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# Earthworm avoidance of silver nanomaterials over time☆

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ABSTRACT

Avoidance behaviour offers a highly relevant information as it reveals the ability to avoid (or not) possible toxic compounds in the field, hence it provides information on reasons for the presence/absence in the field. The earthworm *Eisenia fetida* was used to study avoidance behaviour to four silver forms (three nanomaterials (NMs) and one salt) over four time points (24, 48, 72 and 96 h), using OECD standard soil. Avoidance behaviour depended on both exposure material and concentration, but in general changed little with exposure duration. Avoidance was highest for the salt ($AgNO_3$) for all exposure durations and showed a continuous higher avoidance with time (based on $EC_{50}$ values). The AgNMs avoidance was in the order NM300K<AgNM-non coated = AgNM- PVP coated. It was not possible to identify one soil solution fraction that correlated with $EC_{50}$ across materials.



## 1. Introduction

Earthworms are abundant, represent a large biomass, and are crucial to soil fertility in many terrestrial ecosystems (Scott-Fordsmand et al., 2000). Terrestrial environments may receive a large number of pollutants via direct applications or as contaminants in other products, including also nanomaterials (NMs). Studies have shown that silver nanomaterials (AgNMs) cause toxicity to worms, with effects on survival, reproduction and enzyme activity (Bicho et al., 2016; Gomes et al., 2015a; Hayashi et al., 2013a, 2013b, 2016; Heckmann et al., 2011; Ribeiro et al., 2015). Fewer studies have dealt with the avoidance behaviour for an organism exposed to silver nanomaterials. Avoidance behaviour is considered a highly differentiated measure in relation to other toxicity endpoints in soil organisms (Brami et al., 2017a; Hund-Rinke et al., 2003; Liang et al., 2017; Yeardley et al., 1996). It provides information on reasons for the presence/absence of organisms at field sites (e.g. a contaminated site) as organisms may be absent both due to direct toxic population effects (mortality and reduced reproduction) or because the worms simply avoid an area where they can detect the pollutant. As mentioned, few studies have reported on the avoidance behaviour of worms when exposed to NMs, and most of these studies only reported avoidance behaviour at one time point (usually 48 h, which is the requirement for the standard test guideline, i.e. not allowing to follow time changes) e.g. (Liang et al., 2017; Shoults-Wilson et al., 2011). For example, avoidance was more pronounced for Cu Nanomaterials (CuNMs) than $CuCl_2$ exposure for *Enchytraeus albidus* (Amorim and Scott-Fordsmand, 2012). From a logical point of view avoidance responses may be time dependent, both because the chemical/material may change status over time and because the organisms may over time alter its behaviour (positive and negative) to the chemical. The novelty of this study is that very few studies have reported avoidance over the time issue and employing different ("but comparable") nanomaterials. The studies that do report on time, only in few cases showed a time-dependent response (Amorim et al., 2008).

The main objective of this study is for *Eisenia fetida* to determine the concentration dependent avoidance over time for four Silver (Ag) forms in OECD soil. For each form and time-point 6–8 exposure concentrations were used. The four silver forms were, three nanoparticles (NM300K, Ag-PVP (PolyVinyl Pyrolidone and AgNM-non-coated) and Silver nitrate ($AgNO_3$). The exposure durations were 24, 48, 72 and 96 h. All time-points and material consisted of individual experiments, i.e. no resampling, with 4 replicates.

## 2. Materials and methods

### 2.1. Test materials

Four materials were tested: $AgNO_3$ and three nanomaterials

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(NM300K, AgNM-PVP (polyvinylpyrolidone coatedl) and AgNM-non-coated (a non-coated nanomaterial)). The $AgNO_3$ (high grade > 99.8 percent purity) was purchased from Merck (Darmstadt, Germany). The NM300K (<20 nm, TEM (Transmission Electron Microscopy) 17 ± 8 nm) was dispersed in 4% polyoxyethylene glycerol triolaete and poloxyethylene sorbitan mono-laurate (Tween 20), obtained from The Joint Research Centre, EU (Klein et al., 2011). The AgNM-PVP and AgNM-non-coated were 20–30 nm AgNMs (99.9% metal basis) AgNM-PVP capped with 0.2 wt%/wt polyvinylpyrolidone (TEM 25 ± 5 nm) and AgNM-non coated (TEM 26 ± 4 nm), both obtained from American Elements (Weyburn Ave, Los Angeles). Characterization data (adapted from Gomes et al., 2017) for each chemical is summarized in Table 1.

### 2.2. Test organisms

The test organism used was adult *Eisenia fetida* (Oligochaeta: Lumbricidae) obtained from ECT Oekotoxikologie GmbH (Germany). The adult worms all had a visible clitellum with a wet weight between 300 and 600 mg. The worms were acclimatised in uncontaminated test soil for 24 h (h) prior to the test. The worms assigned to a test vessel were of the same weight range and were randomly chosen without bias. The tests were carried in 20 °C (±1) with 16 h light/8 h dark regime.

