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Bioturbation of Ag_2S-NPs in soil columns by earthworms

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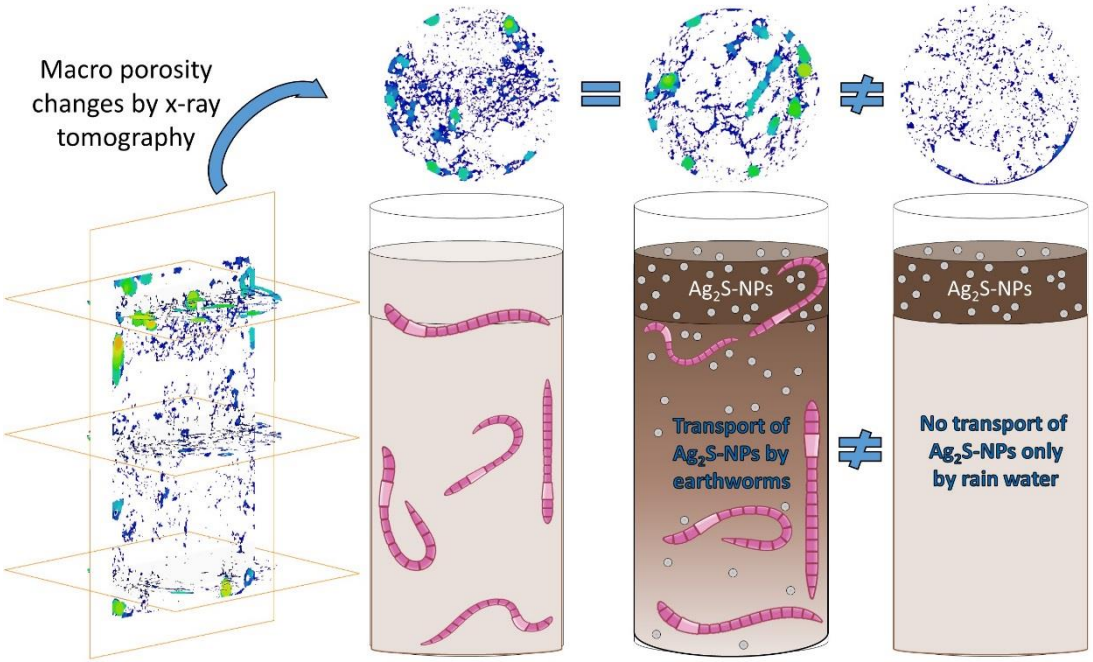

Keywords

Bioturbation, earthworms, nanoparticles, transport, soil

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 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 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.

Capsule

Earthworm bioturbation plays a more important role than rainfall in the vertical transport of Ag_2S-NPs in soil

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 (Devliegher and Verstraete, 1997; Heemsbergen et al., 2004). The activity of earthworms can lead to a complete mixing of the soil over a few years (Müller-Lemans and van Dorp, 1996) and this process can displace strongly adsorbed contaminants or nutrients (Sizmur and Hodson, 2009; Zorn et al., 2005). Apart from moving soil, earthworms create burrows, which may represent preferential routes for the transport of rain water including dissolved nutrients or contaminants (Farenhorst et al., 2000). In turn, burrowing activity of earthworms can be affected by exposure to contaminants, as shown for imidacloprid (Capowiez et al., 2006) and carbaryl (Gupta and Sundararaman, 1991). 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 (Kim et al., 2010; Lombi et al., 2013). The low solubility of Ag_2S-NPs may lead to relatively low bioavailability of Ag for soil organisms (Baccaro et al. 2018) and plants (Doolette et al., 2015), suggesting lower toxicity compared to pristine Ag-NPs or ionic Ag (Levard et

al., 2013; Wang et al., 2016). However, earthworm behavioural alterations may not be directly linked to the uptake of chemicals but to e.g. sensing and detection of the Ag (Shouts Wilson et al. 2011, Mariyadas et al. 2018). For instance, avoidance of Ag-NPs by different earthworm species has been observed (Brami et al., 2017; Mariyadas et al., 2018; Shoults-Wilson et al., 2011; Velicogna et al., 2016) 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 distance of only a few centimetres under saturated flow conditions (Cornelis et al., 2013). Interaction of NPs with air-water interfaces reduces their mobility even more in non-saturated soils (Fujita and Kobayashi, 2016). 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% (Rahmatpour et al., 2018). 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 compare 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 characterization

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 Supplemental Materials, 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.

