

Influence of Earthworms on Extractability of Metals from Soils Contaminated with Al₂O₃, TiO₂, Zn, and ZnO Nanoparticles and Microparticles of Al₂O₃

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Abstract

Nanoparticles (NPs) of Al₂O₃, TiO₂, Zn, and ZnO, and with microparticles (MPs) of Al₂O₃ were introduced into garden soil. Adult *Dendrobaena veneta* earthworms were added to half of the samples. Concentration of the metals was determined after 1 and 10 days in soil eluates (water and EDTA) and in the earthworms before and after gut cleansing.

The study showed the influence of soil and earthworm activity on the bioavailability of aluminum and titanium originating from NPs. Extractability of Al by water or EDTA increased after 10 days from aluminum oxide MP- and NP-treated soils. The presence of live earthworms completely abrogated this increase. Water-extractability of Ti from TiO₂ NP-treated soils increased after 10 days, while EDTA-extractability remained unchanged. The presence of earthworms was associated with a decrease in Ti extractability. The highest extractability was observed for Zn, with negligible effects of time and earthworms.

Al, Ti, and Zn were not substantially accumulated in the earthworm tissues.

It was concluded that interaction of the nanoparticles with the soil and activity of the earthworms can affect the bioavailability and, potentially, toxicity of metals originating from nanostructures.

Keywords: nanoparticles, Al₂O₃-nanoparticles, TiO₂-nanoparticles, earthworm, metal bioavailability

Introduction

The pace of nanotechnology implementation has increased rapidly in the last two decades, bringing a growing risk of creating a new generation of waste (nanowaste) and new potential threats to the environment [1]. Nanoparticles are incorporated into commonly used prod-

ucts (catalysts, pharmaceuticals, cosmetics, fillers, electronic devices, etc) [2]. Nanoparticles used on an industrial scale include Ag, Al-O_x, Fe-O_x, SiO₂, TiO₂, and ZnO [3]. In particular, aluminium oxide is widely used as an abrasive agent or insulator, due to exceptional dielectric and abrasive properties [4]. TiO₂ is used as a pigment in paints, plastics and inks and ZnO as a pigment and semiconductor. Rising production and use of engineered nanomaterials increases the likelihood of environmental exposure [5].

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This can result in rapid distribution of the nanoparticles throughout the environment with unknown consequences.

Nanoparticles may be up to 100 nm in size; compared to other materials, they have unique physicochemical properties resulting from a high ratio of surface area to volume or weight, and exhibit unusual interactions with media. Nanoparticles encountered by living organisms may be metabolized or otherwise altered in size, shape, and/or composition. The resulting interactions of such nanostructures with the biosphere may have unpredictable consequences [6]. Since the surface area-to-volume ratio is much greater for NPs than for MPs, nanoparticles may be much more cytotoxic. Some work has been done on the toxicity of NPs to animals: ZnO, Al₂O₃, and TiO₂ to the nematode *Caenorhabditis elegans* [4], ZnO to fish cells [7], ZnO, TiO₂, SiO₂, and C60 NPs to *D. magna* [8, 9], CuO NPs to zebrafish [10], the toxicity of ZnO and Al₂O₃ NPs to plants [11, 12], ZnO NPs to freshwater microalgae [13], and the toxicity of NPs to bacteria [8, 14-16]. Nanoparticle toxicity can be, among other things, attributed to soluble toxic ions, so the release of ions also deserves careful consideration [17].

The present study investigated the impact of earthworms on extractability of metals from soils contaminated with nanoparticles containing aluminum, titanium, and zinc. Earthworms are abundant in soils and are crucial in the turnover of organic matter and in building soil structure [18]. Annual rates of earthworm cast production range from 5 to more than 25 tons·ha⁻¹ [19]. Earthworms take up soil particles and process them in the alimentary canal with digestive juices, improving organic matter degradation; they also mix soil layers and increase aeration [20]. By ingesting organic detritus these organisms enhance the bioavailability of soil nutrients such as C, N, and P [21]. Aristotle called earthworms the “intestines of the earth.”

