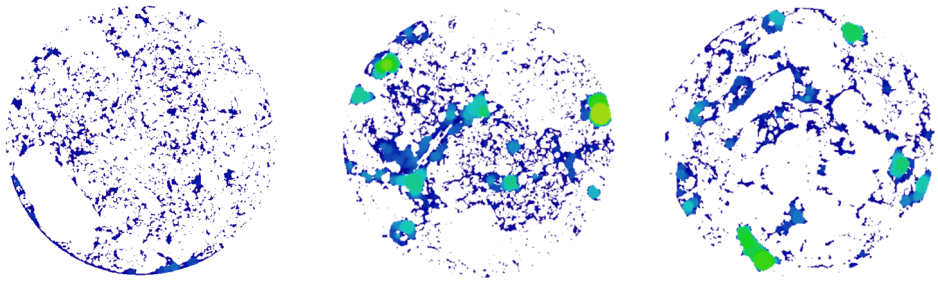
without worms worms Ag_2S-NPs + worms

Time 7 day



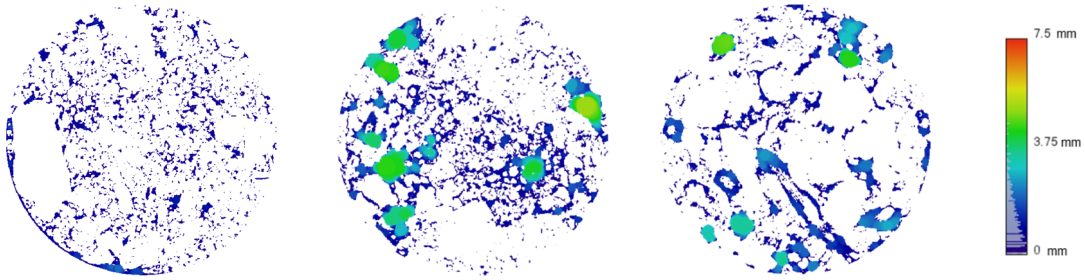
without worms worms Ag_2S-NPs + worms

Time 14 days



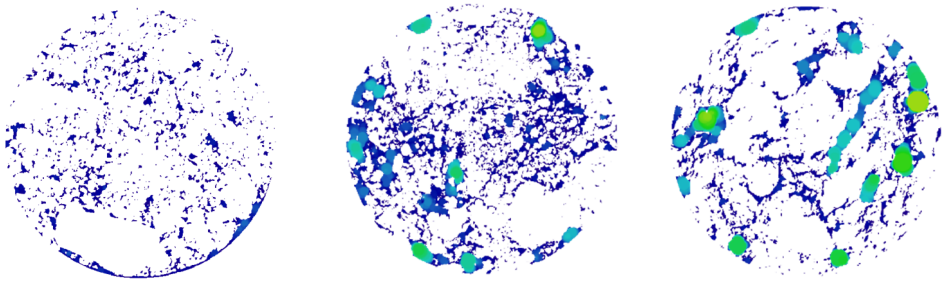
without worms worms Ag_2S-NPs + worms

Time 21 day



without worms worms Ag_2S-NPs + worms

Time 28 day



without worms worms Ag_2S-NPs + worms

Table S10

Two-way ANOVA between Ag soil concentrations in the middle and bottom depths of Kooijenburg soil columns with and without earthworms (2 layers x 2 treatments x 4 replicates). When the interaction leads to significant p value, “presence of worms” and “layer” factor are not reported.

Treatment without artificial rain			
	Interaction	Presence of worms	layers
7 days without earthworms vs 7 days with earthworms	0.046	-	-
14 days without earthworms vs 14 days with earthworms	0.1605	0.0156	0.1644
21 days without earthworms vs 21 days with earthworms	0.9494	0.0015	0.9579
28 days without earthworms vs 28 days with earthworms	0.4541	<0.0001	0.4408
Treatment with artificial rain			
	Interaction	Presence of worms	layers
7 days without earthworms vs 7 days with earthworms	0.0060	-	-
14 days without earthworms vs 14 days with earthworms	0.0296	-	-
21 days without earthworms vs 21 days with earthworms	0.0018	-	-
28 days without earthworms vs 28 days with earthworms	<0.0001	-	-

Table S11

Two-way ANOVA between Ag soil concentrations in the middle and bottom depths of Kooijenburg soil columns with and without earthworms (2 layers x 2 treatments x 4 replicates).

Treatment without artificial rain			
	Mean square	F value	P value
Without earthworms	0.1398	0.822	0.5590
With earthworms	1.774	5.194	0.0006
Treatment with artificial rain			
	Mean square	F value	P value
Without earthworms	0.1431	1.667	0.1575
With earthworms	2.069	2.642	0.0316

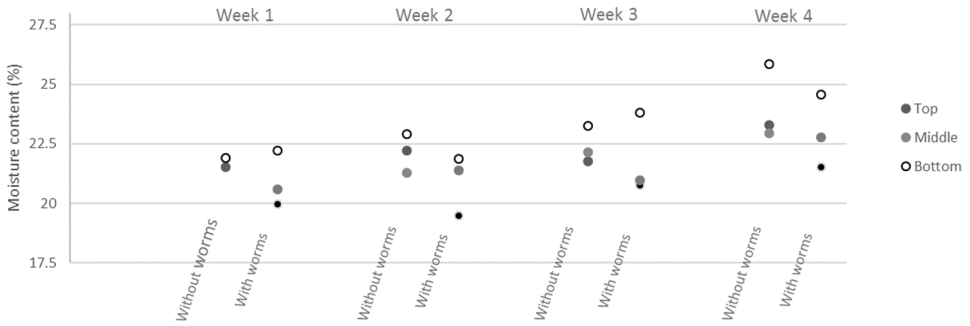
Table S12

Two-way ANOVA between Ag soil concentrations in the middle and bottom depths of Kooijenburg soil columns with and without the application of ARW over time.