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 (Rutgers et al., 2016) 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 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 of 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 (Cornelis et al., 2010). 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). 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 deviation) 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 deviation). 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 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 $[\mathrm{Ag}]_l$ (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 $D_{l:l+1} = v_{l:l+1}h$ (Rodriguez, 2006). The so-called bioturbation rate k_{bioturb} (s^{-1}) is given by

$$k_{\mathrm{bioturb},l:l+1} = \frac{v_{l:l+1}}{d_l} \quad (1)$$

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

$$[\mathrm{Ag}]_{l,t+1} = [\mathrm{Ag}]_{l,t} + k_{\mathrm{bioturb},l:l+1,t}\delta t\left([\mathrm{Ag}]_{l+1,t} - [\mathrm{Ag}]_{l,t}\right) + k_{\mathrm{bioturb},l-1:l,t}\delta t\left([\mathrm{Ag}]_{l-1,t} - [\mathrm{Ag}]_{l,t}\right) \quad (2)$$

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}) (Rodriguez, 2006) 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). 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 absence of rain did not change significantly over time (Figure 1, Table S3). In contrast, the Ag concentrations in the earthworms increased significantly over time when ARW was applied (Figure 1, Table S3), resulting in a significant interaction between two factors, time and treatment (Table S4). 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).

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 (Capowiez et al., 2011) and in the distribution of pore sizes (Porre et al., 2016). 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). 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). Porosity and pore distribution were always significantly different from the columns without earthworms (Tables S7B and S7C). 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). 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 supplemental information (paragraph S9).

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).

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). 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). 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).

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

353 that transport of Ag_2S-NPs via percolating water through the soil is negligible
354 relative to the displacement caused by earthworm bioturbation.
355
356 **Bioturbation rate**
357 The fits of the bioturbation model to the resultant Ag concentrations, with
358 worms and Ag_2S-NPs, with and without ARW are shown in Figure 7. The log
359 of concentrations was taken before fitting to provide better sensitivity to the
360 lower concentrations in the deeper soil layers. The fit resulted in a
361 bioturbation fitting parameters of $\beta = 4.80 \times 10^{-12} \pm 0.99^{-12}$ m^4 s^{-1} and $\beta =$
362 $3.56 \times 10^{-12} \pm 0.65^{-12}$ m^4 s^{-1} (value ± 95% confidence interval) for the
363 treatments without and with rain, respectively. The corresponding soil
364 turnover rate of $v = 0.39 \pm 0.04$ cm day^{-1} (Equation 3) for the treatments
365 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 $k_{\text{bioturb}} = 1.68 \times 10^{-6} \pm 0.14 \times 10^{-6}$
367 s^{-1} were calculated for the treatments with the application of rain (value ±
368 95% confidence interval). The model indicated that complete mixing –
369 defined as concentrations in separate layers being within 0.01 mg kg^{-1} of
370 each other – could (hypothetically) be reached after approximately 100 days
371 in stable conditions and after 150 days when rain was applied.
372
373 **Discussion**
374 Although only the top layer of the soil columns was treated, earthworms did
375 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 (Baccaro et al., 2018) 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 (Ortega Hidalgo et al., 2017). 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 kg_{soil} $kg_{earthworm}^{-1}day^{-1}$ 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 they results 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 Baccaro et al. (Baccaro et al., 2018), 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 supplemental material (Figure S13). 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 (Sizmur et al., 2011). Earthworms can transport and increase the availability of metals (Leveque et al., 2014), 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 (Bystrzejewska-Piotrowska et al., 2012; Lemtiri et al., 2016). 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 (Covey et al., 2010). 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 solubility and their rapid attachment to soil surfaces and/or air/water interfaces (Cornelis et al., 2014). 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. (Makselon et al., 2018) 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 (Löv et al., 2018). 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.

