Study of the ability of *Eisenia fetida* to bioaccumulate metals from the substrate as a promising method for the restoration of soils contaminated with nanoparticles Cu, Zn and Mo

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Abstract. The widespread introduction into production and distribution of nanoparticles and their accumulation in natural environments creates a danger of contamination of fertile soils. Moreover, the accumulation of nanoparticles in soil differs significantly from the behavior of larger particles. Based on the foregoing, the purpose of this study was to determine the ability of worms to accumulate Zn, Cu, and Mo nanoparticles from the substrate in order to assess the possibility of using it in soil restoration. In our study, we used nanoparticles Cu, Zn, Mo at a concentration of 50, 200, 500 and 1000 mg/kg of dry soil. A standardized artificial soil (70 % quartz sand, 20 % kaolin, and 10 % ground peat) was used as a substrate. The test objects were *E. fetida* worms. According to the results of the study, we established a different ability of the worm's organism to cumulate metals with variable valency. At the same time, the level of Cu in the body of *E. fetida* increased along with dosages and exposure times, while the absorption and accumulation of Zn and Mo occurs to a certain level, with a subsequent slowdown in its accumulation rate. The data obtained show the promise of using worms in the technology of restoration of soils contaminated with nanoparticles.

1. Introduction

Nanoparticles are used in various industrial fields. However, this contributes to the ingress and accumulation of nanoparticles in various habitats, and primarily in the soil. At the same time, it is necessary to search for new approaches to the restoration of such anthropogenically contaminated soils, since nanoparticles can have an adverse effect on animals, including soil ones [1]. At the same time, at low concentrations, metal nanoparticles can have a positive effect, which can be successfully applied in agriculture when developing fertilizers. All nanoparticles are potentially toxic to soil and soil organisms at sufficiently high levels, as they are capable of accumulating for a long time [2].

When developing technologies for soil bioremediation, researchers turn their attention to earthworms *Eisenia fetida*, since they have a known ability to bioaccumulate various metals, which can be applied in practice.

Among heavy metals, Zn and Cu play an important role in the vital functions of soil organisms, including worms. Their biological role is associated with the activity of a number of enzymes and the formation of adaptive reactions [3]. Molybdenum is also an important element for organisms, is involved in a number of enzymatic reactions. Molybdenum compounds can be released into the environment when they are used in alloys, flame retardants, catalysts, mining, fertilizers, and plant emissions [4]. It is important to note that the range between the deficit, sufficiency and toxicity of metals is quite small.
Pollutants from various sources ultimately end up on the surface of the soil, and their future fate depends on its chemical and physical properties. The length of stay of polluting components in soils is much longer than in other objects of the biosphere [5].

Ecotoxicity of metals depends on the amount of chemical substance taken into the body, and their bioaccumulation is an important indicator for assessing the risk for organisms living in the soil, as well as for their predators. Unfortunately, there is little information about the effect of various concentrations of nanoparticles in the soil on invertebrates, and it remains unclear to what extent these organisms accumulate metals. According to Suthar et al. [6], earthworms are able to accumulate a significant amount of metal in tissues, which can serve as a useful indicator of biological pollution due to the rather consistent relationship between the concentration of substances in earthworm and soil and can be used for the restoration of soils contaminated with nanoparticles.

Based on the foregoing, the purpose of this study was to determine the ability of worms to accumulate Zn, Cu, and Mo nanoparticles from the substrate.

2. Materials and methods

2.1. Chemicals and substrates

Zn (d = 70 ± 2 nm), Cu (d = 50 ± 0.5 nm) and Mo (d = 48 ± 1.3 nm), manufactured by Advanced Powder Technologies LLC (Russia).

Standardized artificial soil (OECD, 1984) prepared by mixing 70 % quartz sand (dry weight) (Inesco LLC, Hydrotorf, Russia), 20 % kaolin (Novokaolinovsky GOK, Chelyabinsk, Russia) and 10 % of ground peat, (organic nitrogen – 5.8 %, Lama Torf LLC, Volokalamsk, Russia), the pH was adjusted to 6.0 ± 0.5 with powdered calcium carbonate (CaCO3, Mineralprom LLC, Syzran, Russia).