Treatment without earthworms			
	Interaction	Presence of ARW	layers
7 days without ARW vs 7 days with ARW	0.8330	0.0960	0.3854
14 days without ARW vs 14 days with ARW	0.4890	0.2440	0.7973
21 days without ARW vs 21 days with ARW	0.9900	0.3259	0.8505
28 days without ARW vs 28 days with ARW	0.8789	0.6915	0.2841
Treatment with earthworms			
	Interaction	Presence of ARW	layers
7 days without ARW vs 7 days with ARW	0.6869	0.4997	0.0025
14 days without ARW vs 14 days with ARW	0.8379	0.8384	0.0304
21 days without ARW vs 21 days with ARW	0.0217	0.3964	0.0184
28 days without ARW vs 28 days with ARW	0.0569	0.0954	0.0037

Figure S13

Moisture content at three depths in the Kooijenburg soil columns of the treatment with Ag_2S-NPs and with the application of ARW.



Chapter 6

General discussion

Discussion

The increasing application of metal nanomaterials (NMs) in everyday goods will result in their release into the environment, which raises concerns regarding their potential harm for the organisms present. More than a decade of studies brought to the awareness that the environmental risk assessment paradigm used for conventional chemicals may be applicable to NMs but there are some essential aspects of the current approach that still face big challenges. The inadequacy of the current paradigm mainly relates to the fact that NMs are present in a wide variety of forms, resulting in very different physicochemical properties (e.g. size, shape, charge, etc.). Additionally, the numerous transformations which NMs can undergo after their release in the different environmental compartments lead to uncertainty regarding the actual forms of the NMs to which the organisms are exposed. Transformed NM forms (aged NMs) can have a completely different identity and therefore different bioavailability and toxicity when compared to the original NMs. Identifying and assessing the influence of the major transformations of NMs in the environment on their uptake by biota is essential in order to be able to distinguish, quantify and model the form specific exposure, e.g. as particles and/or as ions released from the particles of pristine and aged metal NPs over time. This information is crucial to understand if there is need of NM-specific environmental risk assessment or whether the conventional regulations and methods regarding bulk materials can be safely applied to NMs. Additionally, knowledge on the form and amount of the actual exposure to NMs is needed at environmentally realistic concentrations and exposure time because of the non-persistent nature of NPs.

The research about the ecotoxicology of NMs performed until now used pristine NMs, as manufactured, at rather high concentrations and did not take into account their environmental

transformations. This produced an extensive amount of data that are often not applicable to realistic environmental scenarios. NanoFASE, the overarching EU project of which this thesis is part, therefore set out to investigate the environmental transformations of NMs, their fate in the environment and the final uptake in relevant organisms.

In light of these current needs, the present thesis aimed to contribute to the exposure assessment of pristine and aged NMs in soil organisms under environmentally relevant conditions. This has been performed by the quantification and the modelling of the toxicokinetics (uptake, metabolism and excretion) of pristine and aged NMs in soil invertebrates exposed to environmentally relevant concentrations after short and prolonged time of exposure. The thesis also aimed to evaluate the effect of soil organisms on the fate of aged NMs in the soil under environmentally relevant conditions.

For metal NMs it has been reported that dissolution can be one of the most relevant transformations which affects their uptake and that toxicity can be mainly driven by the released ions [1, 2]. However, no accepted evidences have been provided that NMs are actually not taken up as the original NM, because the performed studies based all the analysis (in the exposure media and in the organism) of the NM on total concentrations (particles and ions) [3-6]. A single study showed particulate Ag in earthworms exposed to Ag-NMs [7], although there was no confirmation that the internalised NMs were actually the same as the ones the worms were exposed to, while also particulate Ag was detected in worms exposed to ionic Ag (from $AgNO_3$).

In the present thesis silver nanoparticles (Ag-NPs) have been used as model for NMs because they are widely used in the production of goods. Ag-NPs referred to the pristine form of Ag-NPs (as manufactured) which are known to dissolve [8]. The main environmentally relevant form of Ag-NPs is the sulfidized form (Ag_2S-NP), which is formed in waste water treatment

plants (WWTP) and retained in the sludge. In many European countries such sludge, potentially containing Ag_2S-NPs, is spread on agriculture fields where Ag_2S-NPs can interact with the soil biota. Ag_2S-NPs are known to have a relatively low dissolvability which, together with the dissolvable pristine form (Ag-NPs), represent the perfect contrasting models of different particulate forms of the same metal, to study the relations between dissolution of nanomaterials in soil and their uptake in soil organisms over time. Additionally, low natural background of Ag and available analytical techniques allow to measure different forms of Ag at environmentally relevant concentrations, also in complex matrices.

The results obtained in the present thesis contribute to the exposure assessment of NMs but in the same time also raise issues to be taken into account for future research. The next paragraphs provide the discussion of the main findings and their future implications.

Quantification and comparison of the Ag uptake of different Ag forms (Ag-NP, Ag_2S-NP and Ag^+) over time

In both **chapter 2** and **chapter 4**, *Eisenia fetida* were exposed to Ag-NP, Ag_2S-NP and Ag ions in natural soil for 28 and 270 days, respectively. In **chapter 2**, results, based on the total Ag (particulate and ionic Ag), showed accumulation in the earthworms for both Ag-NPs and $AgNO_3$ in the same order of magnitude as reported in previous other studies [4, 9, 10]. However, Makama et al. [7] reported twenty times lower Ag accumulation in *Lumbricus rubellus* exposed to 250 mg Ag-NP kg^{-1} dry weight soil for 28 days. In **chapter 2**, comparison between accumulation of total Ag (particulate and ionic Ag) from Ag-NP and $AgNO_3$ over time highlighted no difference between the uptake patterns after 28 days, indicating that in the Ag-NP exposure, Ag was as bioavailable as Ag ions in the $AgNO_3$ exposure. This was in line with

results from Laycock et al. [11] who exposed earthworms to ZnO nanoparticles and Zn ions and traced the uptake using stable isotope labelling. They demonstrated that the uptake in *L. rubellus* of the two different Zn forms were undistinguishable after 72 hours and suggested that Zn-NPs dissolved and became bioavailable for the earthworms similarly to the ions in the ionic exposure. However, Diez Ortiz et al. [4] reported a higher uptake of Ag in the ionic exposure than the Ag-NP exposure in the same earthworm species after 168 hours. Both studies used LUFA 2.2. soil with moisture content equal to 45-50% water holding capacity, hence the differences in bioavailability between Ag-NPs and ZnO-NPs were not caused by differences in soil properties. A plausible alternative explanation for the differences in uptake patterns may be that ZnO-NPs dissolved in the soil solution at a higher rate than Ag-NPs which makes Zn bioavailable faster than Ag for the earthworm [12-14]. This may also apply to different forms of Ag-NMs, since in case of hardly dissolvable Ag_2S-NPs in this thesis the bioaccumulation was around twenty times lower.