Although many studies have investigated contaminant uptake by earthworms as possible bioindicators for evaluation of hazardous chemicals in soils [22-26], the fate of the metals introduced to soil as nanoparticles is still unknown. Also, no information exists on the impact of earthworms on the bioavailability of such metals.

The presented study was undertaken to compare the water solubility and bioavailability of metals in soils contaminated with different metallic (Zn) and metal oxide (Al₂O₃, TiO₂, and ZnO) nanoparticles. For Al₂O₃ NPs comparison with microparticles was performed. We assessed changes in element extractability with water and EDTA (bioavailability) caused by earthworms, by comparing metal content in water soluble and bioavailable fractions of the soils with and without earthworms and by measuring accumulation of the metals in earthworms.

Materials and Methods

Soil Preparation and Exposure of Earthworms

For experiments adult earthworms *Dendrobaena veneta* (Eco-Farma, Zielin, Poland), averaging 1.625±0.198 g per animal, were used. This species belongs to the 2nd class

of earthworms, living in the soil layer up to 30 cm. Earthworms were introduced into soil samples in glass boxes (25 cm × 24 cm × 6 cm) containing 800 g of soil (Universal Garden Soil, Castorama, Poland) mixed with 10 g of specific nanoparticles at the final concentration 32 g·kg⁻¹ of dry mass. Soil humidity was 60%, the pH of the soil averaged 5.5. To each box 200 mL of water was added. All nanoparticles (as a powder): Al₂O₃<10 μm, Al₂O₃<50 nm, Zn<50 nm, ZnO<100 nm, and TiO₂<100 nm were purchased from Sigma-Aldrich. The final metal content was 17 g·kg⁻¹ of Al (soil with aluminium oxide micro- and nanoparticles), 19 g·kg⁻¹ of Ti (soil with titanium oxide), and 32 and 26 g·kg⁻¹ of Zn (soil with zinc and zinc oxide, respectively). In the control soil (without the addition of any chemicals) the metal content (determined by ICP MS after microwave digestion) was: Al 949±79 mg·kg⁻¹, Ti 88±5 mg·kg⁻¹, Zn 12±3 mg·kg⁻¹.

Three experimental variants (all in three replicates) were performed for each nanoparticle. In *variant A*, soil samples were obtained for further analysis immediately after completion of preparation. In *variant B*, the boxes were left for 10 days at room temperature. In *variant C*, 15 adult earthworms were added, and the boxes were left for 10 days at room temperature. The time of exposure was chosen on the basis of experimental data (unpublished) obtained at the Department of Ecology (Faculty of Biology, University of Warsaw). Each experimental variant included a control, comprising a box with soil without any nanoparticles and microparticles. The pH of the soil was controlled during experiments, and except for samples contaminated with Zn and ZnO, the pH of the soil samples remained constant (5.5±0.3) over the study period. The pH of the soil with Zn-NPs and ZnO-NPs averaged 6.5±0.2.

Sample Preparation and Analysis

After 10 days the earthworms were hand collected, rinsed in distilled water, and either analyzed directly or kept in plastic Petri dishes with moist filter paper (two animals per dish) at 20°C for 3 days to permit complete evacuation of the gut contents. The animals were rinsed daily in distilled water and placed on fresh filter paper. The earthworms were killed by freezing, oven dried for 48 h at 60°C and ground using a ceramic pestle and mortar. The soil samples were also dried and ground.

Before analysis of the total metals in the earthworms, samples were digested using the microwave laboratory system ETHOS 1 with ATC-400-CE automatic temperature control (Milestone, Italy). For this purpose about 250 mg of dried sample was weighed into a Teflon container and 2.5 mL of HNO₃ and 0.5 mL of HClO₄ were added.

Metals were extracted from soil samples by elution with water and 0.05 mol·L⁻¹ EDTA. For this purpose, 1.00 g of sample was mixed with 10 mL of extracting agent and shaken on a reciprocating shaker for 1 h at room temperature. Afterward the suspension was filtered and centrifuged for 30 min at 30,000 rpm. Concentration of metals in earthworms and soil extracts was measured using an ELAN 6000 ICP mass spectrometer (PE-SCIEX, Concord, Canada).

