Transformation-dissolution reactions partially explain adverse effects of metallic silver nanoparticles to soil nitrification in different soils

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ABSTRACT

Risk assessment of metallic nanoparticles (NP) is critically affected by the concern that toxicity goes beyond that of the metallic ion. This study addressed this concern for soils with silver (Ag)-NP using the Ag-sensitive nitrification assay. Three agricultural soils (A,B,C) were spiked with equivalent Ag doses of either Ag-NP (d=13 nm) or $AgNO_3$. Soil solution was isolated and monitored over 97 days with due attention to accurate Ag fractionation at low (~10 µg L^{-1}) Ag concentrations. Truly dissolved (<1 kDa) Ag in the $AgNO_3$-amended soils decreased with reaction half-lives of 4 to 22 days depending on the soil, denoting important Ag-ageing reactions. In contrast, truly dissolved Ag in Ag-NP-amended soils first increased by dissolution and subsequently decreased by ageing; the concentration never exceeding that in the $AgNO_3$-amended soils. The half-lives of Ag-NP transformation-dissolution were about 4 days (soils A&B) and 36 days (soil C). The Ag toxic thresholds (EC10, mg Ag kg^{-1} soil) of nitrification, either evaluated at 21 or 35 days after spiking, were similar between the two Ag forms (soils A&B) but were factors 3 to 8 lower for $AgNO_3$ than for Ag-NP (soil C), largely corroborating with dissolution differences. This fate and bio-assay showed that Ag-NPs are not more toxic than $AgNO_3$ at equivalent total soil Ag concentrations and that differences in Ag-dissolution at least partially explain toxicity differences between the forms and among soils.

INTRODUCTION

In the last decades, engineered silver nanoparticles (Ag-NPs) are used in increasing amounts and in an increasing number of applications, ranging from textile and pharmaceutics to crop protection products (Nowack et al. 2011; León-Silva et al. 2016). This is a consequence of the unique physical and chemical properties of nanoparticles, which are defined by the European Commission as particles with at least one size dimension smaller than 100 nm, for 50% or more of the particles in the number size distribution (European Commission 2011). For example, nanoparticles have a high surface area to volume ratio compared to bulk sized materials, leading to a higher reactivity (Cornelis et al. 2013). As a result, Ag-NPs offer additional advantages compared to their bulk equivalents in many applications, but it also implies a potentially altered environmental risk. The rising emissions of Ag-NPs to the environment can occur through various indirect routes, for example, spillage, leakage, wearing out of consumer goods such as textile, but also via direct input in pesticide use (Cornelis et al. 2012). The ionic form of Ag, Ag^{+}, is generally considered as the bioavailable form and environmental limits are therefore established with toxicity tests using soluble Ag salts. Nevertheless, it is unsure whether these limits can be transferred to nanoscale forms, (i.e., if dissolution alone governs toxicity of Ag-NPs) or whether an additional toxic effect of Ag-NPs compared to Ag salts is present. This is the so-called 'nano-effect' that explains toxicity beyond the truly dissolved metal ion or its small molecular complexes. This nano-effect could be caused by direct uptake of the nanoparticles (Morones et al. 2005) or a local effect near the biological membranes (Li et al. 2010).

Risk assessment of Ag-NPs has been focused largely on freshwater systems (Notter et al. 2014), whereas terrestrial systems are also under elevated risk due to the large scale application of sewage sludge, which can contain up to 4.4 mg Ag kg^{-1} (Gottschalk et al. 2009). Moreover, solid

media such as soil can be considered as the major sink for metals and NPs (Sun et al. 2014; Sun et al. 2016). In Europe, the soil Ag concentration is expected to increase with 1.6 µg Ag kg^{-1} $year^{-1}$ in sewage sludge-amended soils (Gottschalk et al. 2009). A long-term field study in Spain found a doubling of the Ag level in a soil amended with sewage sludge for 15 years compared to the non-amended soil (Marguí et al. 2016); also in another study (Yang et al. 2014) a clear accumulation of Ag in the surface layer was found in sewage sludge-amended soils, however, no Ag-NPs could be observed in these soil samples with TEM-EDX. A large fraction of Ag-NPs in sewage sludge is readily transformed into insoluble silver sulfide (Ag_2S) under the prevailing anaerobic circumstances (Levard et al. 2012). As a result, only a minority of the initial Ag-NPs will end up in the environment as untransformed Ag-NP. Furthermore, in soil, ionic Ag will adsorb onto the soil solid phase resulting in lower Ag concentrations in pore water than in freshwater. This low mobility was also found in the before-mentioned long-term studies: Ag concentrations were below the limit of detection for two leaching tests performed on the sewage sludge-amended soils (Marguí et al. 2016) and Ag was nog found in enriched concentrations in the deeper soil layers (Yang et al. 2014). It is, therefore, impossible to transfer conclusions on the risk of Ag-NPs nor the occurrence of a nano-effect from aquatic to terrestrial systems.

Notter et al. (2014) conducted a metastudy on the possible nano-effect of Ag-NP: more than 95% of the data was from aquatic studies, of which only 4% suggested a possible nano-effect, while just eight soil toxicity studies were present, in which one study (earthworm avoidance) suggested a nano-effect. Most studies were performed at environmentally irrelevant high doses. In Europe, the soil Ag concentration ranges between <0.002 and 4 mg Ag kg^{-1} (Reimann et al. 2014), while toxicity tests were conducted between 15 and 2000 mg Ag kg^{-1} (Lee et al. 2012; Schlich et al. 2013). Following Notter's metastudy, more soil studies assessing Ag-NP toxicity were

