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The effect of soil properties on the toxicity and bioaccumulation of Ag nanoparticles and Ag ions in *Enchytraeus crypticus*

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ABSTRACT

Standard natural Lufa soils (2.2, 2.3 and 5 M) with different organic carbon contents (0.67–1.61%) and pH_{CaCl2} (5.5–7.3) were spiked with ionic Ag ($AgNO_3$) and polyvinyl pyrrolidone (AgNP-PVP) and citrate (AgNP-Cit) coated Ag nanoparticles (NPs). *Enchytraeus crypticus* were exposed for 21 days to assess effects on survival and reproduction. Soil, pore water and animals were analyzed for Ag. AgNP-Cit had a strong increasing effect on soil pH, leading to high enchytraeid mortality at concentrations higher than 60–100 mg Ag/kg dry soil which made it impossible to determine the influence of soil properties on its toxicity. LC50s were lower for $AgNO_3$ than for AgNP-PVP (92–112 and 335–425 mg Ag/kg dry soil, respectively) and were not affected by soil properties. $AgNO_3$ and AgNP-PVP had comparable reproductive toxicity with EC50s of 26.9–75.2 and 28.2–92.3 mg Ag/kg dry soil, respectively; toxicity linearly increased with decreasing organic carbon content of the soils but did not show a clear effect of soil pH. Ag uptake in the enchytraeids was higher at higher organic carbon content, but could not explain differences in toxicity between soils. This study indicates that the bioavailability of both ionic and nanoparticulate Ag is mainly affected by soil organic carbon, with little effect of soil pH.

1. Introduction

The numbers of consumer products that contain nanoparticles (NPs) have been increasing dramatically recently. Silver nanoparticles (AgNPs) are among the most widely used NPs in various products including cosmetics, paints, textiles and many others (Vance et al., 2015). As a consequence, there is increasing concern about their release from products which may lead to exposure of the receiving environment and posing harm to ecosystems. While most previous work focused on effects in aquatic environments, there is increasing concern about the effects of AgNPs on soil organisms since recent studies showed that washing of paints with rainwater and sedimentation (depending on the water chemistry; Topuz et al., 2015) may lead to the spread and accumulation of AgNPs also in soils.

The stability of AgNPs is affected by the environmental conditions in the soil solution (Klitzke et al., 2015). Therefore, evaluation of the toxicity of $AgNP_S$ in soil should also consider their speciation in pore water. Moreover, data on the effect of soil properties, such as organic carbon content and pH of the soil, on the toxicity of Ag nanoparticles to soil organisms are lacking. Since Ag may complex with organic matter, altering its speciation and bioavailability (Cornelis et al., 2012), soil organic matter content may affect the toxicity of AgNPs. Soil pH may also affect the speciation of silver by its influence on the processes of dissolution, agglomeration and adsorption (Liu and Hurt, 2010). In addition to the effect of soil properties, there are still doubts about the form of Ag causing toxicity. While some studies report an effect of nano-specific properties (Colman et al., 2013; Eom et al., 2013), others are pointing at a dominant role of ionic silver in causing the toxicity of AgNPs (Heckmann et al., 2011; Gomes et al., 2013; van Der Ploeg et al., 2014; Diez-Ortiz et al., 2015).

Although some studies have been performed with nematodes (e.g., Yang et al., 2014; Starnes et al., 2015), isopods (e.g., Tourinho et al., 2015) and springtails (Waalewijn-Kool et al., 2014), so far most studies have been done with earthworms to assess AgNP toxicity in soil; see Table S1 in Supporting Information. Heckmann et al. (2011) observed no effect of polyvinyl pyrrolidone-coated AgNPs (AgNP-PVP) on the survival and reproduction of the earthworm *Eisenia fetida* at concentrations as high as 1000 mg Ag/kg dry soil, while LC50 of citrate-coated AgNPs (AgNP-Cit) to *Eisenia andrei* was higher than 2000 mg Ag/kg dry soil (Kwak et al., 2014). In several other studies, AgNPs coated with different materials were found to be more toxic to different earthworm species, leading to effect concentrations lower than 1000 mg Ag/kg dry soil (Table S1). This concerned among others reproductive toxicity (Schlich et al., 2013) and the avoidance response of