The particle size and morphology were assessed using transmission electron microscope LEO 912AB equipped with a Proscan High Speed Slow Scan CCD-camera. TEM analysis was performed using $1 \text{ mmol}\cdot\text{L}^{-1}$ water suspensions.

Statistical Analysis

Analysis of variance (ANOVA) and Pearson correlation coefficient (significance level <0.05) were used to estimate differences in the extractability of elements between experimental variants. Because of the high number of calculated coefficients, obtained by comparison of variants A/B, B/C,

and A/C for five contaminants, data are not presented. Values were considered as different when $P < 0.05$.

Results and Discussion

Characterisation of Nano- and Microparticles

In all of the investigated, commercially available chemicals, most of the particles have sizes in agreement with the declaration of the suppliers (Fig. 1). The morphology of particles was different for particular reagents. MPs of Al_2O_3 and Zn-NPs are mainly polyhedral, TiO_2 are spherical, with

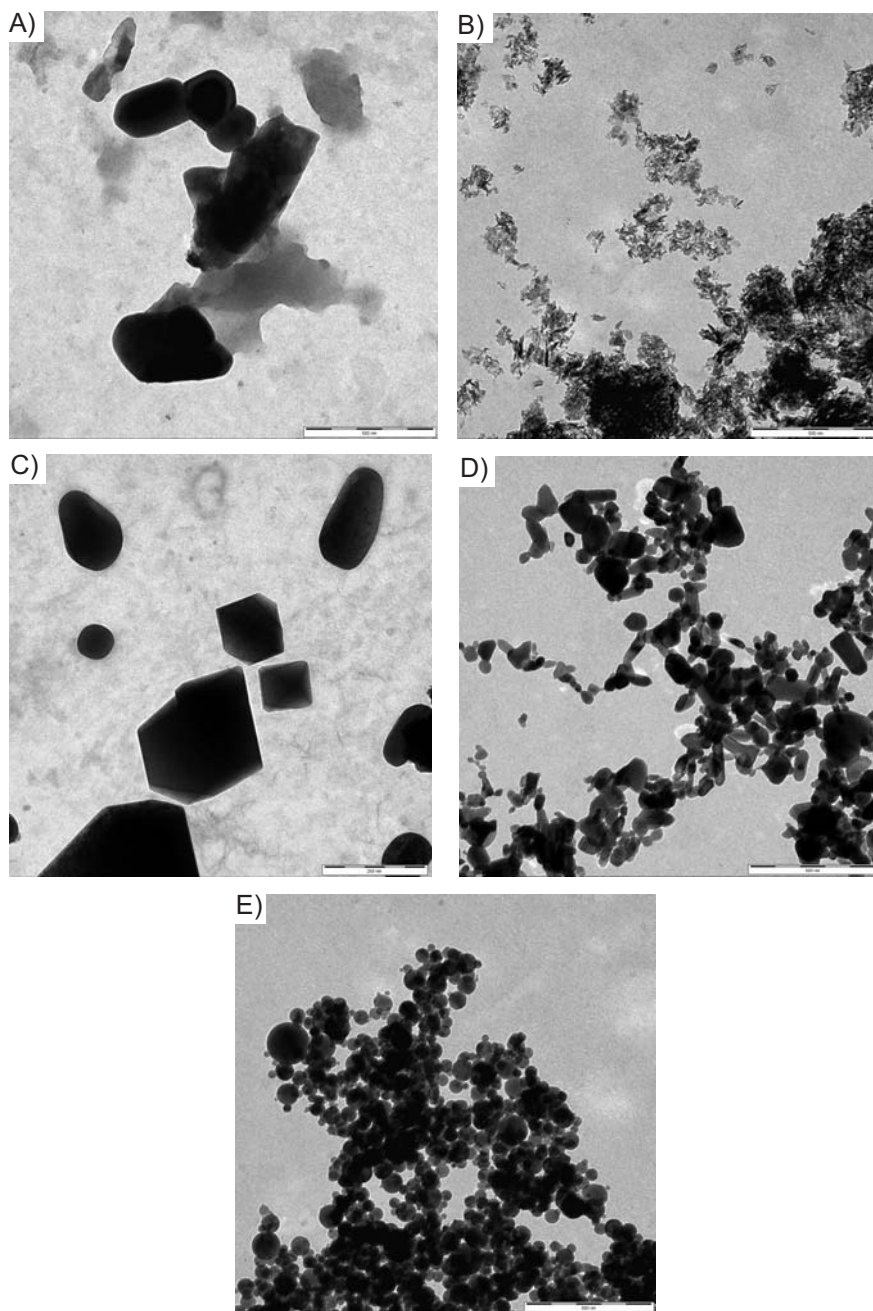


Fig. 1. TEM images of Al_2O_3 MPs (A), Al_2O_3 NPs (B), Zn NPs (C), ZnO NPs (D), and TiO_2 NPs (E) suspensions. Scale bar is 500 nm (A, B, D, E) and 200 nm (C).

very regular shapes. Al_2O_3 NPs are disk-shaped while ZnO-NPs have a stick shape.

Extractability of Al, Zn, and Ti from the Soil

A comparison of variants A (no incubation) and B (10 days without earthworms) shows (Fig. 2) that 10-day incubation resulted in more aluminum extracted with water from the control samples (by 65%), and for the soils treated with Al_2O_3 in the form of MPs (by 21%) and NPs (by 278%). EDTA extraction was also greater in these conditions for NPs than for MPs (Fig. 2), increasing during 10 days by 40%. The presence of earthworms did not affect extractability with water or EDTA (variants A and C) in the control