conducted, showing mixed results. Judy et al. (2015) found enriched Ag concentrations in *Medicago truncatula* grown on biosolid-amended soils compared to control soils, coinciding with lower shoot biomass and lower nodulation; however, the biosolid also contained Zn-NPs and TiO_2-NPs so results cannot be attributed solely to Ag-NPs. In contrast, in a recent study by Sillen et al. (2015) a higher biomass was obtained in maize grown on Ag-NP amended soils compared to control soils with no added Ag-NPs, although changes in microbial community and activity were noted. The importance of organic matter (OM), and thus also the use of 'real' soil, as a factor affecting Ag-NP toxicity was also investigated by several researchers: Calder et al. (2012) observed a mitigation of Ag-NP toxicity to *Pseudomonas* bacteria in soil compared to sand attributed to particle aggregation caused by humics; Peyrot et al. (2014) also observed a alleviation of Ag-NP toxicity, assessed using enzyme activity, in OM-amended soils compared to unamended soils at equal Ag-NP doses, however, no mode of action was found. In this study, Ag-NP toxicity was also compared with ionic Ag, as Ag-acetate, generally resulting in a greater toxic effect of Ag-NPs on enzyme activity, but this was only significant at low Ag concentrations. It is thus crucial to conduct risk assessments in soil with a sensitive test, preferably using different end-points (Samarajeewa et al. 2017), and at relevant concentrations, including a measurement of Ag^+ in the pore water to confirm/reject a possible nano-effect.

Against this background, and as part of the REACH Substance Evaluation for silver, the current study was set up to measure the relative toxicity in soil of Ag-NP compared to the soluble Ag salt, and to relate this to fate and dissolution rate of the Ag-NPs. The Ag-NPs are expected to transform rapidly in soil: Ag^+-ions will be released through dissolution, but particles can also aggregate or sorb to soil particles, which will reduce their reactivity and lead to a lower toxicity than the untransformed NPs. A nitrification assay was selected to assess the toxicity, since this

has been reported as the most sensitive test for Ag (Langdon et al, 2014 and Langdon et al. 2015) The tests were performed in three agricultural soils, representing major soil types in Europe, to address the effect of varying dissolution rate on the relative toxicity. It has been established for Cu-NPs that a difference in toxicity between the metal salt and the metal nano-form can be largely explained by the difference in dissolution rate (Qiu and Smolders 2017); it is thus hypothesized that dissolution processes are also the explanatory factor for observed Ag effects. The nitrification in soil is affected by the free metal ion activity as shown for Zn for example (Mertens et al. 2007). The Ag dissolution was measured in soil solution at different moments in time after spiking, through a fractionation of the Ag in the pore water. By measuring the truly dissolved Ag, defined as the fraction <1kDa, it can be assessed if the toxicity is determined by the truly dissolved Ag fraction alone or if particles >1 kDa in the solution phase also contribute to toxicity (nano-effect). Special attention was devoted to the validity of this fractionation method since detection and sample preservation of Ag is notoriously difficult. This resulted in a better sensitivity for truly dissolved Ag compared to other studies (Diez-Ortiz et al. 2015), which already highlighted the importance of this fractionation. In addition, this data was to construct a compartment model, allowing, for the first time, to calculate transformation-dissolution of NPs in different soils and calculate half-lives.

MATERIALS AND METHODS

Chemicals

$AgNO_3$ (Heraeus, 63.49% Ag, purity >99.9%) was used as soluble Ag-salt, and a Ag-NP aqueous suspension (Heraeus, Silberpulver typ300-30, 37% Ag) was used as Ag-nanoform. The mass percentage of Ag in both products was verified with Inductively Coupled Plasma – Mass Spectrometry (ICP-MS, Agilent 7700). Prior to the measurements, the Ag-NPs were dissolved by hot HNO_3 digestion, followed by dilution in a 3% HNO_3 and 1.5% HCl matrix. For the ICP-MS

133 measurements of all samples (soil digest, pore waters and NP digests), an extensive rinsing
134 procedure of the sample uptake system was performed before each analysis sequence to avoid
135 carry-over and adhesion to the plastic tubing sequentially flushing with methanol, 25% NH_4OH,
136 30% HCl and 30% HNO_3 for 20 minutes, intermittently rinsing 5 minutes with ultrapure water.
137 The Ag mass percentage was confirmed for $AgNO_3$, however only 34% Ag was measured for the
138 Ag-NP. Therefore, this latter concentration (34%) and not the reported Ag content in the stock
139 solution (37%) was used to calculate the nominal doses. The particle size of the Ag-NP was
140 measured using Single Particle ICP-MS (Agilent 8800, Agilent Technologies) on a 12.5 ng L^{-1}
141 sample. Mean particle size was determined at 12.6 nm, but a fraction of the Ag-NP was smaller
142 than the size detection limit (12 nm) and thus not taken into account for particle size
143 determination. Granulometry of the original Ag-NP suspension (100x diluted) was further
144 determined by TEM (Tecnai Spirit microscope, FEI) using ParticleSizer software, and showed
145 the suspension consisted mainly of single primary particles which were spherical and relatively
146 homogeneous in size and shape. The mean primary particle size was 9.4 nm and the volume
147 specific surface area was 0.63 m^{-1}.
148
149 *Soils*
150 Soil samples from three European, arable soils were collected from the plough layer (0-20 cm).
151 The Rots soil was sampled in 2007, Poelkapelle in 2015, and Lufa 2.2 in 2016, all soils were air-
152 dried, sieved over 4 mm and stored in darkness until use. The topsoil characteristics are
153 summarized in Table 1. The soils were selected to have properties in the P10-P90 interval of
154 European agricultural soils, that is a pH value between 4.4 and 7.4, % Organic Carbon (OC)
155 content between 0.9 and 3.9% and cation exchange capacity (CEC) between 8.0 and 30.3 $cmol_c$
156 kg^{-1} (Reimann et al., 2014). These soil parameters have been shown to influence the toxicity of

Ag in soils: Langdon et al (2014) found a positive correlation between EC10, determined from potential nitrification rate (PNR), and pH and %OC, indicating a strong effect on sorption/complexation of Ag impacting the bioavailability. The soils were dried in a thin layer at 25°C in a plant growth cabinet with continuous illumination. After drying, all soils were sieved through 4 mm. The sieved and dried soils were stored in the dark.