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E. fetida to AgNP-PVP (Shoults-Wilson et al., 2011), and the responses of the antioxidant system, acid phosphatase and ATPase of *E. fetida* to AgNPs (Hu et al., 2012). The cytotoxicity of AgNP-PVP in *E. fetida* was determined by Schlich et al. (2013). AgNPs with different coatings also did affect the growth and reproduction of the earthworm *Lumbricus rubellus* (van Der Ploeg et al., 2014; Makama et al., 2016).

Compared to earthworms, relatively few studies have been performed on the toxicity and bioaccumulation of AgNPs in enchytraeids (Gomes et al., 2013; Bicho et al., 2016; see Table S1). Enchytraeids are ecologically relevant soil organisms owing to their role in decomposition and bioturbation processes in soils and are commonly preferred for toxicity testing due to their sensitivity to a wide range of stressors (Didden and Römbke, 2001). One of the most commonly used species is *Enchytraeus crypticus* (Castro-Ferreira et al., 2012), which tolerates soils with total organic carbon contents down to 1.5% and pH in the range of 4.3–6.8 (Vasickova et al., 2015). Moreover, metal uptake in *E. crypticus* is exposure concentration dependent (He and van Gestel, 2013) and can be higher than in earthworms exposed to the same soils (Luo et al., 2014). It therefore is worth to investigate the toxicity and bioaccumulation of Ag and AgNPs also in this species.

However, data are still lacking in the literature on: 1) the toxicity of AgNPs with different capping agents, which have potential to affect their fate and effects, 2) the source of Ag (Ag ions and/or AgNPs) that is causing the toxicity, 3) the characterization of pore water and organisms in terms of Ag concentrations to evaluate the possible exposure pathways and to determine bioaccumulation levels, and 4) the effect of organic carbon content and pH of the soil on the toxicity of AgNPs.

Therefore, this study aimed at: a) determining Ag bioaccumulation and the lethal and reproductive toxicity of PVP and citrate coated AgNPs to *E. crypticus* in three different soils, b) assessing the effect of Ag source on Ag uptake and toxicity, including $AgNO_3$ as a ionic Ag form, c) relating Ag bioaccumulation and toxicity to soil properties (e.g., organic carbon content and pH), d) relating toxicity and Ag uptake by the animals to the speciation of AgNPs in the soil pore water, and e) relating toxicity to Ag body concentrations in the test animals.

2. Materials and methods

2.1. Test organisms

Enchytraeus crypticus (Enchytraeidae; Oligochaeta; Annelida) has been cultured for over 10 years in the laboratory of the Department of Ecological Science, Vrije Universiteit, Amsterdam, The Netherlands. *E. crypticus* were cultured in agar media prepared with soil extract and kept in a climate room at constant temperature (16 °C), relative humidity (75%) and dark conditions. The culture was fed twice a week with a mixture of oatmeal, dried baker's yeast, egg yolk powder, and fish oil. For the experiments, adult *E. crypticus* with white spots in the clitellum region were selected.

2.2. Test compounds

Polyvinyl pyrrolidone (AgNP-PVP) and Citrate (AgNP-Cit) coated silver nanoparticles and ionic silver in the form of $AgNO_3$ were used in this study. $AgNO_3$ served as an ionic control to distinguish possible effects originating from the nanoparticles from those of the released Ag^+ ions. AgNP-Cit and AgNP-PVP were purchased from NanoSys GmbH (Switzerland) and obtained as suspensions in water at concentrations of 1 g/L and 10 g/L AgNP, respectively. $AgNO_3$ (Sigma-Aldrich, > 99%) was dissolved in deionized water (Milli-Q). Nominal size of AgNP-Cit and AgNP-PVP (1 g/L) provided by the manufacturer was around 25 nm; Z-average sizes of AgNP-Cit and AgNP-PVP (1 mg/L) in deionized water measured by DLS were 61 ± 0.6 nm and 42 ± 0.4 nm, respectively. Detailed characterization of the AgNPs used has been reported in Topuz et al. (2014).