To understand if potential belated dissolution of Ag-NPs and Ag_2S-NPs could play a role in the long-term accumulation in the earthworms during an environmentally relevant period of time [15], earthworms *E. fetida* were exposed to Ag-NP, Ag_2S-NP and Ag ions in the same natural soil used in **chapter 2** but the experiment was conducted for 270 days (**chapter 4**). The uptake patterns among the organisms exposed to pristine Ag-NPs and ionic Ag remained similar after nine months of exposure. Interestingly, Ag body concentrations increased after the 28^{th} day of exposure, indicating that no steady state was reached in the short-term experiment. This showed that both forms of Ag were still bioavailable. In case of Ag_2S-NPs in the long-term experiment, the uptake was again significantly lower than its pristine form. However, the Ag accumulation from Ag_2S continued over time, indicating that Ag from Ag_2S-NPs also remained bioavailable over time.

In **chapter 5**, earthworms were exposed to Ag_2S-NP (10 mg Ag kg^{-1} dry weight soil) just applied in the upper 2 cm of the soil column in presence and in absence of daily application of artificial rain water for 28 days. In both treatments, earthworm *L. rubellus* accumulated higher amount of Ag compared to the short-term experiment (**chapter 2**) and long-term experiment (**chapter 4**) in which exposure was distributed evenly over the soil compartment, and in particular four times and two times higher in the experiment in presence and in absence of rain, respectively. Together with the results of **chapter 2 and 4**, this study showed that the bioavailability of Ag from Ag_2S-NPs is dependent on the experimental conditions.

Finally, we demonstrated that there is no difference between pristine Ag-NPs and Ag ions uptake in both short- and long-term exposure scenarios. Furthermore, we showed that accumulation from Ag_2NPs is different from that from pristine Ag-NPs which illustrates the need to test environmentally relevant forms of NMs in ecotoxicological testing. Additionally, we found that although Ag_2S-NPs were taken up to a lower extent than Ag-NPs even in the long term study, their accumulation curve did not reach a steady-state suggesting that Ag_2S-NPs continued to be bioavailable also after nine months, potentially related to belated dissolution. A closer analysis of the time dependent bioavailability of different forms of NPs is crucial to explain such results.

Bioavailability of Ag in the soil

There is no consensus how to measure bioavailability of metal NPs in soil. In general, salinity, texture, pH, concentration, nature of mobile organic compounds and degree of saturation determine NP bioavailability in the soil [16].

Bioavailability is not only related to the NP form and to the soil characteristics but it is also closely connected with the feeding behaviour and the physiology of each soil organism species [17]. Earthworms ingest soil and soil solute and, being soft-bodied invertebrates, absorb soil solute dermally. However uncertainties remain regarding the main uptake route (dietary and/or dermal) for both metal salts and metal NPs [11, 18]. Some studies [19, 20] brought evidences that toxicity is related to the concentration of the metal NPs in the soil pore water which would suggest that such concentrations could represent a surrogate of the bioavailable fraction of NPs for soil organisms [21]. In light of this assumption, in **chapter 2 and 4**, the concentrations of Ag were analysed in the soil pore water of soil spiked with Ag-NPs, Ag_2S-NPs and Ag ions. In **chapter 2** (short-term exposure), the total Ag concentration in soil pore water in the soil spiked with pristine Ag-NPs and Ag ions were not statistically different. This suggested that Ag-NP release ionic Ag similarly to $AgNO_3$, leading to a similar exposure scenario over 28 days. In case of hardly dissolvable Ag_2S-NP, the Ag concentrations in soil pore water were below the detection limit indicating a limited release of ionic Ag in this short-term experiment. However, concentrations of Ag in pore water in the long-term experiment (**chapter 4**) showed no differences amongst treatments over time. Based on these results and other studies [22-24], we propose that in case of pristine NPs a fast dissolution took place in the initial phase of exposure (from hours to days). Then, the process is slowed by aging of the NP surface in the soil and soil pore water Ag concentrations can decrease by adsorption and complexation of ions to soil particles [16, 23, 25]. For Ag_2S-NP, the surface is already sulfidized therefore the dissolution rate is slower and constant. These newly released ions are relatively available to the organisms, hence. Although the overall concentrations of released ions may be lower, this release continues longer over time and their bioavailability may also be prolonged in time. The underlying assumption that the Ag in the soil pore water is in the form of ions is corroborated by the fact

that the analyses of Ag in pore water by spICP-MS, which identified particulate Ag in pore water to be less than 0.5% of the total Ag amount (for Ag-NP and ions).

Bioavailability appeared to be governed by fluxes of released and re-adsorbed Ag ions in the soil solution which cannot be fully described by the total (ions and particulate) Ag concentrations in the pore water at defined moments only. The rates of release and re-adsorption are dependent on Ag form, type of soil and its conditions (e.g. moisture and organic matter content) which are likely not to be static. In the long term study (**chapter 4**), the regular addition of water could have affected bioavailability of NP/ions and therefore explain the differences between the Ag concentrations in the soil pore water at day 28 in the experiment with Ag_2S-NPs in **chapter 2** (< limit of detection, stable conditions) and the ones in the long-term study (between 24 and 68 µg Ag L^{-1}) (**chapter 4**). However, in **chapter 5**, although the Ag concentration in soil pore water resulted to be below the detection limit, the Ag uptake in the earthworms exposed to Ag_2S-NPs was higher in the presence of daily rain application as compared to the ones exposed without this daily rain application.