Pore water fractionation method

Pore water ultrafiltration was performed to determine truly dissolved Ag, defined as the fraction <1 kDa. The accuracy of the fractionation method was verified. Several preliminary experiments were performed using the Cu^{2+} filter saturation method described by Cornelis et al (2010). However, that method failed by yielding large fractions of truly dissolved Ag for fresh Ag-NP suspensions. This is unexpected as the 1 kDa should retain particles as small as 1 nm. After various other tests (see supporting information), only tests with repeated use to saturate Ag sorption to the filter were adopted. During further testing, a comparison was made between unfiltered samples, samples filtered over 0.45 µm (Chromafil PET-45/25 polyester membrane filters) and samples filtered with an ultrafiltration device (1 kDa filter, Microsep, Pall Corporation) at 3800 g for 15 min. In addition, multiple filtration runs were conducted (one to three times) on freshly diluted samples of $AgNO_3$ or Ag-NP to prevent loss due to sorption on the filter membrane, which would result in an underestimation of the truly dissolved Ag concentration. The method with three successive runs of ultrafiltration proved to be the most accurate method, with a full recovery of ionic Ag combined with a relatively low recovery of Ag-NPs in the filtrate (Table 2). This method was, therefore, used for the determination of truly dissolved Ag in the pore water.

Transformation-dissolution test

For each of the three soils, 4500 g of soil was pre-incubated for 1 week at 20°C and at 60% moisture content, by addition of demineralized water to the air-dried soils until the desired moisture content was reached. After the incubation period, one third (1500 g) of each soil was spiked with $AgNO_3$, one third with Ag-NP - both at 50 mg Ag kg^{-1}-, and the last third was wetted with demineralized water to function as control treatment. A low Ag dose was chosen that was relevant for the toxicity tests, i.e. near EC50. The soils were thoroughly mixed after spiking and were incubated in closed 2.5 L pots with sufficient headspace to ensure aerobic conditions. At day 1, 4, 7, 14, 35 and 97 days after spiking, pore waters were sampled in duplicate by centrifugation (2000 g, 30 min.) in a double chamber system, using about 50 g soil. For the Poelkapelle soil, which has a heavy soil texture impeding extraction of large pore water volumes, four sampling replicates were taken and the small volumes of pore water were combined to two sampling replicates. After centrifugation, samples were filtered over 0.45 µm to obtain the total dissolved Ag concentration, conventionally defined as < 0.45 µm, .

Toxicity test

A range finding test was used to determine the spiking concentrations. The concentrations for this range finding test were based on the formula of Langdon et al. (2014). In this formula, the soil pH and %OC are used to predict the EC10 value for the potential nitrification rate (PNR), which is the nitrification potential in soil at saturated substrate concentrations. In practice, this was determined as the nitrification rate observed in the first 7 days after adding NH_4 as the substrate. The range finding test resulted in the selection of six (Rots and Poelkapelle) or seven (Lufa 2.2) different Ag concentrations for spiking. These concentrations ranged between 4 and 2500 mg added Ag kg^{-1} for the Rots soil, between 0.5 and 670 mg added Ag kg^{-1} for the Lufa 2.2 soil and

between 10 and 4600 mg added Ag kg^{-1} for the Poelkapelle soil, with a factor 3.2 between subsequent doses. Soil samples were pre-incubated for one week at 20°C at a moisture content of 60% of the water holding capacity. Then, the soils were spiked with stock solution $AgNO_3$ or Ag-NP to obtain seven or eight concentrations, including a control with demineralized water but no added Ag. For each treatment 50 g soil was spiked in triplicate. All subsamples were thoroughly mixed after spiking and incubated again for one week at 20°C before the toxicity tests were started. The soils amended with $AgNO_3$ were not leached prior to spiking. Metal toxicity I salt spiked soil can be largely affected by the counterion, especially when the metal cation is not very toxic on a molar basis, for example in the case of lead (Smolders et al. 2014). For Ag, however, this effect is lower as shown by no or very small differences in Ag toxicity to nitrification between leached and unleached soils (Langdon et al. 2014). After this equilibration time, the soils were amended with 100 mg NH_4-N (kg fresh soil)$^{-1}$ using a 80 mg $(NH_4)_2SO_4$ L^{-1} stock solution. This day of NH_4 spiking is termed day 0 of the toxicity test but is 7 days after Ag spiking. At day 0, 7, 14 and 28, the soil nitrate concentration was measured colorimetrically in a centrifuged soil extract, using 1 M KCl (1:2.5 w:v), after 2 h end-over-end shaking. Two endpoints were determined: (i) the PNR between days 0 and 14, which is calculated as the slope of the linear regression of soil nitrate concentration versus time for 0, 7 and 14 days for each replicate, and (ii) the substrate induced nitrification rate (SIN), which is calculated as the difference in soil nitrate concentration between 0 and 28 days, both expressed in mg NO_3-N (kg fresh soil)$^{-1}$ day^{-1}. In general, sensitivity of the nitrification assay to toxicants decreases with time when the NH_4 substrate gets depleted. Therefore, the PNR is expected to be more sensitive than the SIN, because depletion of the NH_4 substrate happens within 28 days in most soils. For the Lufa 2.2 and Poelkapelle soil and for both Ag forms, the PNR and SIN approached 0 mg NO_3-N (kg fresh soil)$^{-1}$ day^{-1} between 100 to1000 mg Ag kg^{-1}. At the largest doses (>1000 mg Ag kg^{-1})

in the $AgNO_3$ spiked soils, the added NO_3^- via the $AgNO_3$ interfered with the analytical precision. For example, when 1000 mg Ag kg^{-1} is added, there is theoretically about 130 mg NO_3-N kg^{-1} soil on top of the typical background of 60 mg NO_3-N kg^{-1} soil, by which low nitrification yields of, for example 10 to40 mg NO_3 kg^{-1}, are difficult to detect. Indeed, a large variability of PNR or SIN values, including negative values, was observed beyond that concentration. Therefore, all $AgNO_3$ treatments with > 1000 mg Ag kg^{-1} were excluded for PNR and SIN data analysis. At days 7 and 28 after start of the toxicity test, the soils were also sampled for dose confirmation. The soils were oven dried at 105°C and crushed with a mortar and pestle. Duplicate samples of 50 mg soil were weighed and digested with 1 mL HNO_3 (65%, Suprapur) for about 3 h and diluted to 10 mL before ICP-MS analysis. Measured doses were used in all subsequent data analysis.

