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Toxicokinetics of zinc-oxide nanoparticles and zinc ions in the earthworm *Eisenia andrei*

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ABSTRACT

The toxicokinetics of zinc in the earthworm *Eisenia andrei* was investigated following exposure for 21 days to ionic zinc ($ZnCl_2$) or zinc oxide nanoparticles (ZnO-NPs) in Lufa 2.2 soil, followed by 21 days elimination in clean soil. Two concentrations were tested for both $ZnCl_2$ (250 and 500 µg Zn g^{-1}) and ZnO-NPs (500 and 1000 µg Zn g^{-1}), corresponding to EC_{25} and EC_{50} for effects on reproduction. Based on the measured internal Zn concentrations in the earthworms over time of exposure, the kinetics parameters k_a – assimilation rate constant (g_{soil} $g^{-1}{}_{body\ weight}$ day^{-1}) and k_e – elimination rate constant (day^{-1}) were estimated using a one-compartment model for either total Zn concentrations in the soil or porewater Zn concentrations. In the $ZnCl_2$ treatments, k_a was higher for total Zn concentrations in soil, whereas in the ZnO-NP treatments, k_a was higher for porewater Zn concentrations. The value of k_e did not differ between the two Zn forms ($ZnCl_2$ vs ZnO-NPs) for either EC_{50} or EC_{25} when related to total Zn concentrations in soil, but for EC_{50}, k_e related to porewater Zn concentrations was significantly higher for $ZnCl_2$ than for ZnO-NPs. It is concluded that differences in kinetic parameters between treatments were connected with exposure concentrations rather than with the form of Zn. Zinc was efficiently regulated by the earthworms in all treatments: a 2-fold increase in exposure concentration resulted in a less than 2-fold increase in internal concentration, and after transfer to uncontaminated soil the internal Zn concentrations in the earthworms returned to ca 111 µg g^{-1} dw in all treatments.

1. Introduction

The increasing usage of engineered nanoparticles (NPs) is associated with the risk of uncontrolled release into the environment (Caballero-Guzman and Nowack, 2016; Ju-Nam and Lead, 2008). Once released into the environment, NPs can enter wastewater and sewage sludge, which may in turn be used as fertiliser on crop fields. Since ZnO-NPs are among the most widely used nanoparticles, the risk of release into the soil environment is high. However, due to the complex nature of the soil, the exact amount of NPs in the environment is difficult to determine due to various limitations (Caballero-Guzman and Nowack, 2016). After reaching the soil environment, NPs can potentially be taken up by soil-dwelling organisms (García-Gómez et al., 2014). Thus, to date several studies on ZnO-NPs have been performed aimed at examining their influence on soil invertebrates, among them earthworms (Fernández et al., 2014). Most of these studies indicated that ZnO-NPs are not as toxic as the ionic form of this metal (Kwak and An, 2015). Hooper et al. (2011) showed a 50% decrease in reproduction by ZnO-NPs compared to complete inhibition by $ZnCl_2$ treatment when exposing *Eisenia veneta* to 750 µg Zn g^{-1} soil. Moreover, no increased mortality was observed in comparison with control when earthworms were exposed to 0.1, 1, 1000, and 5000 µg Zn g^{-1} of ZnO-NPs in sand for 14 days (Cañas et al., 2011).

Some studies suggest that the toxicity of NPs may be connected with ions released after their dissolution (Heggelund et al., 2014; Kool et al., 2011), rather than with exclusive effect of nanoparticles itself. And recent studies on the metabolomics and genotoxicity of silver nanoparticles (Ag-NPs) confirm that toxicity results from the simultaneous action of nanoparticles and released metal ions (Garcia-Reyero et al., 2014; Li et al., 2015). Probable differences in the pathway and mode of action for ZnO-NPs and Zn^{2+} ions could be indirectly connected with differences in the intracellular compartmentalisation of zinc in the earthworms *Eisenia fetida* exposed to either nano or ionic zinc forms (Li et al., 2011). Li et al. (2011) showed that zinc in ionic form was distributed mainly in cell membranes and tissues, while zinc derived from NPs was stored in organelles and cytosol. Moreover, despite the lower toxicity of ZnO-NPs in comparison with ions, internal Zn concentrations in earthworms were higher for the former (Heggelund

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et al., 2014; Hooper et al., 2011). Thus, different mechanisms may be responsible for the regulation of Zn concentrations in animals exposed to different forms of the metal.