2.2. Test organisms

The E. fetida worms used in the studies were grown in the nursery of the Laboratory of Agroecology of Technogenic Nanomaterials of the All-Russian Research Institute of Beef Cattle Breeding of the Russian Agricultural Academy, (Orenburg, Russia), purchased at BiOEra-Penza LLC (www.bioeragrup.ru). E. fetida was cultivated in horse manure without any drugs and chemicals at a temperature of 22 ± 2 °C. In the experiment, worms weighing 350 to 450 mg were used.

2.3. Testing

The test was conducted according to guidelines for testing bioaccumulation of chemicals in OECD terrestrial oligochaetes (1984) in four concentrations. Doses of nanoparticles were selected taking into account the load of 50 mg/kg – background, 200–500 mg/kg – increased and 1000 mg/kg – threshold.

The starting lyosols of nanoparticles were prepared by adding a test metal (dry powder) at concentrations of 50, 200, 500 and 1000 mg/kg in deionized water (10 ml), followed by dispersion on a sonicator (USDN, f – 35 kHz, N –300 W, Russia) for 30 minutes.

Next, the prepared Zn, Cu, and Mo lyosols were mixed with moist artificial soil for each repetition and concentration (humidity 45–50 %), then brought with distilled water to a moisture content of 75–80 % and mixed with a mixer. For each concentration, we used plastic containers of 5 × 4 × 4 cm (length, width, height) filled with 500 g of dry artificial soil.

Before staging the experiment, the worms were washed with distilled water and placed for 3 days in plastic containers with a moist filter paper substrate to cleanse the digestive tract. After 3 days of exposure, the worms were washed and weighed on filter paper.

10 healthy worms were added to each container. The test was carried out for 14 days, at t air 20 ± 2 °C, in five replicates.

2.4. Determination of the concentration of metals in E. fetida

The concentration of zinc, copper, and molybdenum in E. fetida was determined at the beginning, on days 7 and 14 of the experiment. For this, 5 worms we selected from 5 replicates, cleaned intestinal contents, and determined metal content.
Laboratory analysis was carried out at the independent accredited Testing Center and the Center for Collective Use of the Federal State Budgetary Scientific Institution “Federal Scientific Center for Biological Systems and Agrotechnologies of the Russian Academy of Sciences” (No. RA.RU No. 21PF59 dated 02.12.15).

2.5 Determination of the degree of absorption and the rate of accumulation of metals in the body E. fetida

The metal absorption was calculated by the formula: \( R = \frac{(M_a - M_b)}{M_e} \times 100\% \), where \( M \) is the mass, mg, \( a \) – at the end of the experiment, \( b \) is at the beginning, \( e \) is the mass of the worm [7].

Accumulation rate: \( S = \frac{\Delta C}{\Delta T} = \frac{(C_{\text{worm2}} - W_{\text{worm1}})}{(t_{n2} - t_{n1})} \) [8], where \( C_{\text{worm}} \) is the concentration of the substance in the worm on the sampling day, \( t_n \) – during sampling days [8].

2.6 Statistical analysis

Statistical analysis was performed using standard ANOVA techniques, followed by the Tukey test (SPSS ver. 17.0). The differences were considered statistically significant at \( P < 0.05 \).

3. Results and discussion

The background concentration of the studied trace elements in the natural substance E. fetida had a certain constant level (Table 1), which changed after a 14-day incubation in artificial soil. In particular, in the control (without adding nanoparticles), the concentration of Zn increased by 43.3 % (\( p \leq 0.05 \)), Cu by 61.1 % Mo 87.5 % compared to the background values, which is associated with the trophometabolic activity of E. fetida and the presence of the tested metals in the substrate: Zn – 0.65 mg/kg, Cu – 1.6 and Mo – 0.026 mg/kg.