Table 1
Characteristics of the Lufa soils used for the tests determining the toxicity to *Enchytraeus crypticus* of silver nanoparticles and silver nitrate. Characterization was provided as average ± standard deviation by the supplier (LUFA-Speyer, Germany, 2009) (for more detailed information, see: http://www.lufa-speyer.de/).

Parameters	Soils		
	Lufa 2.2	Lufa 2.3	Lufa 5 M
organic carbon in % C	1.61 ± 0.15	0.67 ± 0.04	0.98 ± 0.05
pH (0.01 M $CaCl_2$)	5.5 ± 0.1	5.8 ± 0.7	7.3 ± 0.1
cation exchange capacity (meq/100 g)	10.0 ± 0.7	7.3 ± 1.0	16.1 ± 4.2
maximum water holding capacity (g/100 g)	43.3 ± 2.6	35.6 ± 1.7	39.8 ± 2.1
weight per volume (g/1000 mL)	1236 ± 32	1334 ± 6	1291 ± 45
soil type (German DIN)	Loamy sand	Silty sand	Loamy sand
soil type (USDA)	Sandy loam	Loamy sand	Sandy loam

2.3. Test Soils

To evaluate the effect of soil properties, the toxicity experiments were conducted in three natural standard soils, namely, Lufa 2.2, Lufa 2.3 and Lufa 5 M. Lufa 2.2 is a standard soil type widely used in soil ecotoxicology since it provides favorable conditions for toxicity studies. Lufa 2.3 soil was selected to have different organic carbon content but similar pH as Lufa 2.2 soil, and Lufa 5 M soil to have a different pH but intermediate organic carbon contents. The properties of the test soils are summarized in Table 1. Soils were air dried at 40 °C before being used in the experiments.

2.4. Toxicity experiments

Effects of the three silver forms on the survival and reproduction of *E. crypticus* were determined according to OECD (2004), but using an exposure duration of 3 weeks as recommended by Castro-Ferreira et al. (2012). Test concentrations were selected based on LC50/EC50 values previously reported for earthworms and enchytraeids (see Table S1). AgNPs was tested at 25.6, 64, 160, 400, 1000 and 2500 mg Ag/kg dry soil.

The pH_{CaCl2} of soils spiked with AgNP-Cit at 400 mg Ag/kg dry soil was around 8.50 and all organisms died in a few hours. Also 160 mg Ag/kg dry soil led to improper conditions especially for Lufa 5 M, which had a higher measured pH_{CaCl2} (7.14 ± 0.002) than the other soils. Therefore, AgNP-Cit was tested only at concentrations of 25.6 and 64 mg Ag/kg dry soil.

$AgNO_3$, which often is more toxic than AgNPs (Table S1), was tested at 10.6, 25.6, 64, 160, 400, 1000 mg Ag/kg dry soil.

The soils were spiked in glass jars with the different Ag forms dissolved in demineralized water, at the same time adjusting soil moisture content to 50% of the water holding capacity (WHC). After spiking, the soils were mixed to achieve an as homogeneous distribution of the Ag as possible. For the 2500 mg Ag/kg concentration of AgNP-PVP, spiking had to be performed in steps, in between which the excess amount of suspension was evaporated to keep the moisture content of the soil at 50% of the WHC. Control soils received demineralized water only. Four replicates were prepared for each test concentration and control. For each replicate, 30 g moist soil was placed in a 100 mL glass jar and equilibrated overnight. Toxicity tests started with the introduction of 10 adult *E. crypticus*, with clearly visible clitella and approximately 1 cm length, to the jars. A few grains of oat meal were added for food. The jars were covered with perforated aluminum foil for air transfer and incubated at 20 ± 1 °C and 12:12 h light:dark. Water content and food availability were checked twice a week and adjusted if necessary. After 21 days, the soil from each test jar was transferred to a plastic box (250 mL) to collect the surviving animals, which were kept overnight in petri dishes filled with ISO 6341 dilution solution (ISO, 1996)