Data analysis

The transformation-dissolution data yielded exponentially decreasing Ag data for the $AgNO_3$ spiked soils while a rise followed by a decline were observed for the Ag-NP spiked soils. This revealed a transformation-dissolution-fixation pattern which was modelled with compartmental analysis. The total dissolved Ag (<0.45 µm) and truly dissolved Ag (<1 kDa) concentration dynamics were fitted with a three-compartment model shown in Figure 1, consisting of Ag-NP, dissolved (total or truly dissolved) Ag and sorbed Ag ($Ag_{(s)}$). For the soils spiked with $AgNO_3$, [Ag-NP] = 0, and reversible first order kinetics were assumed yielding

$$[Ag] = \frac{k_b}{k_f + k_b}[Ag]_{t=0} + \frac{k_f}{k_f + k_b}[Ag]_{t=0}e^{-(k_f + k_b)t} \quad (1)$$

where [Ag] is the Ag concentration in the pore water (µg Ag L^{-1}), $[Ag]_{S,t=0}$ is the initial Ag concentration in the pore water (µg Ag L^{-1}), k_b is the first-order rate constant for the desorption of

Ag, k_f is the first-order rate constant for the sorption of Ag, and t is the time (days). The reaction half-live, i.e. the time to reach the point at which the dissolved Ag is half of the difference between initial and equilibrium concentration, equals

$$t_{1/2} = \frac{\ln(\frac{k_f - 2k_b}{2k_f})}{-(k_f + k_b)} \quad (2)$$

For the soils spiked with Ag-NP and first order transformation-dissolution and reversible first order, this reads

$$[Ag] = \frac{k_b}{k_f + k_b}[Ag]_{t=0} + \frac{(k_{diss} - k_b)}{k_f - k_{diss} + k_b}[Ag]_{NP,t=0}e^{-k_{diss}t}$$

$$- \frac{k_{diss}k_f}{(k_f + k_b)(k_f - k_{diss} + k_b)}[Ag]_{t=0}e^{-(k_f + k_b)t} \quad (3)$$

where $[Ag]_{t=0}$ is the initial pore water concentration of Ag in the Ag-NP amended soils, k_{diss} is the first-order rate constant for the dissolution of Ag-NPs. The half-live for dissolution of Ag-NP equals

$$t_{1/2} = \frac{\ln(2)}{k_{diss}} \quad (4)$$

The first-order rate constants of forward sorption (k_f) and backward desorption (k_b) were first fitted on the data obtained from the soil spiked with $AgNO_3$ for each soil separately. Secondly, the dissolution rate constant (k_{diss}) was fitted using the soil-dependent k_f and k_b values.

The data from the toxicity test, i.e., the PNR or SIN, were first converted to their value relative to the average of the control value (Y, in %). Subsequently, these relative responses, Y, were statistically analyzed using a log-logistic dose-response model fitted with JMP (JMP Pro 12, SAS Institute) to derive the 10% inhibition concentration (EC10, equation (5)) or 50% inhibition concentration (EC50, equation (6)) as

$$Y = \frac{100}{1 + \frac{1}{9} * (\frac{dose}{EC10})^{\beta}} \quad (5)$$

$$Y = \frac{100}{1 + (\frac{dose}{EC50})^{\beta}} \quad (6)$$

where 'dose' refers to the measured total Ag concentration in soil, and β denotes the slope of the dose-response relationship. The statistical significance of the effect of the Ag form (i.e., $AgNO_3$ or Ag-NP) was tested by the introduction of a dummy variable D_i to discriminate the two treatments (0 for $AgNO_3$, 1 for Ag-NP). The EC50 or EC10 value of the Ag-NP treatments was statistically different from $AgNO_3$ when λ was significantly ($p < 0.05$) different from zero, e.g. for the EC10 model

$$Y = \frac{100}{1 + \frac{1}{9} * (\frac{dose}{EC10 + \lambda D_i})^{\beta}} \quad (7)$$

RESULTS

Transformation-dissolution of Ag-NP

For all soils spiked with $AgNO_3$, the total dissolved Ag concentration (<0.45µm) in the pore water decreased with time (Figure 2) and obeyed the trends predicted by the compartmental model. The reaction half-lives (in days), based on the total dissolved concentration, for the Ag^+ ageing could be inferred from k_f and k_b (see data analysis) and equalled 9 (Rots), 2 (Lufa 2.2) and 20 days (Poelkapelle; details not shown). In the Ag-NP-spiked soils, the total dissolved Ag concentration initially increased; this increase was, however, rapidly followed by a concentration decrease, analogous to the $AgNO_3$ scenario. The truly dissolved Ag concentrations were lower than corresponding total dissolved Ag in the pore water (Figure 2). The few exceptions are ascribed to analytical errors by sample contamination. The reaction half-lives based on the truly

dissolved Ag concentrations for the Ag^+ ageing equalled 22 (Rots), 4 (Lufa 2.2) and 8 days (Poelkapelle; details not shown). The fraction of truly dissolved Ag (<1 kDa) to total dissolved Ag (<0.45 µm) in the pore water was lower in the AgNP than in the corresponding $AgNO_3$ treatments (data not shown). This was especially prominent in the first days after Ag-spiking. Differences in pore water Ag concentrations between soils were also observed, whereby Ag concentrations in the Rots and Lufa 2.2 soils showed similar trends. In the Ag-NP-spiked Poelkapelle soil, truly dissolved Ag was above Ag background concentration only at days 4 and 7. In this soil, the Ag background concentration in the control treatment was around 1 µg Ag L^{-1}, about tenfold above that in the Lufa 2.2 and Rots control soils. This is likely an artefact: this soil contains the largest clay and silt content and the pore water volumes obtained were very small, requiring large dilutions to obtain sufficient sample volume for the ICP-MS measurements.