Many toxicokinetic experiments have been conducted in order to understand the uptake and elimination of different metals by different earthworm species (Giska et al., 2014; Li et al., 2009; Nahmani et al., 2009), including *Eisenia andrei* (Smith et al., 2010). However, the toxicokinetics of Zn after ionic and nanoparticle exposure has rarely been studied in earthworms (Laycock et al., 2016).

Our aim was to compare the toxicokinetics of zinc in the earthworm *E. andrei* after exposure to the metal present in soil in the form of either zinc oxide nanoparticles (ZnO-NPs) or ions derived from a soluble salt ($ZnCl_2$). Two different concentrations, covering the EC_{50} and EC_{25} for effects on earthworm reproduction (Heggelund et al., 2014) were chosen. Based on the measured internal Zn concentrations in the earthworms over time, toxicokinetics parameters, i.e. assimilation and elimination rate constants, were calculated and compared between treatments.

2. Materials and methods

2.1. Test species

Earthworms of the species *E. andrei* were obtained from a laboratory culture at the Department of Ecological Science of Vrije Universiteit in Amsterdam. The earthworms were fed with horse manure free of any pharmaceuticals and cultured at 20 °C in darkness. The experiment used 4-month old adult individuals with well-developed clitellae.

2.2. Soil spiking procedure

Loamy sand soil (LUFA-Speyer 2.2, Germany) was used. Soil came from two deliveries with pH_{CaCl2} 5.4 ± 0.2, total organic carbon content 1.59 ± 0.13%, cation exchange capacity 9.7 ± 0.4 meq. 100 g^{-1}, and maximum water holding capacity (WHC) 43.5 ± 2.8% (w/w). The experiment used 30 nm ZnO-NPs as powder without any surfactant or coating (Nanosun Zinc Oxide P99/30, Microniser, Australia). Transmission electron micrographs and particle size distribution, determined by Waalewijn-Kool et al. (2012), showed that the primary particle size of the nanoparticles was in agreement with the values provided by the manufacturer. Full details and results of particle characterization can be found in Waalewijn-Kool et al. (2012). Soil spiked with zinc chloride salt ($ZnCl_2$, Merck, Germany) was used to represent treatments with ionic Zn. Two concentrations of ZnO nanoparticles (nominal: 500 and 1000 µg Zn g^{-1} dry soil, designated as ZnO-NP 500 and ZnO-NP 1000, respectively), two concentrations of $ZnCl_2$ (nominal: 250 and 500 µg Zn g^{-1} dry soil, designated as $ZnCl_2$ 250 and $ZnCl_2$ 500, respectively), and one control without added zinc were tested. Test compounds were introduced into soil using different methods. Four days before starting the exposure (in order to obtain equilibration), soil was spiked with $ZnCl_2$ as aqueous solutions prepared with an amount of water sufficient to achieve a soil moisture content of 50% of WHC. After spiking, to achieve homogenous distribution, the soil was mixed with a kitchen robot. Shortly before starting the exposure (day 0), ZnO nanoparticles were added to a small portion of dry soil (i.e. 250 g which was 10% of the soil used per treatment) as dry powder and then mixed by shaking 4–6 min in a closed jar. Subsequently, the remaining soil (2300 g) and enough water to reach a soil moisture content equivalent to 50% of WHC were added and homogenised carefully with the kitchen robot under a fume hood. Thus, a shorter equilibration time was used for treatments with ZnO nanoparticles than for $ZnCl_2$ to take into account differences in their solubility (Romero-Freire et al., 2017) and to ensure that the earthworms from the ZnO-NP treatments were exposed mainly to zinc in the form of nanoparticles.