soils, nor in soils treated with MPs and NPs. After incubation with earthworms, less aluminum could be extracted with water compared to samples left alone (variant C versus variant B). The amounts were reduced by 38% (control), 27% (MPs), and 76% (NPs), and were comparable to the amounts obtained from un-incubated samples. Thus, earthworms appeared to reduce aluminum present in the water-soluble fraction, indirectly proving that speciation of aluminum is changed in the gut of earthworms. Earthworm activity has been shown to increase the level of

organic carbon in the soil [27], arguing for an increase of aluminum complexed with organic compounds as the mechanism of the observed changes in aluminum extractability. It is probable that this complexing may reduce the aluminum phytoavailability and, consequently, aluminum toxicity for plants. The role of organic ligands in the complexation of aluminum is well documented. More than 90% of the Al in the soil solutions can be bound into complexes with organic ligands [28] and fulvic acids presumably participate in the transfer of Al and contribute to its solubilization.

Our results also confirmed the influence of particle size on the bioavailability of the metals. MPs are considerably larger than NPs, and the specific surface area of NPs is significantly higher than that of MPs. As observed, efficiency of extraction in the case of nanoparticles was clearly higher than for microparticles, where efficiencies were similar to control samples. It must be stressed that extractability of aluminum from NPs is higher than for MPs, in spite of the probable agglomeration of nanoparticles [29].

Changes in metal solubility and bioavailability, as a consequence of NP-soil interaction and earthworm presence, were much more evident in the case of TiO_2 nanoparticles. Titanium extractability (Fig. 3) with water increased