Soil as NP reactor: environmental transformations driving the uptake of metal NPs

In **chapter 2**, the accumulation data for dissolvable Ag-NP, not dissolvable Ag_2S-NP and Ag ions from $AgNO_3$ already showed that dissolution is a relevant transformation in the soil driving the uptake in soil organisms. In the same chapter, based on spICP-MS analysis of earthworm tissues it was concluded that only ~ 17% (average during the uptake phase) of the Ag in the earthworms exposed to pristine Ag-NP was present in particulate form. In case of Ag_2S-NPs, this percentage was up to ~40 % (average during the uptake phase). This was a clear evidence that the main form present in the earthworms is the ionic form. Ag_2S-NPs are present as particle

to a higher extent than Ag-NPs likely because they are less dissolvable [26]. However, detection of the particles within the organisms does not unequivocally confirm the uptake of the Ag as nanoparticle. Particle formation may have followed uptake of the Ag ions as shown to occur in a previous study [7]. Therefore, in chapter 2, imaging techniques (Scanning Electron Microscopy, SEM) and elemental analysis (Energy Dispersive X-ray analysis, EDX) were applied to identify Ag nano-objects in the tissue of earthworms exposed to Ag-NP. This revealed that Ag-NPs were associated with sulphur and chloride. Additionally, the nano-objects showed shapes (not spherical) and sizes (75-200 nm) different from the original particles that were spiked in the soil, indicating that the Ag-NPs in the tissues were not the same as the ones the worms were exposed to. This suggested that ions were taken up and biogenic transformation or formation of original Ag-NPs took place within the earthworms, likely as product of the metal detoxification pathway.

In **chapter 3**, by the exposure of earthworms to bimetallic Au core-Ag shell NPs it was confirmed that the Ag form which was mainly present in the earthworms was the ionic form (95% and 96% of the Ag and Au internalized by the earthworm, respectively) and that dissolution is the main transformation affecting the uptake of Ag in the earthworm in the soil. Also the low uptake of Au-NPs (shown not to be subject to dissolution [27, 28]) in earthworm is a further element supporting the finding that dissolution is the main factor driving uptake of metal NPs.

In **chapter 3**, complexation also represent a relevant transformation. Uptake of ionic Au in combined exposure with Ag was depending on the form in which Ag was included. In the exposure with soil spiked with Au^+ and Ag-NPs, the accumulated amount of Au^+ was lower than in the exposure with Au^+ only and higher than the one in the exposure together with Ag^+, while Ag accumulations was not statistically different between the treatments. This indicates

that Ag^+ and Au^+ interact in the soil and Au^+ became less available depending on the form of Ag [29, 30] or that Ag^+ and Au^+ compete at the uptake sites. However this is not applicable when Au is present as Au-NPs, indicating that in those treatments uptake of Au was particulate. This indicates that further studies should be performed to elucidate the behaviour of NPs in the presence of other metals and other contaminants, since they may influence the ultimate bioavailability of the NPs and their corresponding ions [31-33].

Organism as NP reactor: bio-mediated transformation and bio-mediated transport of NPs

Both **chapter 2 and 3** revealed the transformation and/or formation of new particulate metal forms within the earthworm. In **chapter 2**, by the use of spICP-MS it was possible to quantify the Ag in its particulate form and this revealed it to equal ~27% of the total amount of Ag accumulated in earthworms exposed to Ag-NPs after 28 days. However, the principle on which this analytical technique is based only allows the quantification of one element (metal) particle per time. From this, it follows that any other element associated with the Ag particles is not detected and quantified. Therefore, an imaging technique coupled with elemental analysis, SEM-EDX (Scanning Electron Microscopy coupled with Energy Dispersive X-ray) was applied and revealed that particles were new Ag nano-objects having a different shape (not spherical) and different size (75-200 nm) from the original particles. These Ag nano-objects were associated with sulphur and chloride, likely as products of a biogenic formation of clusters following a metal detoxification pathway. In earthworms, metals are primarily sequestered intracellularly by metallothioneins (MT) and compartmentalized once they enter the chloragogenous tissue. Previous studies identified metal- and sulphur-rich granules within this tissue and showed that these granules are indeed concentrated compartments of MT proteins

[34, 35]. The biogenic formation of particles was corroborated by the spICP-MS and SEM-EDX analysis of earthworm tissue exposed to $AgNO_3$. Also in this case, in earthworms exposed to Ag ions only, ~10% of the total Ag accumulated was present as particulate matter and association with sulphur and chloride was found. The low concentration of Ag in the earthworms exposed to Ag_2S-NPs did not allowed the visualization of any particles by SEM for this treatment group (too few to detect) but it is likely that also for this form the particles measured in the organisms were newly created or transformed within the earthworm. Indeed, the size of those particles as measured by spICP-MS resulted to be three times bigger than the initial spiking stock solution. It is therefore possible that also Ag_2S-NP dissolved (less than Ag-NPs) and were taken up by the earthworms as ions, while up to 40% of these ions were transformed into nano-objects.

However, from the results of the experiment presented in **chapter 2** of the thesis it was still not clear to which extent the earthworms exposed to Ag-NP transformed internalized particles or created new particles from accumulated Ag ions. When earthworms were exposed to bimetallic particles Au core-Ag shell, as described in **chapter 3**, a different technique was utilized, spICP-TOFMS which allowed the quantification of multi-elements single particles. This study was based on the assumption that Ag shell would interact with the soil and soil solution and would dissolve (partially or totally) and that the Au core would remain intact and its presence within the organisms would represent the true uptake as particles. Only 5.2% and 4.0% of Ag and Au in the worms were part of bimetallic nanoparticles. The Au core size of the bimetallic nanoparticles detected within the earthworm tissue had not changed, while the outer Ag shell was thicker when compared to the original sizes. This suggests that part of the dissolved Ag ions available in the cellular environment precipitated around the bimetallic particles. The addition of the new layer of Ag on the Ag shell could concur during bio-corona formation [36].