### 2.3. Test soil and spiking procedure

The standard OECD artificial soil (<2 mm) was prepared according to the OECD guideline 222 (OECD, 2016) consisting of 70% dry quartz sand (sieved to <2 mm, from- Dansk sand, Denmark), 20% kaolin clay (from Borup kemi, Denmark), 10% Spaghnum (dried at 105 °C, Kekkilä Garden Denmark), and 0.3–1.0% calcium carbonate (Sigma- Aldrich) added to have an initial pH of 6.0 ± 0.5 (measured in soil water as described for the chemical analyses). Final water content was 50% of water holding capacity (WHC).

For $AgNO_3$, all four replicates for one replication were mixed together, whereas for NM300K all replicates were mixed individually (except for the lowest concentrations, which was mixed as all replicates). There was no correction for dispersant.

The AgNM-PVP and AgNM-non-coated dry powder nanomaterials were weighed and mixed thoroughly with quartz sand (5 g) and immediately added and mixed into the pre-moistened OECD soil batches (with remaining % constituents). Pre-moisture was half of the water, i.e. 25% of WHC. The spiking was done individually per replicate, except for the lowest concentration where all replicates were spiked as one batch. All spiking was done 1 day prior to starting the experiment.

Based on preliminary experiments, the nominal test concentrations (mg Ag/kg dry soil) were 0, 0.01, 0.1, 1, 5, 20, 64 and 128 mg Ag/kg dry soil for NM300K, 0, 0.5, 5, 9, 20, 64, 128 and 512 mg Ag/kg dry soil for AgNM-PVP and AgNM-non coated, and 0, 0.01, 0.1, 0.5, 1, 5, 20 and 32 mg Ag/kg soil for $AgNO_3$.

### 2.4. Experimental procedure

The avoidance test was performed according to the standard avoidance protocol (ISO 17512–1:2008), with the exception of duration. The two-chambered test containers were used (Hund-Rinke et al., 2005). Each test container consisted of a circular plastic box (ø 12 cm; 13 cm in height) and a removable metal wall to divide the container into two equal sections. The control soil (250 g dry weight) was introduced on one side of the test container followed by the addition of the 250 g of contaminated soil on the other side. After the addition of soil, the wall was gently removed and ten adult earthworms were placed onto the line of contact of the soil treatments. The test containers were covered with lids (pinholes were made on the lids to allow air exchange) and the containers were incubated at 16:8 h light/dark photoperiod cycle at a temperature of 20 (±2) °C for 24, 48, 72 and 96 h. Each time point was an individual test, and not a resampling from one test. Each test concentration had four replicates per treatment with 10 adult worms per replicate. At the end of the test, the removable wall was inserted and each side of the test container was independently searched for worms.

### 2.5. Chemical analysis

The total Ag soil concentration in the soil and in soil:water solution was measured by using the flame atomic absorption spectroscopy (AAS, Perkin Elmer 4100, Ueberlingen, Germany). The soil for the analysis (1 g dry weight) was digested using 7N $HNO_3$ (nitric acid) and heating up to 110 °C until all brown fumes were gone (Scott-Fordsmand et al., 2000).

For the soil-solution the highest exposure concentrations were analysed, by extracting the supernatant of a soil: water (1:5) solution, i.e. 5 g soil and 25 ml deionized water. The soil/water solution was mixed in a lab-shaker for 5 min at 250 rpm and then centrifuged for 20 min at 2000 rpm (Sigma (3–18), Germany). Filtering (Minisart-plus®, Microsep Advacne®) was done using four fraction size filters (Kilodalton): >200 KDa, 30–200 KDa, 3–30 KDa and <3 KDa. This solution was digested the same way as the dry soil. The filter-selection were to some extend based on availability from the producers, but included filters that in principle should be able to detect smaller agglomerated (i.e. >200 Kda), discriminate free particles (i.e. below 30 KDa) and free ions (i.e. below 3 KDa). Although the filter selection could have been different, this approach does provide a standardised size fractionation.

### 2.6. Data analysis

The avoidance endpoint was expressed as the percentage of worms that avoided the treated soil in the test container from the

**Table 1**
Summary of the characteristics of Ag (nano)materials used (details from (Gomes et al., 2017)).

| | $AgNO_3$ | AgNM-non-coated | AgNM-PVP | NM300K |
|---|---|---|---|---|
| Supplier | Sigma Aldrich | AG-M-03M-NM.020N (American Elements) | AG-M-03M-NMCP.020N (American Elements) | JRC repository |
| State | Powder | Powder | Powder | Suspension |
| Solubility | Water soluble | Not dispersed | Not dispersed | Dispersible |
| Coating | – | – | 0.2%w/w PVP | – |
| Nominal size (nm) | – | 20–30 | 20–30 | 15 |
| TEM[a] (nm, n = 60) | – | 26 ± 4 | 25 ± 5 | 17 ± 8 |
| Purity | >99% | 99% | 99% | 10.2% w/w Ag |
| Morphology | – | Spherical | Spherical | Spherical |

[a] Transmission Electron Microscope.