2.3. Toxicokinetics experiment

The toxicokinetics test followed OECD Guideline 317 (OECD, 2010), with 21 days of exposure in Zn contaminated soil (uptake phase) followed by 21 days of elimination in control soil (elimination phase, also described in the literature as the decontamination phase). Three replicates (i.e. glass jars filled with approximately 60 g of wet soil) were prepared per sampling point for each Zn treatment and control. Food was added at the beginning of each phase by mixing 7 mg dry weight of horse dung per 1 g dry weight of soil prior to introducing the soil into the test jars. Earthworms, one individual per jar, were randomly allocated to treatments. Before starting the exposure (day 0) and after 1, 2, 4, 7, 10, 14, 17, and 21 days (uptake phase) and 22, 23, 25, 28, 31, 35, 38, and 42 days (elimination phase), three individuals were sampled from each Zn treatment. At days 0, 7, 14, 21, 28, 35, 38, and 42, three individuals were sampled from the control treatment. Once a week, soil moisture content was checked by weighing the jars, and moisture loss was replenished with deionised water when necessary. The jars were also aerated by this procedure. At each sampling point, the collected earthworms were rinsed with tap water, blotted dry on filter paper, and weighed to the nearest 0.0001 g. Then, the animals were kept individually in Petri dishes lined with moistened filter paper to void the gut content. After 24 h, the earthworms were rinsed, blotted dry and weighed again, then killed by freezing at −20 °C.

2.4. Chemical analysis

Frozen animals were freeze-dried for 48 h and then weighed to the nearest 0.0001 g. Soil samples collected before the exposure (day 0) from each treatment were dried for 48 h at 50 °C. For analysis of the total zinc concentrations, both individual earthworms and soil samples (ca 100 mg dried soil) were digested in 2 mL of a 4:1 mixture of HNO_3 (65% p.a., Sigma-Aldrich) and HCl (37% p.a., Sigma-Aldrich). Digestion was performed using Teflon bombs, closed tightly and placed in an oven (CEM MDS 81D) at 140 °C for 7 h. To determine the level of analytical precision, three blanks and three samples of a certified reference material (for earthworms: *Dolt-4 Dogfish Liver*, National Research Council of Canada, with a certified Zn concentration of 116 ± 6 µg g^{-1}; for soil: *ISE sample 989 of River Clay* from Wageningen, the Netherlands, with a certified Zn concentration of 1060 µg g^{-1}) were run with the samples. After digestion, the samples were complemented with demineralised water (8 mL) and Zn concentrations were analysed using flame Atomic Absorption Spectrometry (AAS) (Perkin Elmer AAnalyst 100) and expressed in µg g^{-1} dry weight (dw). Measured zinc concentrations in the reference materials were within ± 0.5% and ± 3.5% of the certified concentrations for the *Dolt-4 Dogfish Liver* and *ISE sample 989 of River Clay*, respectively.

At the beginning (day 0) and end (day 21) of the uptake phase, three soil samples (ca 49g) per treatment were taken, saturated to 100% WHC and equilibrated for 48 h. Soil porewater was collected via centrifugation (Centrifuge Falcon 6/300 series, CFC Free) for 45 min with a relative force (RCF) of 2000 × *g* over two round filters (cat. no. 1001-047, Ø 47 mm) and a 0.45 µm membrane filter (cellulose nitrate, cat. no. 10401112, Lot G9942878, Ø 47 mm) placed inside the centrifuge tubes. Approximately 10 mL of soil porewater per sample was collected and analysed using flame AAS (Perkin Elmer AAnalyst 100) for Zn concentration. Additionally, Zn concentration was determined after ultrafiltration from an aliquot of the porewater: to obtain particle-free extracted porewater, samples were centrifuged in a 3 kDa ultrafiltration device (Amicon Ultra-15 Filters, Millipore) for 45 min at 3000 × *g*. The ultrafiltration was only done for soil samples taken at day 0.

To measure the pH_{CaCl2} of the test soils, samples (ca 5g dw) were taken at days 0, 7, 14, and 21 and shaken in plastic tubes with 25 mL 0.01 M $CaCl_2$ for 2 h at 2000 rpm. After overnight settling of the floating particles, pH was measured using a pH-meter (inoLab® pH 7110, WTW).