However, it could also be that the dissolution of the Ag shell occurred completely outside the earthworm and that some naked Au particles were taken up together with Ag ions. Then naked Au particles could act as seed for the precipitation of dissolved Ag ions. Another study successfully applied bimetallic NPs (au core – Ag shell) to assess transformation in complex aqueous media [37] showing that the increase or decrease of the Ag shell is dependent on the concentration of the NPs.

Both biogenic formation (formation of nano-objects from ions in biological matrices) and biotransformation (transformation of nanoparticles by biological activity) of NPs have been reported in other studies. Formation of nano-objects from ions within biota has been already reported in rats [38], plants [39-41] mostly as part of detoxification processes. The biogenic formation of nanomaterials is also intentionally exploited by the green synthesis of NMs which is a growing field of nanotechnology [42] focussing on the production of NMs by chemical reduction in bacteria [43], plants [44], fungi [45], or algae [46]. Biotransformation of NPs are reported in plants [47, 48], in rats [49], in algae [50], and cells [36]. The experiments reported in **chapter 2 and 3** demonstrated that the earthworms processed both metal NPs and the related dissolved ions into nano-objects with a new identity, different size and chemical composition.

The organisms as reactors, able to actively change the physico-chemical characteristics of NPs, is complementary with the concept that organisms may act as reactors by actively influencing the environmental fate of NPs. In **chapter 3**, the burrowing activity of earthworms (*L. rubellus*) was shown to have more influence than artificial rain fall on the vertical transport of non-dissolvable Ag_2S-NPs in soil columns, while the presence of Ag_2S-NP at the concentrations used, did not affect the behaviour of the earthworms. Up to 9% of the total amount of Ag that was spread on top of soil columns was transported to the bottom of the column (10-12 cm

deeper) after 28 days. Therefore, it was concluded that the earthworm burrowing has a great impact on the transport of non-dissolvable (or hardly dissolvable) NPs. Regardless the daily addition of artificial rain water with slight acidic pH (5.1) (dissolution is promoted by lower pH), no Ag was detected at the deepest layer of the columns without worms, indicating limited transport. Based on these results it was concluded that earthworms (and other soil organisms) are rather active drivers in the environmental fate of NPs, more than just final receptors of NPs. While this may already be known for conventional chemicals [51], the current work highlights the importance for nanomaterials as well. The results of **chapter 5** corroborate the relevance of earthworm bioturbation for the transport of nanomaterials in the soil [52]. Additionally, the proposed bioturbation model, built on the experimental data, offers a simple method to predict the concentration of the nanomaterials in the different soil layers based on earthworm density. Altogether these findings (**chapter 2, 3 and 5**) highlighted the importance of the ability of the earthworms to transform NPs and affect their transport in the soil. The concept that organisms are reactors of NMs, highly influencing their fate in the environment needs to be taken into account and extended to all the biota for the environmental risk assessment of NMs.

Biodynamic models to predict toxicokinetic processes of metal NPs in earthworm

One of the biggest challenges of the environmental risk assessment of NPs is the identification and quantification of the exposure. Compared to conventional chemicals, the assessment of NP exposure requires not only the quantification of the NPs in the exposure media but also the identification and quantification of the forms in which they are present.

As shown by the results of the concentration of Ag in the soil pore water in **chapter 4**, dynamic measures of bioavailability are needed for dissolvable NPs in the soil [17]. However, an

indication of the extent of bioavailability can be derived from the rate at which organisms take up ions from the environment [53, 54].

In **chapter 2**, spICP-MS was applied to distinguish the concentrations of particulate Ag from those of Ag ions internalized by the earthworms over time and kinetic rate constants were quantified for uptake and excretion of the particulate and ionic form of Ag-NP, Ag_2S-NP and $AgNO_3$. The toxicokinetic model used is a one-compartment model that considers the earthworm as one single compartment able to take up different forms of Ag ($k_{1\ particulate}$, $k_{1\ total}$, expressed in mg Ag kg^{-1} dry soil /mg Ag kg^{-1}wet body weight * day^{-1}) and to eliminate different form of Ag ($k_{2\ particulate}$, $k_{2\ total}$, expressed in day^{-1}). The results showed that the rate constants related to Ag-NP and $AgNO_3$ exposures did not differ significantly from each other, particularly due to the fact that earthworms exposed to $AgNO_3$ biogenically formed particulate form of Ag (**chapter 2**). The uptake rate constants for total Ag are substantially higher than those of the particulate form, indicating that uptake occurred mainly as the ionic form, regardless of the form the worms were exposed to. For Ag_2S-NPs, the uptake rate constants were significantly lower than the ones for the other forms. In contrast, elimination rate constants (k_2) were not different among Ag forms, likely due to the biogenic formation and biotransformation processes, resulting in a similar form of Ag present within the earthworms.

In **chapter 4**, the same one-compartment model used in chapter 2 was applied to describe the bioaccumulation data of the long-term exposure. Statistical differences were found for the uptake kinetic rate constants (k_1) between Ag-NP and Ag_2S-NP and between $AgNO_3$ and Ag_2S-NP. The same elimination kinetic rate constants (k_2) of chapter 2 were set for chapter 4 because the excretion capacity is mainly dependent on the physiology of the earthworm and therefore assumed not to be different. In order to comprehend if the toxicokinetic model based on the short-term exposure can provide parameters that can be used to predict longer and more realistic