| Concentration NP, mg/kg | Zn (7 days) | Zn (14 days) | Cu (7 days) | Cu (14 days) | Mo (7 days) | Mo (14 days) |
|-------------------------|------------|--------------|-------------|--------------|-------------|--------------|
| background              | 53.3±3.8   | 6.25±0.57    | 0.45±0.018  |              |             |              |
| 0                       | 57.8±4.3   | 94.1±7.2b    | 9.3±1.3     | 16.4±2.3b    | 0.7±0.03b   | 3.62±0.23b   |
| 50                      | 67.2±4.3   | 102±6.8b     | 18.6±2.3b   | 24.6±3.3b    | 2.62±0.4b   | 4.59±0.51b   |
| 200                     | 73.6±6.2   | 119±7.2b     | 19.5±1.5b   | 27.5±8.3b    | 7.26±6.8b   | 26.8±1.8b    |
| 500                     | 119±9.3b   | 134±9.2bab   | 29.8±3.1ab  | 58.3±4.5ab   | 67.3±4.2b   | 38.6±2.3ab   |
| 1000                    | 252±12.3b  | 122±9.3bab   | 36±2.6ab    | 72±6.2ab     | 73.1±5.4ab  | 45±3.2ab     |

Note: *a* – significant difference with the control (\( p \leq 0.05 \)), *b* – is a significant difference with the initial concentration (\( p \leq 0.05 \)).

A general tendency toward an increase in the concentration of metals in the body of E. fetida depending on the dose of introduced nanoparticles into the substrate was noted. So, on the 7th day of the experiment, against the background of Zn nanoparticles, its concentration in E. fetida increased by 16.26 % at a dose of 50 mg/kg, by 33.5 % at a dose of 1000 mg/kg. Thus, the first week of incubation leads to the accumulation of Zn in the body of E. fetida. However, when extending the exposure time, the dynamics of the accumulation of trace elements is preserved only for low and medium dose values of nanoparticles. Thus, a maximum dose load of 1000 mg/kg reduces the concentration of Zn in the body of E. fetida by 106 % compared with a seven-day exposure.

The level of Cu in the body of E. fetida increased in parallel with the increase in dosages and exposure times: at 50 mg/kg by 73.9 % (\( p \leq 0.05 \)), at 200 mg/kg by 91.9 % (\( p \leq 0.05 \)), at 500 mg/kg by 89.2 % (\( p \leq 0.05 \)), at 1000 mg/kg by 91.3 % (\( p \leq 0.05 \)).

The presence of Mo nanoparticles in the medium on the 7th day of incubation leads to a significant increase in the concentration of Mo in the body of E. fetida from 90.1 % to 169 %. Extending the exposure time to 14 days at a dose of nanoparticles from 200 to 1000 mg/kg is accompanied by a decrease in concentration from 64.8 to 57.4 %, respectively. However, compared to the background and zero concentration of nanoparticles, the metal level remains high.
It is worth noting that incubation of E. fetida in artificial soil without introducing nanoparticles (control) compared to the background also leads to a progressive increase in the concentration of trace elements in E. fetida.

According to studies, the ability of worms to absorb and accumulate metals introduced into the substrate in nanoforms was different (Table 2).

In particular, if the rate of accumulation and absorption of Zn was 2.9 mg/kg/day and 1.27 mg/kg/day, respectively in the control over 14 days of exposure; then there was a dose effect in these indicators at a dose of 50 mg/kg by 17.14 % and 7.2 %, at 200 mg/kg by 36.9 % and 29.4 %, at 500 mg/kg – 49.1 % and 51.3 % and at 1000 mg/kg – by 40.8 % and 46.1 %. It is interesting to note that at a dose of 1000 mg/kg, the accumulation rate was 7 days (28.4 mg/kg/day), then the subsequent period (7-14 days) was characterized by metal excretion at a rate of 18.64 mg/kg/day.

Table 2. Accumulation rate (S) and degree of absorption (R) metals in E. fetida over 14-day exposure on artificial soil.

| Concentration NP, mg/kg | S, Zn mg/kg/days | R, Zn mg/ % | S, Cu mg/kg/days | R, Cu mg/ % | S, Mo mg/kg/days | R, Mo mg/ % |
|------------------------|------------------|------------|------------------|------------|------------------|------------|
| 0                      | 2.9±0.31         | 1.27±0.08  | 0.69±0.05       | 0.20±0.06  | 0.45±0.07       | 0.10±0.06  |
| 50                     | 3.5±0.54*        | 1.38±0.07  | 1.26±0.08*      | 0.35±0.08  | 0.59±0.03       | 0.14±0.03  |
| 200                    | 4.6±0.34         | 1.8±0.16   | 5.08±0.4*       | 1.35±0.2*  | 3.77±0.41*      | 0.88±0.07* |
| 500                    | 5.7±0.62         | 2.61±0.09* | 3.69±0.2*       | 0.83±0.07* | 5.45±0.62*      | 1.51±0.2*  |
| 1000                   | 4.9±0.33         | 2.3±0.17*  | 4.70±0.51*      | 1.44±0.08* | 5.1±0.67*       | 1.32±0.18* |

Note: * – significant difference with the control (p≤0.05).