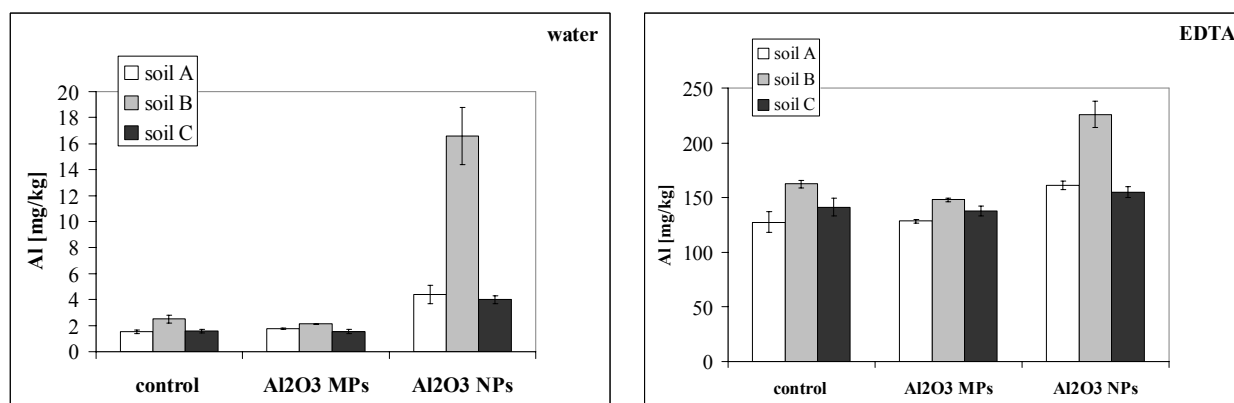


Fig. 2. Water and EDTA-extractable Al contents of the soil samples ($\text{mg}\cdot\text{kg}^{-1}$). A – soil without earthworms, no incubation; B – soil without earthworms, incubation period 10 days; C – soil with earthworms, incubation period 10 days. (Results are presented as means \pm standard deviations, $n=3$).

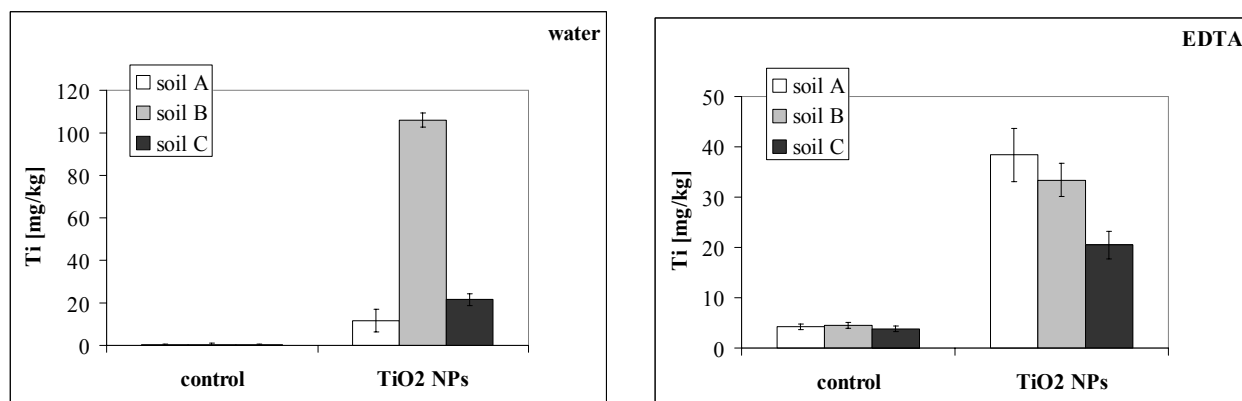


Fig. 3. Water and EDTA-extractable Ti contents of the soil samples ($\text{mg}\cdot\text{kg}^{-1}$). A – soil without earthworms, no incubation; B – soil without earthworms, incubation period 10 days; C – soil with earthworms, incubation period 10 days. (Results are presented as means \pm standard deviations, $n=3$).

greatly (9 times) after 10 days of incubation, but did not change for EDTA. Earthworm presence was associated with a reduction of water-soluble titanium in the soil by 80% (variants B and C). Likewise, titanium extractability with EDTA was decreased by 49%. These results again indicate the importance of earthworms in the reduction of bioavailable titanium, possibly by relatively stable binding of titanium to protein structures, mediated by the earthworm's digestive juices.