term of exposure, the kinetic rate constants of the short-term study were used to predict the bioaccumulation of Ag in the earthworm after 270 days. The outcome of the comparison as reported in **chapter 4**, was that the toxicokinetic rate constants derived by the experimental data from the short-term exposure predicted the modelled data obtained after nine months exposure for Ag-NP and $AgNO_3$ but under-predicted those for Ag_2S-NP. This was demonstrated by the calculation of confidentiality intervals for each modelled curve of each Ag form (Ag-NP, Ag_2S-NPs and $AgNO_3$) for both short- and long-term exposure. The underestimation of the bioaccumulation of Ag_2S-NP in earthworms exposed for nine months by the kinetic rate constants of the short-term experiment can be explained by the dynamic bioavailability of Ag ions in the soil solute and their fluxes in the organisms. Indeed, by considering that the concentrations of Ag in pore water for all the treatments did not differ (**chapter 4**) and that earthworm mainly took up Ag as ions (**chapter 2 and 3**), it was concluded that Ag-NP dissolved rather fast in the first hours or days of exposure and because of surface aging and adsorption of the Ag ions on the soil particles reached steady state between the soil phases similar to the one of ions from $AgNO_3$. In the case of Ag_2S-NP the dissolution is slower but constant over time. Therefore, the short-term uptake kinetic rate constants may relatively underestimate the uptake of Ag_2S-NP in the long-term study because it did not take into account the late dissolution of Ag_2-NPs. A comparison of toxicokinetics for *L. rubellus* under different conditions has been also studied by Giska et al. [55] who reported that kinetic parameters derived from a laboratory toxicokinetic experiment in earthworms exposed to different metals were relevant for the field situation. This thesis work showed that toxicokinetic models are suitable to study the uptake of NPs and provide knowledge regarding their bioavailability.

Implication for environmental risk assessment of NMs and future perspectives

In this thesis, we have shown that the use of aged NM (in this thesis Ag_2S-NPs) instead of pristine NM as manufactured (Ag-NPs) in ecotoxicological testing is crucial for a proper exposure and risk assessment of NM in the environment. Therefore, identification of transformations and the resultant main form of a specific NM that reaches a specific environmental compartment is the first step towards the exposure assessment and this should be integrated in future studies [56, 57]. Our data demonstrated that the bioaccumulation of pristine Ag-NP is not different to that of Ag ions and that the bioaccumulation of Ag_2S-NPs is lower than Ag ions, even after a long-term exposure. Therefore, we showed that their potential uptake and consequent risk can be conservatively covered by the ones of the ionic Ag. This represents a useful starting point to evaluate the uptake in soil organisms of other dissolvable metal NP known to reach the soil in their sulfidized form (e.g. CuO-NP, ZnO-NP) [58, 59]. This thesis provided unequivocal evidence that dissolution is the major transformation of Ag-NP in soil which is of high importance for the standardization of the battery of tests needed for measuring physicochemical NM properties relevant to environmental risk assessment [60]. However, as also this thesis showed, the type of environmental compartment, its conditions and the time of exposure highly affect the behaviour of sulfidized NPs. For instance, copper sulphide nanoparticles (CuS-NPs) have been reported to be more dissolvable than CuO-NPs [61] and not to show significant dissolution [62] with different conditions in aquatic environment. Therefore, future research should be focus on the quantification of dissolution rates of aged metal NMs within complex environmental matrices and over long time frames.

Finally, in this thesis we demonstrated that earthworms were reactors of NMs because they biogenically formed and transformed NMs and affected their fate in the soil. This showed that

the biota is a driving element in the fate of NMs rather than only a final receptor and their influence needs to be taken into account when modelling the fate of NMs. Therefore, this concept should be extended to all the biota of a specific environmental compartment.

Overall conclusion

This thesis quantified and modelled the exposure and bioaccumulation of pristine and aged Ag-NP and Ag ions in earthworms. It can be concluded that the toxicokinetics of all the studied forms of Ag-NPs are governed by the fluxes of dissolved ions from the soil to the earthworms. The bioaccumulation of Ag-NPs is not different than that of Ag ions and the bioaccumulation of Ag_2S-NPs is lower than that of Ag ions after a short-term exposure, and the pattern remained the same after a long-term exposure. Therefore both Ag-NP and Ag_2S-NP potential uptake in earthworms are conservatively covered by the uptake of the ionic Ag ($AgNO_3$). This thesis also provides an additional proof of concept of how the soil organisms can affect the fate of contaminants (Ag_2S-NP in this thesis) by their internal processes and by their activities in the soil.

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Chapter 7

Summary

Chapter 1 introduced the objectives of the thesis and background information regarding the production of nanomaterials (NMs) and their release in the environment. The concepts of environmental and bio-mediated transformations of NMs were defined and their implication on the NM exposure assessment is explained. Silver nanoparticles (Ag-NPs) were selected as model NM because they are widely produced, undergo transformations that represent all the most relevant transformations of NMs in soil and specific analytical methods are available to quantify them at relatively low concentrations. A short review regarding previous toxicological studies of Ag-NPs in soil organisms and the importance of performing toxicokinetic studies was presented. Finally, selected model organisms (earthworms *Eisenia fetida* and *Lumbricus rubellus*) were briefly described. **Chapter 2** reported a short-term (28 days) toxicokinetic study in *E. fetida* exposed to Ag-NPs, aged Ag-NPs (Ag_2S-NPs), and $AgNO_3$. A one-compartment model was applied to calculate separately the kinetic constants for uptake and elimination of particulate and ionic forms of Ag. The uptake and elimination rate constants for earthworms exposed to pristine Ag-NP or $AgNO_3$ were not significantly different from each other. Uptake rate constants of (hardly dissolvable) Ag_2S-NPs which resemble the environmental relevant form of Ag-NPs was significantly lower. spICP-MS analysis demonstrated that ~85% (average of both Ag-NP and $AgNO_3$ treatments) of the Ag within the earthworms was present as ionic Ag, regardless of the actual form of Ag that the earthworms were exposed to. Indeed, the biogenic formation of particulate Ag (~10 % of total Ag accumulated overtime) in earthworms exposed to $AgNO_3$ led to a kinetic pattern of particulate Ag body burden similar to pristine Ag-NPs. NP size analysis and imaging techniques showed evidences that the particles in the tissues were not the same as those to which worms were exposed, highlighting that biotransformation and/or biogenic formation took place also in the case of the Ag-NP exposure. **Chapter 3** investigated the influence of dissolution on the uptake of metal NPs in earthworms by the use