Positive dynamics was also noted during the accumulation of Mo over a seven-day exposure. So, at a dose of 10 mg/kg, the accumulation rate was 0.3 mg/kg/day, absorption – 0.06 mg/%, at a dose of 40 mg/kg S – 10.8 mg/kg/day, R – 2.09 mg/%, at a dose of 500 mg/kg S – 9.5 mg/kg/day and R – 2.2 mg/%, at a dose of 1000 mg/kg, 5.1 and 1.32. Over a period of 7–14 days, accumulation at concentrations from 200 to 1000 mg/kg decreased by 64.8 and 47 %, which was expressed in a decrease in the rate of accumulation (–7.05 and –4.08 mg/kg/day) and absorption (–1.6 and –1.12 mg/ %), respectively.

The constancy in the accumulation of Cu was expressed in the accumulation of Cu at a rate of 5.08 and 4.7 mg/kg/day at doses of 200 and 1000 mg/kg with a significant difference with the control. A similar dynamics was established for the degree of absorption of copper in the body of E. fetida.

According to the results of our study, E. fetida worms have the ability to accumulate metals, but this depends on the dose, type and time of exposure to nanoparticles. So, the excretion of nonspecific elements is slower, and in some cases absent.

According to our data, incubation in a medium with Zn and Mo nanoparticles leads to an increase in the absorption and accumulation of the corresponding metal to a certain level, followed by a decrease in the accumulation rate. This made it possible to determine the dose load of metal nanoparticles and establish a threshold concentration of the level of trace elements for Zn – 252 mg/kg and Mo – 67.2 mg/kg, while the Cu level in the body of E. fetida increased in parallel with increasing dosages and exposure times.

So, living organisms, incl. earthworms can bind zinc in tissues through the participation of metallothioneins and are capable of rapidly eliminating Zn with an increase in its concentration in soil [9]. E. fetida regulates the content of Zn in the body, provided that its concentration in the soil does not exceed 560 mg/kg [9], with an increase in concentration, a significant accumulation of metal in the body occurs.

At the same time, the absorption of Mo by worms can be a passive process, which is caused by the available amount of molybdenum in the substrate and is not associated with active processes that are regulated by earthworms [10].

Given that the mechanisms of regulation of the concentration of metals in the body include both an increase in the rate of excretion with increasing concentration in the soil, and a toxic effect, in which the
metabolic rate in the worm increases against the background of high concentrations of metals [11]. In particular, according to our data, a decrease in the concentration in the body of the worm is associated both with the ability of the worm to regulate these metals and with the manifestation of toxic effects that reduce the motor activity and trophometabolic activity of the worm.

Short-term exposure when using nanoparticles may not reveal the specific aspects of their interaction with soil organisms. However, not an absolute concentration of the metal, but its accumulation should be considered a key indicator of metals’ accumulation in the context of dose-dependent values.

4. Conclusion

Thus, we established a different ability of the worm organism to accumulate metals with variable valency if the substrate contains the same name nanoparticles. In particular, the level of Cu in the body of E. fetida increased along with dosages and exposure times, while the absorption and accumulation of Zn and Mo occurred to a certain level, with a subsequent slowdown in its accumulation rate. According to a number of published data, the effect of some elements is slow, and their accumulation can continue for a long period (months). Whereas the toxikinetics of the effect of nanoparticles can affect unknown adaptation mechanisms, which is mainly associated with both the regulation of metals in the body of the worm and the manifestation of toxic effects that reduce motor activity and trophometabolic activity of the worm. On the other hand, the skin route is an important channel for collecting water metal ions from soil and not through a gastrointestinal tract [12]. This indicates the specificity of the translocation of dissolved Zn, Mo, and Cu ions from substrate to a worm.

Thus, the above results can become the basis for the development of restoration of soils contaminated with nanoparticles, which will allow using vermicultivation to bind and extract various metals from the soil.

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