The three-compartment model, including dissolution and sorption, described the observations of the Ag-NP adequately. Large differences were found in the half-life of Ag-NP between the different soils (Table 3). At the start of the experiment, the total dissolved Ag concentration in the pore water was higher in the $AgNO_3$-spiked soils than that in the Ag-NP-spiked soils, in all three soils (Figure 2). During the three month incubation period, differences in total dissolved Ag concentration between the $AgNO_3$ spiked soil and Ag-NP-spiked soil decreased. Of special interest is the difference in soluble Ag in the period of the toxicity tests, i.e. between days 7 – 35 after Ag spiking. Statistical analyses of (log-transformed) Ag concentrations versus time (days 7, 14 and 35 data) showed that Ag concentrations in the Poelkapelle was markedly and significantly ($p<0.01$) larger in the $AgNO_3$ than in the AgNP by, on average, factors 7 (total dissolved) and 47 (truly dissolved). In contrast, for soil Rots, the corresponding factors were only factors 1.6 (total

dissolved, $p<0.05$) and 1.2 (truly dissolved, not significant at $p=0.05$) while for soil Lufa 2.2 the factors were 2.5 (total dissolved) and 6.2 (truly dissolved), both statistically significant at $p<0.05$.

Toxicity test

The doses reported below are all based on measured total soil Ag concentrations (details not shown) collected at 14 and 35 days after spiking. There were logically no significant effects of time on the total Ag concentration in soil. The dose confirmation, as expressed by the slopes of the regressions between the measured and nominal Ag dose, showed adequate recovery (86-104%) for the $AgNO_3$ spiked soil. In the Ag-NP spiked soils, the recovered Ag concentration showed larger variation (65-130%), likely because the spiking with the particles was more heterogeneous. The average measured doses between 14 and 35 days after spiking were used in all data analysis**.**

The control treatments (no Ag-spiking) showed a sufficient nitrification with PNR values that fall within expected trends based on soil pH (Table 4) (Smolders et al. 2001). Dose response curves for effect of $AgNO_3$ and Ag-NP on PNR and SIN in the different soils were fitted against the total Ag concentration in soil (mg Ag kg^{-1}) (Figure 3). The Lufa 2.2 soil is the most sensitive to Ag-spiking and the Poelkapelle soil the least sensitive (Table 5). No significant differences in toxicity between the $AgNO_3$ and Ag-NP spiking were identified in Lufa 2.2 and Rots. In contrast, in the Poelkapelle soil a significantly ($p < 0.05$) higher sensitivity of the $AgNO_3$ spiked samples compared to Ag-NP spiking was observed for both the PNR and SIN endpoints. The EC10 and EC50 values based on SIN were larger than those based on PNR for all three soils and both forms of Ag tested, confirming the negative correlation between test duration and sensitivity as toxicity indicator (see above).

DISCUSSION

The general objective of this study was to assess the relative toxicity of Ag-NPs compared to a soluble Ag-salt in soil in order to assess the presence or absence of a nano-effect, and to relate the observations to Ag dissolution rate. The nitrification assay was chosen as a sensitive indicator for Ag toxicity (Langdon et al. 2014).

In none of the soils the ECx values were lower for Ag-NP-spiked samples compared to $AgNO_3$-spiked samples. On the contrary, in the Poelkapelle soil, significantly higher ECx values were calculated for the Ag-NP-spiked samples compared to the $AgNO_3$- spiked samples, indicating a lower toxicity of the Ag-NP than the soluble $AgNO_3$ salt in that soil. This finding that the Ag-NPs are not more toxic than the salt forms at equivalent total concentrations is in agreement with the metastudy by Notter et al (2014), who found that in most studies the nanoforms of metals (nano-Ag, nano-CuO, and nano-ZnO) were not more toxic than a corresponding metal salt.

The results from the pore water fractionation of the Poelkapelle soil support the hypothesis that the measured difference in toxicity between $AgNO_3$ and Ag-NP is related to a difference in dissolution; higher concentrations (~factor 50) of truly dissolved Ag were measured in the $AgNO_3$ spiked samples compared to the Ag-NP-spiked samples during the time frame of the nitrification experiment, and this fraction is generally accepted to be responsible for (potential) toxic responses. In the Rots soil, truly dissolved Ag in the pore water were not different between the two Ag forms (see above), and there was no significant difference in ECx values. In the Lufa 2.2 soil, however, comparable ECx values were derived for both forms while the pore water data revealed differences in Ag-solubility that were over factor 10 at the start of the test (day 7) and

that did not fade out completely at the other times of the toxicity tests. Added to this is the consideration that the truly dissolved Ag in Ag-NP amended soils is potentially overestimated by the three-step size fractionation method: Table 2 shows that the truly dissolved Ag of a pure Ag-NP dispersion increases during each step, potentially related to dissolution reactions taking place during fractionation. Hence; the truly dissolved Ag in the pore water of the Ag-NP amended soil was likely lower than measured. That suggestion, combined with the equal total soil based ECx data show that truly dissolve Ag insufficiently explains the equal toxicity of the two Ag forms. This suggests that nanoparticulate Ag in soil contributes to toxicity in that soil. A more complete understanding or quantification of the contribution of the NP to toxicity would require a transformation dissolution test performed at all doses. We did not perform this for practical reasons. Even with more detailed data, it remains unclear if the pore water Ag, derived from the soil macropores, is the true indicator of the toxic dose since truly dissolved Ag and Ag-NP may adhere to the biofilms of the microorganisms associated with the particles, resulting in local hotspots of toxicity.

The dissolution and ageing reactions observed and described with the three-compartment model correspond to previous studies that highlight the importance of ageing in fate and toxicity test with Ag (Settimio et al. 2014; Diez-Ortiz et al. 2015). These observations confirm the hypothesis that Ag toxicity is driven by Ag dissolution, which conforms to results from aquatic organisms (Wang et al. 2012; Sakamoto et al. 2015). It also highlights that metal NP toxicity studies in soil need to give due attention to the dynamics of transformation-dissolution and that NP toxicity testing needs sufficient incubation to avoid underestimation of its toxic potential.