447 The present study also shows that bio-mediated transport of Ag_2S-NPs may
448 exceed physical chemical transport in soils. Bioturbation therefore has to be
449 considered when discussing NP bioavailability because a higher mixing rate
450 implies a lower local NP concentration in the different strata.
451 In order to predict the bioturbation rate of Ag_2S-NPs due to earthworm
452 activity, the experimental data related to the treatment without rain were
453 fitted using the previously described bioturbation model, yielding a
454 bioturbation rate of $k_{\mathrm{bioturb}} = 2.3 \times 10^{-6} \pm 0.26 \times 10^{-6}$ s^{-1} across the soil column
455 for the experiment with controlled conditions and $k_{\mathrm{bioturb}} = 1.68 \times 10^{-6} \pm$
456 0.14×10^{-6} s^{-1} for the experiment with the rainfall. Complete mixing of the
457 soil column due to bioturbation was predicted to occur within 100-150 days.
458 Treating this dispersion rate as directly proportional to earthworm density
459 resulted in a significant fit of the experimental data (Figure 7).
460 Apart from quantifying the rate at which bioturbation proceeds, validating
461 the model against experimental data is of relevance for predictive models of
462 nanomaterial fate, on which bioturbation may have a large impact. The
463 difficultly in sourcing data for such models makes the simple linear
464 relationship between bioturbation rate and earthworm density, presented
465 here, highly attractive. Indeed, spatially resolved earthworm density data for
466 the EU already exists (Rutgers et al., 2016), and the dependence of
467 earthworm density on land-use and land-management has been quantified
468 (Spurgeon et al., 2013). Nevertheless, the linear relationship between
469 bioturbation rate and earthworm density may have limitations. Earthworm
470 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 (Capowiez and Belzunces, 2001). 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 (Edwards and Bohlen, 1996; Wijnhoven et al., 2006). 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 (Mahdi et al., 2018).

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 (Jarvis et al., 2010).

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 insoluble 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.

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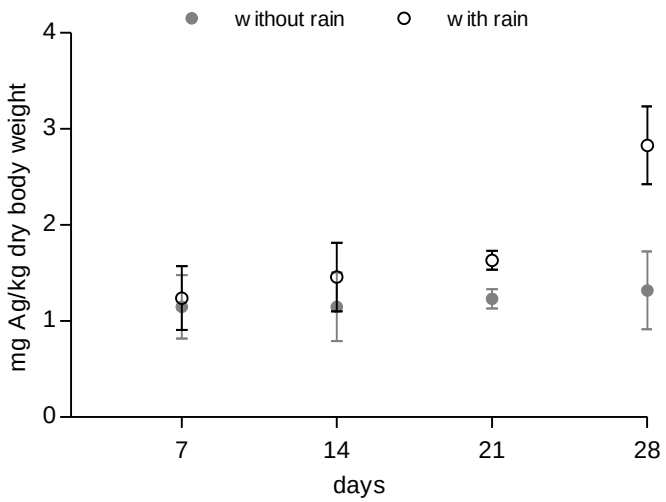

616

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

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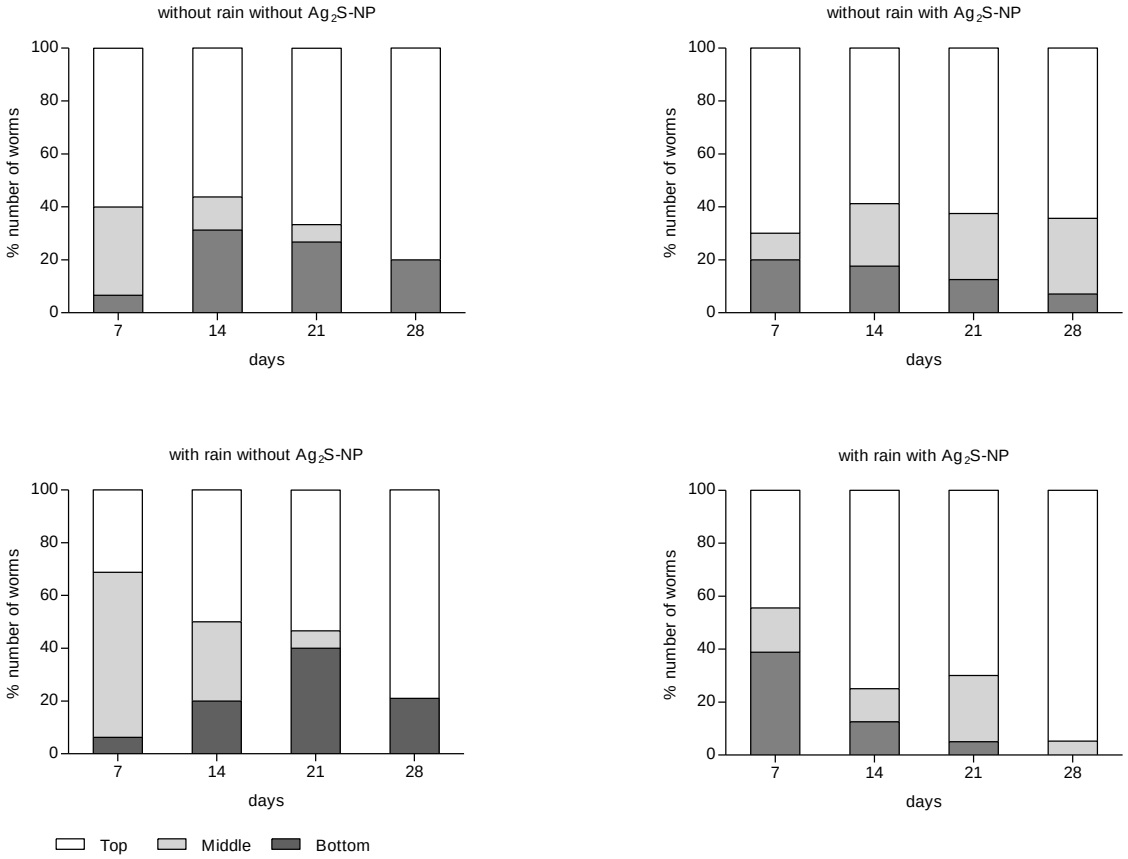