of bimetallic NPs. *E. fetida* specimens were exposed to Au core-Ag shell NPs (Au@Ag-NPs) and to a combination of Au-NPs, Ag-NPs, Ag and Au ions containing natural soil for 28 days. Our hypothesis was that Ag shell would dissolve partially or completely and that Au core would not interact with the exposure media and would therefore behave as a tracer of the particulate uptake. Analysis of earthworm tissues showed that concentrations of Ag in the earthworms were not statistically different in organisms exposed to the different forms of Ag. However, the concentration of Au in the earthworms exposed to $HAuCl_4$ (ionic Au) exceeded around twenty times the Au concentrations in the exposures to particulate Au, which did not differ among each other. Mass measurements by spICP-TOFMS provided evidence that the uptake of the metals in their bimetallic particulate form represents approximately 5 % of the total metal amount. Size measurements by spICP-TOFMS showed that the Au core remained similar after the uptake, while the Ag shell increased in thickness suggesting that biotransformation processes took place at the surface of the NPs (e.g. aggregation, adsorption of Ag ions on the surface of existing particles). The study confirmed that dissolution is the main factor driving the uptake of (dissolving) metal NPs in earthworms. Additionally, different uptake patterns resulted from the co-exposure to Au and Ag-NP and Ag^+, indicating that the Ag form can lead to different interactions with Au in the soil affecting the uptake in the earthworms. **Chapter 4** presented a toxicokinetic study performed to assess the potential impacts of long-term exposure (nine months) on the uptake of pristine Ag-NP, aged Ag-NP (Ag_2S-NP) and ionic Ag in earthworms *E. fetida*. The study was conducted with same species and conditions similar to the short-term experiment which was previously conducted for 4 weeks (chapter 2), in order to allow comparison between the two models. The accumulation of Ag in Ag-NP and $AgNO_3$ exposed earthworms did not statistically differ after nine months exposure. In Ag_2S-NPs exposed earthworms, the internalized concentrations were five times lower compared to the other

treatments. The Ag concentrations in pore water did not reflect the uptake pattern and metallothionein concentrations were not different from the control group. The overall conclusion of this chapter was that even after a prolonged period of time the uptake kinetic rate constants of Ag-NP and $AgNO_3$ were not statistically different, while the one of Ag_2S-NP was statistically significant lower than the other treatments. Additionally, the short-term kinetic rate constants predicted the average bioaccumulation of pristine Ag-NP and $AgNO_3$ in the earthworms exposed for nine months, while the bioaccumulation of Ag_2S-NPs in earthworms was under-predicted somewhat. This was likely because the short-term did not take into account the late dissolution of Ag_2-NPs. Ag bioaccumulation of Ag_2S-NP could not be related to the concentrations of Ag measured at a specific time in pore water. **Chapter 5** reports a study which demonstrated that earthworm bioturbation plays an important role in the vertical transport of Ag_2S-NPs in soil. In the soil columns, daily rainfall from artificial rain water did not lead to displacement of Ag_2S-NPs within 28 days indicating that in the case of hardly soluble metal NPs and unsaturated soil conditions, bio-mediated transport overcomes physical chemical transport. Bioturbation from *L. rubellus* was quantified by assessing the changes of the macro porosity in the soil columns. Results indicated that earthworms burrowing activity was not affected by the presence of Ag_2S-NPs at the experimental concentrations. The study proposed a linear relationship between bioturbation rate and the abundance of earthworms that is applicable to future bioturbation studies. **Chapter 6** presented an overall discussion of the results obtained in the thesis, and concluded with the implication of such findings in the risk assessment of NMs.

Annex

Acknowledgements

Curriculum Vitae

List of publication and conference presentations

SENSE certificate

Overview of completed training activities

Acknowledgments

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I know that for most of the people at the Department of Toxicology I was the weirdo PhD candidate working with earthworms (not for Hans van den Berg). Although some people would react with disgust just to the idea of seeing some earthworms :) still I felt appreciated and

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Back to September 2015, when I started my PhD and before even I have set up my work space at Toxicology Department, Wim at Wageningen Environmental Research (former Alterra) showed me how to keep the first crate of earthworms, to sieve and moisturize the soil, to feed the earthworms and mainly to observe them. Thanks Wim!

I (re)discovered a special affinity with my paranymphs during the PhD trip in Japan in 2018. Thank you for your friendship and for being at my side during the final discussion of my PhD. Diego, I wish you all the best for finalizing your PhD and I hope you are around for some drinks afterwards. Katja, I like your perfect balance between organized and passionate scientist and easy-going and enjoyable person. I think you will go far.

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Curriculum vitae

Marta Baccaro was born on the 26th of November 1985 in Maglie, Italy. She completed her BSc studies in Environmental Sciences at University of Salento with a thesis in Analytical Chemistry with title " Quantification of emission of PCDD/Fs, PCBs and PAHs released by incinerator". Afterwards, between 2012 and 2015, she obtained a MSc degree in Ecotoxicology at University of Siena with a thesis in Ecotoxicology with title "Kinetic studies on the nanoparticle accumulation in zebrafish embryos by ICP-MS" for which she spent six months at the Helmholtz Centre for environmental research – UFZ. Later, at the same institute she conducted an internship where she carried out studies on the effects of mixtures of nanomaterials and other chemicals. In the same year (2015), Marta started her PhD study at the Department of Toxicology, Wageningen University & Research, under the supervision of Prof. Dr. Ir. Ivonne Rietjens and Dr. Ir. Nico van den Brink. During her PhD, she initiated and led the Young Nanoscientists group with members from Europe and USA, she supervised MSc student thesis and assisted practical activities of MSc courses. She also followed a postgraduate education program in Toxicology, which will enable her to register as European toxicologist in the near future. Marta is currently a postdoctoral researcher at the department of Toxicology, Wageningen University and Research.