Fractionation of the pore water allowed the distinction between Ag ions (ionic Ag and small inorganic/organic Ag complexes; assessed as 'truly dissolved silver') and Ag-NPs/Ag-complexes. The fraction of truly dissolved Ag to total dissolved Ag was lower in the first days of incubation of Ag-NP spiked soils than in $AgNO_3$-spiked soils. Since the Ag-NPs are retained by the 1 kDa filter (Table 2), this suggests that nanoparticulate Ag was indeed initially present in the unfiltered pore water. Over time, the fraction of truly dissolved Ag in the pore water decreased for the $AgNO_3$ treatment, likely due to the formation of Ag complexes or colloids via association with organic or mineral colloids that are naturally present. In the Poelkapelle soil, truly dissolved Ag concentrations were close to background concentration. Total dissolved Ag was still detectable in the pore water, indicating that Ag is present in the pore water for example as colloids, not as truly dissolved Ag. It is unsure why the formation of colloids (>1 kDa) is more promoted in the Poelkapelle soil than in the other soils, since the pore water DOC and Ca, factors which can induce colloid formation, are not the largest among the soils. Possibly, due to the relatively high clay content of this soil, local anaerobic spots were present which may have affected the transformation.

The three different soils also showed a different sensitivity to Ag toxicity. The Poelkapelle soil had the highest ECx values among the three soils, which was already predicted by the formula from Langdon et al. (2014). This is likely related to the relatively high CEC, clay and %OC, properties that positively affect the complexation/sorption of Ag^+-ions. Also the dissolution rate is lowest in this soil, as obtained from the three-compartment model. On the contrary, the Lufa 2.2 soil was the most sensitive to Ag, with EC10 values of only 3.8 mg Ag kg^{-1}, which can be explained by the low pH.

Taken together, using PNR and SIN as endpoints of nitrification, no indications of a nano-effect were shown when evaluated on the basis of total Ag in soil. In contrast, in one soil Ag-NP was significantly less toxic than $AgNO_3$. This was attributed to the large factor differences in dissolution rate between the two Ag forms in the period of the toxicity test. In one soil, however, Ag-NP yielded less truly dissolved than the salt while the toxicity of total soil Ag was equal, suggesting that the nanoparticulate Ag contributed to toxicity in soil. Despite this, it is considered adequate to perform a Ag-NP risk assessment using the Ag-salt toxicity data based on this test, these soils and this Ag-NP form.

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DATA AVAILABILITY

Data are available on request. Please submit requests for data to E. Smolders

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List of Figures

Tables

Table 1: Selected topsoil characteristics (0 – 20 cm) and pore water concentration of dissolved organic carbon (DOC), K, Ca, and Fe, sampled after a 2 week incubation period

		Rots	Lufa 2.2	Poelkapelle
Soil characteristics				
Country of origin		France	Germany	Belgium
pH[a]		7.3	5.4	6.0
OC[b]	%	1.30	1.61	3.8
Sand/Silt/Clay[c]	%	20/50/10	76/17/8	17/66/16
CEC[d]	$cmol_c\ kg^{-1}$	14.3	9.7	19.7
Total Ag[e]	mg Ag kg^{-1}	0.4	0.4	0.1
Pore water concentrations[f]				
DOC[g]	mg C L^{-1}	20.9 (1.9)	32.1 (7.3)	12.2 (3.3)
K	mM	0.85 (0.01)	0.06 (0.01)	0.02 (0.01)
Ca	mM	10.16 (0.12)	1.71 (0.38)	1.55 (0.10)
Fe	µM	0.15 (0.09)	59 (1)	1.08 (0.64)

[a]: pH determined in a 1:5 (w:v) 0.01 M $CaCl_2$ suspension; [b]: %OC determined as difference between total (Variomax CN analyser) and inorganic C content (determined as pressure increase after HCl and $FeSO_4$ addition);[c]: particle size analysis by pipette method, after digestion of organic matter with hydrogen peroxide, removal of carbonate and soluble salts with HCl, and dispersion with sodiumhexametaphosphate; [d]: determined at soil pH with Cohex method; [e]: determined with ICP-MS after hot HNO_3 digestion; [f]: pore water was sampled by centrifugation in a double chamber system and measured with ICP-MS after acidification to 1% HNO_3 to determine K, Ca and Fe concentrations; [g]: DOC determined by catalytic combustion after acidification to pH 2 and O_2 purging (Analytic Jena, Multi N/C 2100S); means (n = 2) are reported with standard deviations in parentheses.

Table 2: Recovery of Ag (%) from a $AgNO_3$ solution and a Ag-NP suspension, both at 10 µg Ag L^{-1} in a 5 mM $Ca(NO_3)_2$ background, using different filtration cut-offs and sequential runs

		Recovery (%) – $AgNO_3$		Recovery (%) – Ag-NP	
Filtration	N° of runs	Sample A	Sample B	Sample A	Sample B
none	1	91.5	90.8	100	100
0.45 µm	1	90.4	91.4	45.4	72.6
1 kDa	1	23.0	20.4	4.1	3.7
	2	69.2	71.9	20.0	21.9
	3	101.9	102.3	31.5	32.9

Table 3: First-order dissolution rate constant (k_{diss}) and half-live ($t_{1/2}$) of Ag-NPs in the Rots, Lufa 2.2 and Poelkapelle soils based on the fit of a three-compartment model on measured truly dissolved Ag concentrations, obtained using 1kDa ultrafiltration[a]

	k_{diss}		$t_{1/2}$ (day)
Rots	0.232	n.d.	3.0
Lufa 2.2	0.135	(0.058 - 0.265)	5.1
Poelkapelle	0.019	(0.003 – 0.064)	35.8

[a]Values in brackets give the 95% confidence interval.

n.d.: could not be determined

548 Table 4: Potential nitrification rate (PNR, mg N kg^{-1} day^{-1}) and substrate induced nitrification rate

549 (SIN, mg N kg^{-1} day^{-1}) in the no Ag-spiked control soils[a]

	PNR	**SIN**
Soil	mg N kg^{-1} day^{-1}	mg N kg^{-1} day^{-1}
Rots	6.0 (0.3)	3.1 (0.2)
Lufa 2.2	2.9 (0.4)	2.5 (0.8)
Poelkapelle	5.7 (0.2)	3.0 (0.1)

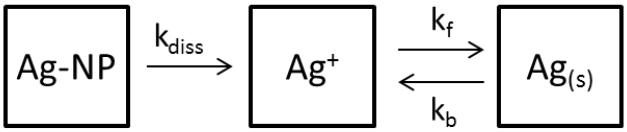
550 [a]Mean values with standard deviation (n = 3) given in parenthesis.