622

623 Figure 2. Depth distribution of earthworms in Kooijenburg soil with or without

624 Ag_2S-NPs and with and without the application of rain at different time points.

625 Columns were sampled at the three different depth (4 cm height)

626

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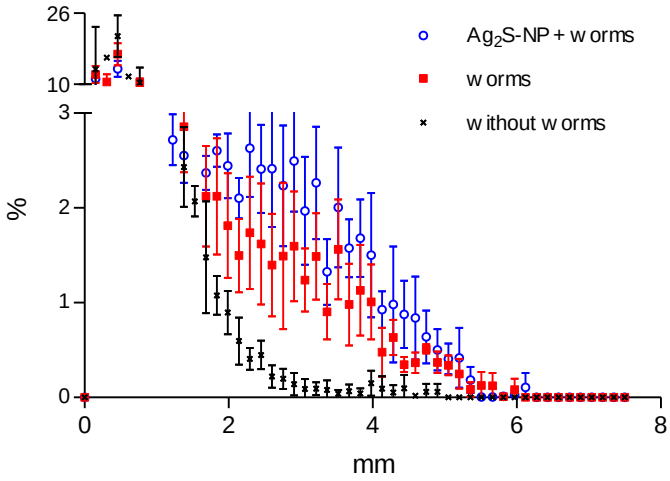
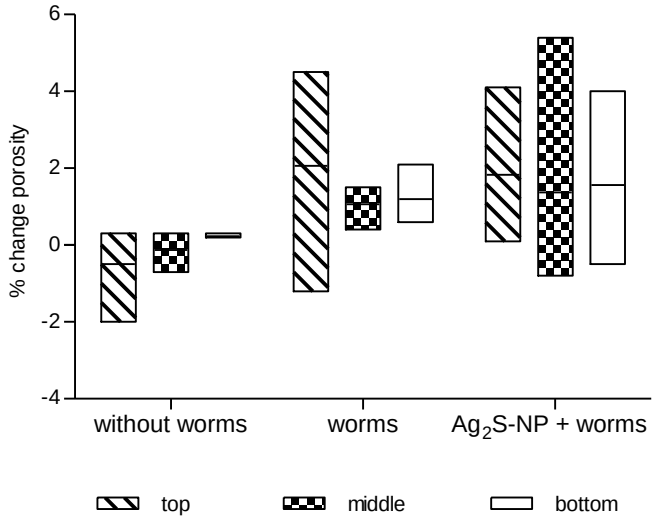