The highest extractability using the investigated extracting solutions was obtained for Zn. Zinc extractability (Fig. 4) with water did not change after 10 day incubation for soils treated with Zn-NPs (variants A and B), but decreased significantly (~75%) for soils treated with ZnO-NPs, irrespective of earthworm presence. As could be expected, zinc oxide is a better source of ionized zinc complexes than metallic zinc, independently of nanoparticle size. Earthworm presence only marginally affected zinc speciation in soil (variants B and C). The results obtained by Udovic and Lestan [22] indicate a minor decrease of Zn in the exchangeable fraction and a slight increase of Zn bound to the organic matter fraction from earthworm cast in comparison to the surrounding soil. Zn was predominantly present in the least accessible residual soil fraction. Our results showed a significant increase of water-soluble Zn without any effect on earthworm activity, in contrast to the results of Cheng and Wong [30], who reported an earthworm-mediated decrease in the concentration of exchangeable Zn and Zn bound to carbonates.

According to our results, earthworm presence moderates the consequences of the increased soil concentration of metals introduced as nanostructures. A higher concentration of metals in water-soluble and EDTA-extractable fractions, after interaction with the soil, can be explained by two possible mechanisms. The first possibility is that the relatively high concentration of metal in the soil is a consequence of NP surface degradation and release of metals. A very complex chemical composition of the soil can increase NP solubility. The second possibility is that the presence of a high concentration of NPs in the soil affects the equilibrium between metals and soil, influencing the extractability of metals. Regardless of the explanation, the presence of NPs

Table 1. Metal concentrations in earthworms ($\text{mg}\cdot\text{kg}^{-1}$). (Results are presented as means \pm standard deviations, $n=3$).

		With the gut contents	After evacuation of the gut contents
Al	control	157.0 \pm 56.2	2.53 \pm 1.29
	Al ₂ O ₃ MPs	185.9 \pm 16.7	1.89 \pm 0.72
	Al ₂ O ₃ NPs	1396 \pm 70	3.69 \pm 1.14
Ti	control	8.91 \pm 1.27	3.05 \pm 0.62
	TiO ₂ NPs	102.8 \pm 11.4	3.76 \pm 0.84
Zn	control	103.6 \pm 23.9	106.9 \pm 10.9
	Zn NPs	3347 \pm 369	138.9 \pm 23.1
	ZnO NPs	3362 \pm 301	158.6 \pm 20.7

in the soil causes an increase of metal as water-soluble and bioavailable species, and earthworm presence moderates this processes. The earthworm-mediated effect on metal distribution in the soil is complex, and both increase (caused by an increase of pH and DOC content) [27] and decrease (as a result of burrowing activity of earthworms) [31] of bioavailability have been described in the literature.

As the bioavailability of heavy metals in soils is strongly affected by pH, a parameter that can be modulated by earthworm activity [32], the pH of the soil was controlled during these studies. For experiments with Al₂O₃ and TiO₂, the pH of the soil samples remained constant over the study period, so it must be concluded that other factors related to earthworm presence contributed to low availability of the metals. For samples treated with Zn and ZnO, the pH was about one unit higher, but for these samples the influence of earthworms was negligible.

Accumulation of Al, Zn and Ti by Earthworms

Earthworms subjected to 3-day cleansing contained only a practically negligible fraction of the aluminium present in their bodies prior to cleansing, namely 1.61% (control), 1.02% (Al₂O₃-MPs), and 0.26% (Al₂O₃-NPs).