551 Table 5: EC50 and EC10 values (mg Ag kg^{-1} soil) and 95% confidence intervals for the PNR
552 over 14 days incubation and SIN over 28 days incubation, for the Rots, Lufa 2.2 and Poelkapelle
553 soils based on measured total Ag concentrations in soil

	EC10 ($AgNO_3$)		EC10 (Ag-NP)		EC50 ($AgNO_3$)		EC50 (Ag-NP)	
	mg Ag kg^{-1} soil							
PNR								
Rots	4.8	(2.3 – 10)	9.0	(4.1 – 17)	49	(30 – 83)	68	(47 – 97)
Lufa 2.2	3.8	(1.2 - 11)	3.8	(0.9 – 14)	36	(8 - 143)	38	(23 – 62)
Poelkapelle	**8.1***	(4.1 – 15)	29	(13 – 59)	**66***	(34 – 128)	242	(165 – 356)
SIN								
Rots	30	(22 – 41)	35	(22 – 54)	113	(91 – 146)	141	(112 – 177)
Lufa 2.2	42	(29 – 72)	37	(33 – 42)	100	(76 – 133)	107	(93 – 114)
Poelkapelle	**45***	(33 – 61)	132	(90 – 189)	**134***	(98 – 182)	397	(269 – 568)

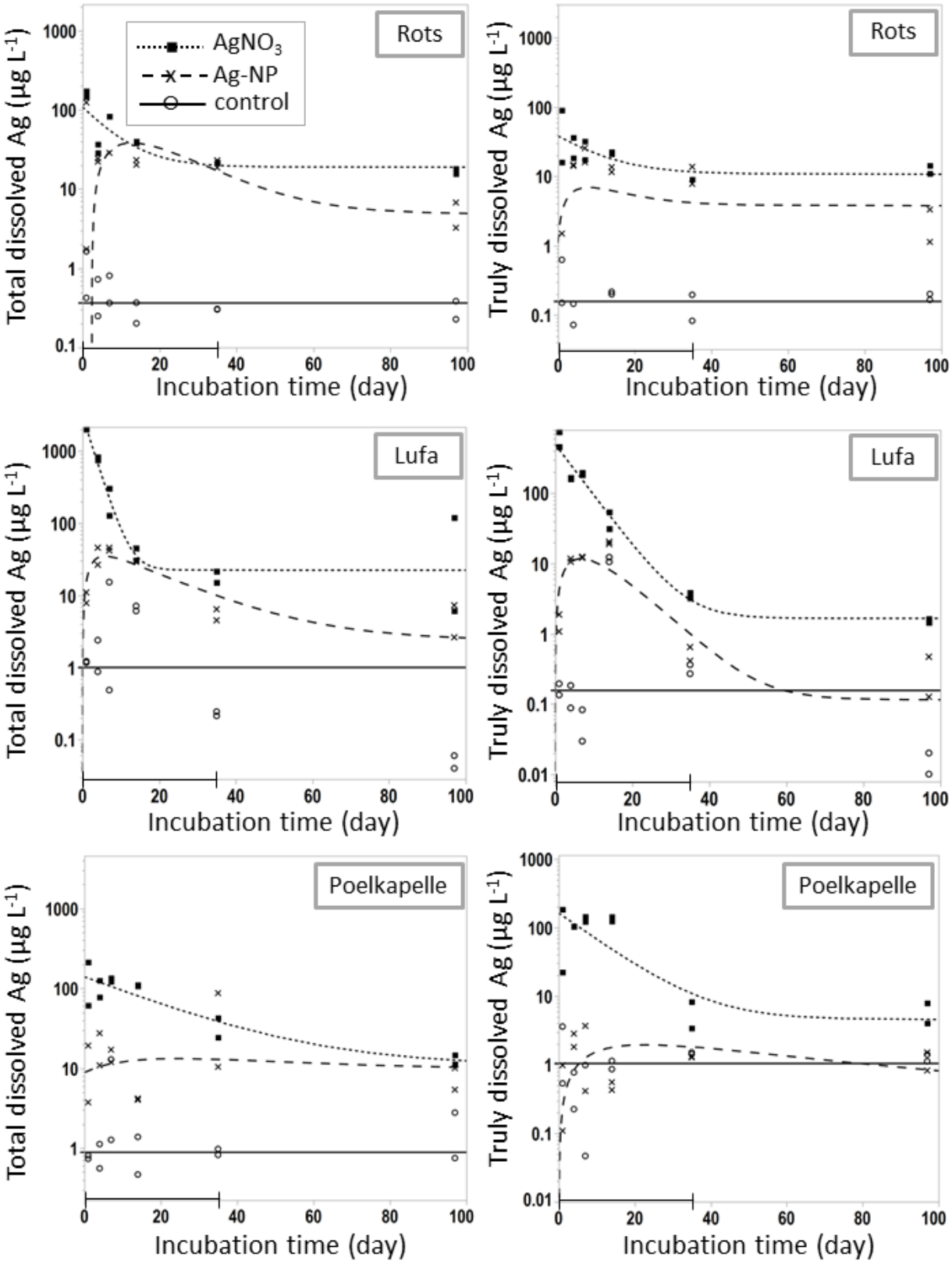
554 *significantly different ($p<0.05$) compared to the Ag-NP