List of publications (this thesis)

Baccaro, M., Undas, A. K., de Vriendt, J., Van Den Berg, J. H. J., Peters, R. J. B., & van den Brink, N. W. (2018). Ageing, dissolution and biogenic formation of nanoparticles: how do these factors affect the uptake kinetics of silver nanoparticles in earthworms?. *Environmental Science: Nano*, *5*(5), 1107-1116. DOI: 10.1039/C7EN01212H

Baccaro, M., Harrison, S., van den Berg, J. H. J., Sloot, L., Hermans, D., Cornelis, G., van Gestel, C. A. M., van den Brink, N. W. (2019). Bioturbation of Ag2S-NPs in soil columns by earthworms. *Environmental Pollution, 252 Part A*, 155-162. DOI: 10.1016/j.envpol.2019.05.106.

Baccaro, M., van den Berg J. H. J., van den Brink, N. W. Particle or ion, what is the influence of dissolution on the uptake of metal nanoparticles in earthworm?. To be submitted.

Baccaro, M., van den Berg J. H. J., van den Brink, N. W. Is a short-term toxicokinetic study able to predict the uptake of metal NPs in earthworms after nine months?. To be submitted.

Other publications

Böhme, S., Baccaro, M., Schmidt, M., Potthoff, A., Stärk, H. J., Reemtsma, T., & Kühnel, D. (2017). Metal uptake and distribution in the zebrafish (*Danio rerio*) embryo: differences between nanoparticles and metal ions. *Environmental Science: Nano*, *4*(5), 1005-1015. DOI: 10.1039/C6EN00440G

van den Brink, N. W., Jemec Kokalj, A., Silva, P.S., Lahive, E., Norrfors, K., Baccaro, M., Khodaparast, Z., Loureiro, S., Drobne, D., Cornelis, G., Lofts, S., Handy, R. D., Svendsen, C., Spurgeon, D., & van Gestel, C. A. M. (2019). Tools and rules for modelling uptake and bioaccumulation of nanomaterials in invertebrate organisms. *Environmental Science: Nano*, 6, 1985-2001. DOI:10.1039/C8EN01122B.

International conference presentations

Biokinetic rate constants of particulate Ag and Ag^+ differ in *Eisenia fetida* exposed to Ag-NP and $AgNO_3$ under environmentally relevant conditions. SETAC Europe 27th Annual meeting, May 2017 – Brussels – oral presentation.

Mobilisation of silver sulphide nanoparticles in soil column by earthworms´ bioturbation. SETAC Europe 28th Annual meeting, May 2018 – Rome – oral presentation.

Ageing, dissolution and biogenic formation of nanoparticles, how do these factors affect uptake kinetics of silver nanoparticles in earthworm?. Japanese Society of Toxicology 45th Annual Meeting, July 2018 – Osaka – oral presentation.

Influence of dissolution on the uptake of metal nanoparticles in soil organisms *Eisenia fetida.* SETAC Europe 29th Annual meeting, May 2019 – Helsinki – oral presentation.

Long term, chronic toxicokinetics of Ag-NP and Ag_2S-NP in earthworms exposed for nine months. International Conference on the Environmental Effects of Nanoparticles and Nanomaterials (ICEENN), September 2019 – Vienna – oral presentation.

SENSE certificate

Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment

D I P L O M A

For specialised PhD training

The Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment
(SENSE) declares that

Marta Baccaro

born on 26 November 1985 in Maglie, Italy

has successfully fulfilled all requirements of the
Educational Programme of SENSE.

Wageningen, 17 January 2020

The Chairman of the SENSE board
Prof. dr. Martin Wassen

the SENSE Director of Education
Dr. Ad van Dommelen

The SENSE Research School has been accredited by the Royal Netherlands Academy of Arts and Sciences (KNAW)

KONINKLIJKE NEDERLANDSE
AKADEMIE VAN WETENSCHAPPEN

Annex

Overview of completed training activities

The SENSE Research School declares that **Marta Baccaro** has successfully fulfilled all requirements of the Educational PhD Programme of SENSE with a work load of 47.2 EC, including the following activities:

SENSE PhD Courses

- Environmental research in context (2016)
- Research in context activity: 'Initiating and developing active network for young researchers in the field of nanotechnology: YoungNanoScientists (YNS, http://nanofase.eu/show/Young%20Scientists_341)'

Other PhD and Advanced MSc Courses

- Cell toxicology, Postgraduate education in Toxicology (2016)
- Epidemiology, Postgraduate education in Toxicology (2016)
- General toxicology, Postgraduate education in Toxicology (2016)
- Pathobiology, Postgraduate education in Toxicology (2017)
- Laboratory animal science, Postgraduate education in Toxicology (2017)
- Immunotoxicology, Postgraduate education in Toxicology (2017)
- Mutagenesis and Carcinogenesis, Postgraduate education in Toxicology (2017)
- Workshop spICP-MS data analysis, RIKILT (2017)
- Engineered Nanoparticles in the Environmental Systems, University of Koblenz-Landau (2018)
- Organ toxicology, Postgraduate education in Toxicology (2018)
- Risk assessment, Postgraduate education in Toxicology (2019)

Management and Didactic Skills Training

- Assisting practicals of the BSc course 'Environmental Toxicology' (2016-2019)
- Organising and member of scientific committee of 3rd NanoSafety Forum for Young Scientists (2018)

Selection of Oral Presentations

- *Quantification of uptake and excretion kinetics of pristine AgNPs in Eisenia fetida at environmental concentrations.* Nanofase, 17-20 October 2016, Lasko, Slovenia
- *Mobilisation of silver sulphide nanoparticles in soil column by earthworms´ bioturbation.* SETAC, 13-17 May 2018, Rome, Italy
- *Ageing, dissolution and biogenic formation of nanoparticles, how do these factors affect uptake kinetics of silver nanoparticles in earthworm?* JSOT, 18-20 July 2018, Osaka, Japan
- *Influence of dissolution on the uptake of metal nanoparticles in soil organisms Eisenia fetida.* SETAC, , 26-30 May 2019, Helsinki, Finland

SENSE Coordinator PhD Education

Dr. ir. Peter Vermeulen

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