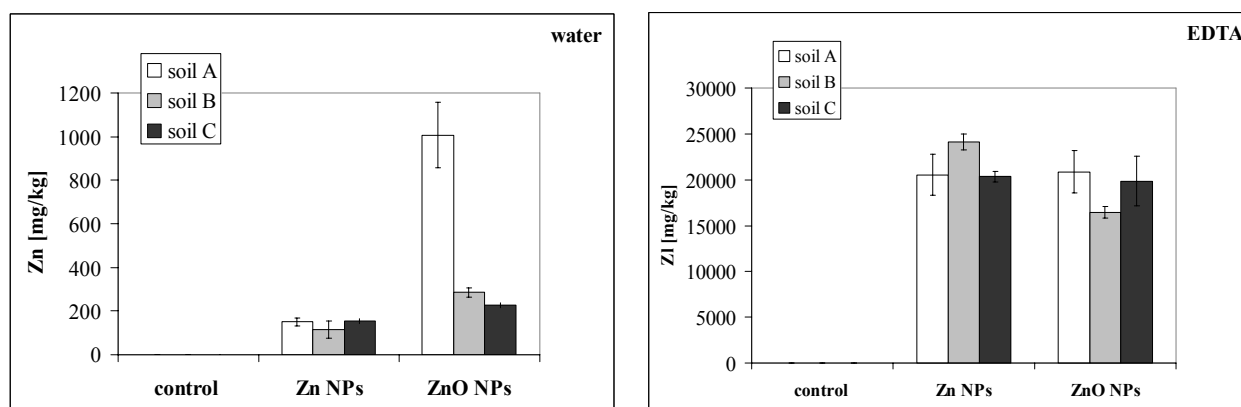


Fig. 4. Water and EDTA-extractable Zn contents of the soil samples ($\text{mg}\cdot\text{kg}^{-1}$). A – soil without earthworms, no incubation; B – soil without earthworms, incubation period 10 days; C – soil with earthworms, incubation period 10 days. (Results are presented as means \pm standard deviations, $n=3$).

The aluminum still remaining in earthworm tissues could be absorbed not only in ionic form, but also as nanoparticles, as suggested by 95% higher content of aluminum originating from nanoparticles than from microparticles (Table 1). While still marginal, an accumulation of aluminum in earthworms may thus be increased by nano-size of the source of the metal. Also, little titanium was present in the cleansed earthworms (3.66% of the original content), not different from the control. Zinc dynamics were substantially different from aluminum and titanium. The level of Zn was the same before and after cleansing in control earthworms, indicating a dynamic equilibrium between the Zn amount in the gut and tissues. In the presence of Zn and ZnO NPs, the fraction of zinc present in earthworm bodies after cleansing was 4.1 and 4.7 %, respectively, in comparison with the level of zinc before evacuation of the gut content.

The minimal retention (at trace levels) of aluminum and titanium by earthworms, and a relatively low retention of zinc indicates that these metals could not be toxic for the animals at the concentrations and forms used over the 10-day period. The concentration of nanoparticles used during our studies greatly exceeded the toxic doses described in the literature for a similar organism – *Eisenia fetida* [33]. The values for TiO₂ and ZnO NPs were several times higher than reported. Nevertheless, all earthworms remained in good condition during the experiments, and no negative effect was noticed during the 3-day observation post-exposure (during gut evacuation), as evidenced by the observed high behavioral activity. However, no toxic effects such as activity of enzymes or DNA damage were investigated. Additionally, a very low level of investigated metals in earthworm bodies in comparison with the relatively high amounts of nano- and microparticles in the soil indicates both a very low accumulation of metals and very efficient evacuation of metals from the alimentary canal.

Conclusions

Earthworm activity substantially affected speciation of aluminum and titanium in the soil and, to a lesser extent, speciation of zinc. Repeated ingestion, digestion, and excretion of soil by earthworms apparently resulted in reduced bioavailability of the metals. It may be concluded that earthworm presence moderates the consequences of the increased soil concentration of metals introduced as nanostructures. It can reduce the negative effect of the nanoparticles, which is particularly important since the increasing use of nanomaterials and rapid distribution of the nanoparticles throughout the environment has been observed. The study shows for the first time a strong dependency between the size of Al₂O₃-particles and levels of water-soluble and EDTA-extractable aluminum.

In further experiments the particles should be characterized in more detail under assay conditions, as in the soil they are very probably present as agglomerates rather than as nanostructures. However, it still can be concluded that one of the consequences of the presence of nanoparticles of

Al₂O₃, TiO₂, Zn, and ZnO in the soil is an increase of metal concentration in the bioavailable fraction and that nanoparticle interaction with the soil and activity of the earthworms can affect the bioavailability and, potentially, toxicity of metals.

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