555 Figure 1: A three-compartment model was used to fit the measured Ag concentration in solution
556 (either total or truly dissolved) during the incubation period. Rate constants for the
557 transformations are given by k_{diss} as the first-order rate constant for the dissolution of Ag-NP, k_f
558 as the first-order rate constant for the sorption of Ag, and k_b as the first-order rate constant for the
559 desorption of Ag.
560



561

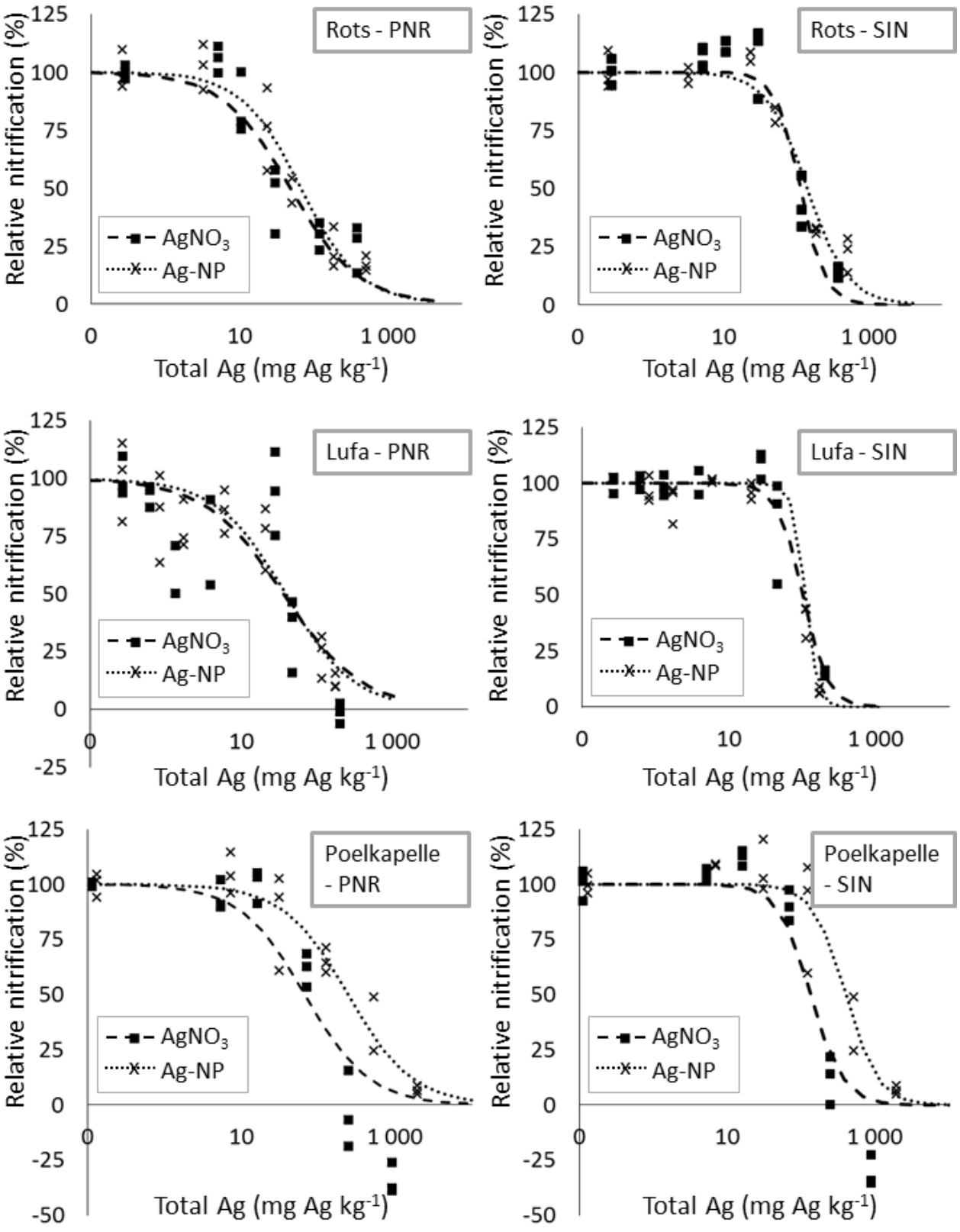
562 Figure 2: Total dissolved (<0.45 µm) and truly dissolved (<1 kDa) Ag concentration in the pore
563 water of the Rots, Lufa 2.2 and Poelkapelle soils over a three month incubation period. Soils were
564 either not spiked (control) or spiked at a fixed dose of 50 mg Ag kg^{-1} with $AgNO_3$ or Ag-NPs.
565 Dotted and dashed lines are model fits for the compartment model, the full line shows the median
566 concentration in the control. Note the different axes. All measurements were performed in
567 duplicate. Bar on the x-axis indicates the time range of the SIN toxicity test (up to 35 days after
568 Ag spiking). Note differences in dissolution between Ag-NPs and $AgNO_3$ in the Poelkapelle soils
569 within that time range.


Rots
AgNO3
Ag-NP
control
Total dissolved Ag (µg L-1)
Truly dissolved Ag (µg L-1)
Incubation time (day)
Lufa
Poelkapelle

570

571

572 Figure 3: Effect of $AgNO_3$ and Ag-NP spiking on the nitrification in the Rots, Lufa 2.2 and
573 Poelkapelle soils. Soil samples were left to equilibrate for 7 days following Ag application.
574 Afterwards, NH_4^+ substrate was added and the potential nitrification rate (PNR) was measured
575 between 0 and 14 days after NH_4^+ addition, and substrate induced nitrification rate (SIN) between
576 0 and 28 days. Nitrification rates are normalized (Relative nitrification, %) to the corresponding
577 control treatments (no Ag added; cfr. Table 4). Lines illustrate the dose-response relationship
578 based on the total measured Ag in soil (mg kg^{-1}).


Rots - PNR
Rots - SIN
Lufa - PNR
Lufa - SIN
Poelkapelle - PNR
Poelkapelle - SIN
Relative nitrification (%)
Total Ag (mg Ag kg-1)
AgNO3
Ag-NP
125
100
75
50
25
0
-25
-50
0
10
1 000

579