628

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



632

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

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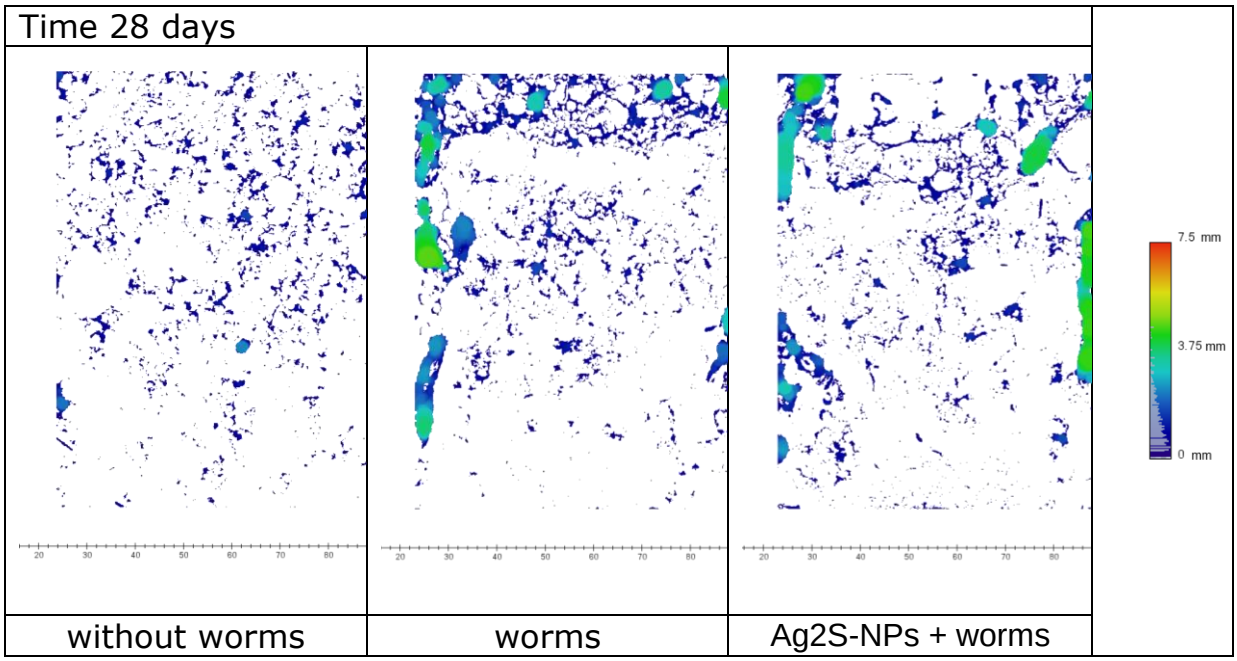


Figure 5. Colour maps of the pore size distribution in longitudinal profile of the Kooijenburg soil columns at the end of the incubation (28 days) with and without Ag_2S-NPs and with and without earthworms.

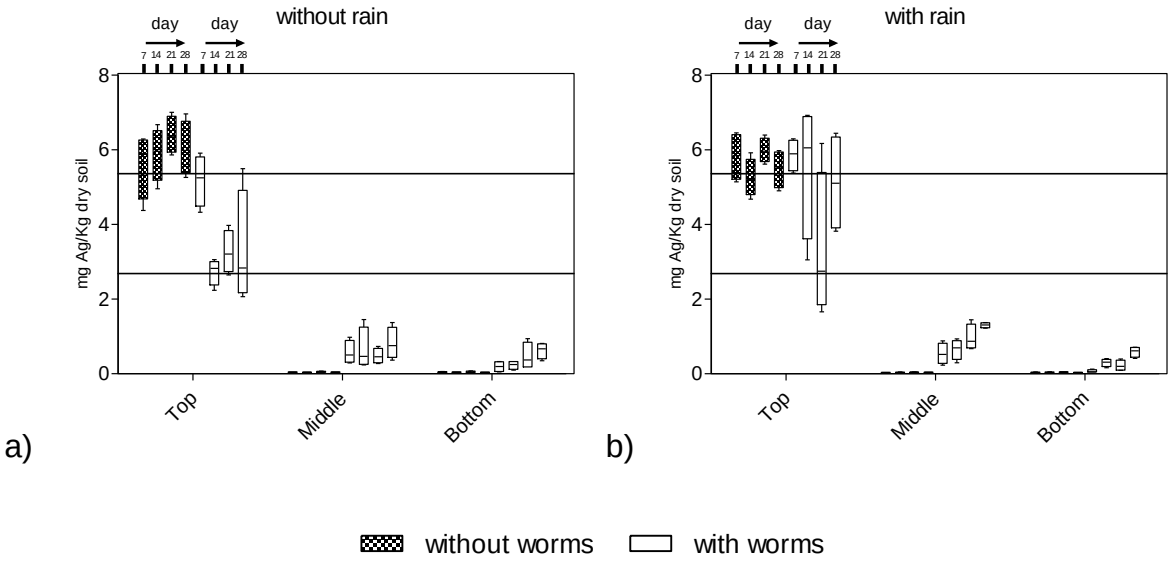
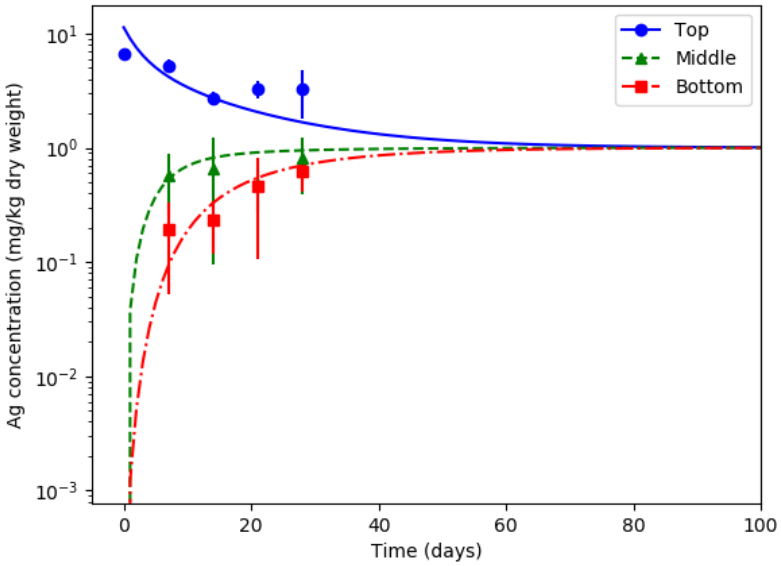
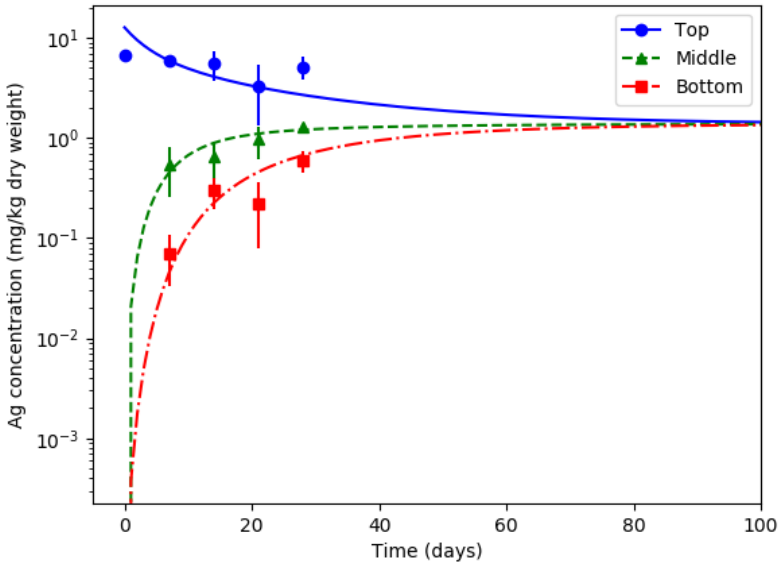


Figure 6. a) Ag concentrations at three depths of columns with Kooijenburg soil, with a top layer spiked with Ag_2S-NPs, with and without earthworms for the treatments without artificial rain water overtime, b) Ag concentrations at

649 three depths of soil in columns with and without earthworms for the
650 treatments with artificial rain water over time.
651



652 a)



653 b)

654 Figure 7. Development over time of experimental Ag concentrations at three
655 different depths in Kooijenburg soil in columns with Ag_2S-NPs spiked layer
656 on top, for the treatments with earthworms (*Lumbricus rubellus*) and Ag_2S-

657 NPs without (a) and with artificial rainwater (b), fitted by the bioturbation
658 model. Concentrations are log-transformed to provide better sensitivity to
659 lower concentrations in the deeper soil layers.