

Impact of molybdenum nanoparticles on survival, activity of enzymes, and chemical elements in *Eisenia fetida* using test on artificial substrata

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Received: 23 October 2015 / Accepted: 17 May 2016 / Published online: 3 June 2016
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Abstract The influence of molybdenum oxide nanoparticles (MoO_3) on the growth and survival of *Eisenia fetida* was established. The activity of antioxidant enzymes and changes in concentration of molybdenum in the body of *E. fetida* were determined. The degree of bacterial bioluminescence inhibition in extracts of substrates and worm was studied using luminescent strain *Escherichia coli K12 TGI*. The enzymatic activity of substrates before and after exposure with nanoparticles and worms was assessed. Nanoparticles have concentrations of 10, 40, and 500 mg/kg of dry matter, and substrata are made of artificial soil (substrate A) and microcrystalline cellulose (substrate B). Spherical nanoparticles MoO_3 , yellow in color, with size 92 ± 0.3 nm, Z-potential 42 ± 0.52 mV, molybdenum content 99.8 mass/%, and specific area $12 \text{ m}^2/\text{g}$ were used in the study. A significant decrease by 23.3 % in weight was registered (for MoO_3 NPs at 500 mg/kg) on substrate A ($p \leq 0.05$). On substrate B, the maximum decrease in weight by 20.5, 33.3, and 16.9 % ($p \leq 0.05$) was registered at a dose of 10, 40, and 500 mg/kg, respectively; mortality was from 6.6 to 73 %. After the assessment of bacterial bioluminescence inhibition in substrates A and B (extracts) and before worms were put, the toxicity of substrates was established at doses of 40 and 500 mg/kg, expressed in inhibitory concentration (IC) 30 and IC 50 values. Comparatively, on days 7 and 14, after exposure in the presence of *E. fetida*, no inhibition of

bioluminescence was registered in extracts of substrates A and B, indicating the reduction in toxicity of substrates. The initial content of molybdenum in *E. fetida* was 0.9 ± 0.018 mg/kg of dry matter. The degree of molybdenum accumulation in worm tissue was dependent on the dose and substrate quality. In particular, 2–7 mg/kg of molybdenum accumulated from substrate A, while up to 15 kg/kg of molybdenum accumulated from substrate B (day 7). Molybdenum concentration decreased by 64.8 and 57.4 % at doses 40 and 500 mg/kg, respectively, on day 14. The reaction of antioxidant enzymes was shown in an insignificant increase of glutathione reductase (GSR) and catalase (CAT) at concentrations of 10 and 40 mg/kg in substrate A, followed by the subsequent reduction of their activity at the dose of 500 mg/kg MoO_3 . The activity of GSR in substrate B against the presence of MoO_3 nanoparticles decreased, with significant difference of 33.5 % ($p \leq 0.05$) at the dose of 500 mg/kg compared with untreated soil. In experiments with substrate A, an increase of catalase activity was registered for the control sample. The presence of MoO_3 nanoparticles at the concentration of 10 mg/kg in the environment promoted enzymatic activity on days 7 and 14, respectively. A further increase of nanoparticle concentration resulted in the decrease of catalase activity with a minimum value at the concentration of MoO_3 of 500 mg/kg. In the experiment with substrate B at the concentration of MoO_3 nanoparticles of 40 mg/kg, enzymatic activity increases on day 7 of exposure. However, the stimulating effect of nanoparticles stops by day 14 of the experiment and further catalase activity is dose dependent with the smallest value in the experiment with MoO_3 having the concentration of 500 mg/kg.

Responsible editor: Henner Hollert

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Keywords Molybdenum nanoparticles · *Eisenia fetida* · Mortality · Growth · Antioxidant enzymes · Chemical elements

Introduction

There is very little data about toxicity of molybdenum in its nanoform. Therefore, the mechanism of its action on soil invertebrates is not clear. It is also not clear how the bioavailability of molybdenum depends on the composition of the test substrate and what adaptive mechanisms are included in it.

Molybdenum is an essential element for living organisms; it participates in many enzymatic reactions (Mendel and Bittner 2006). Molybdenum has five degrees of oxidation (Adriano 2001) so that its range between deficiency, sufficiency, and toxicity is quite narrow (McBride et al. 2000).

Molybdenum is found in natural soil in different concentrations from 0.2 up to 100 mg/kg. The background concentration is from 0.2 to 6 mg/kg (He et al. 2005), the threshold concentration is 129–450 mg/kg, and the toxic concentration is 2378 mg/kg (Van Gestel et al. 1997).

The sources of molybdenum introduction into the soil are alloys, fire-retarding chemicals, catalysts, inhibitors of lubricants and corrosion of rocks, sewage sludge, and fertilizers (Buekers et al. 2010). In the era of nanotechnology, an additional source of contamination of soil and soil organisms appeared. High bioavailability of metal nanoparticles can have a significant impact on soil ecosystems, especially on earthworms, which are key organisms in the soil ecosystem and are known for their high absorption of metals (Nahmani et al. 2007). Given their body size and the fact that they are easy to handle, they are also the ideal organisms for the determination of “safe levels” of metals and other soil contaminants (Žaltauskaitė and Sodienė 2010). From an ecological point of view, the accumulation of metals in earthworms is also important, as they are a source of food for many amphibians, reptiles, birds, and mammals (OECD 2004).

Molybdenum toxicity depends on the amount and form. It can quickly accumulate in earthworms, and its bioaccumulation is mainly dependent on soil pH and the content of organic carbon (Diez-Ortiz et al. 2010).

At present, the bioavailability and range of toxic effect of zinc, copper, and titanium are determined (Diez-Ortiz et al. 2010; Hu et al. 2010; Li et al. 2011; Natal-da-Luz et al. 2011; Van Gestel et al. 2011a, 2011b; Groplova et al. 2011; Tourinho et al. 2012).

Data on the effect of molybdenum on microorganisms (Buekers et al. 2010) and plants (McGrath et al. 2010) were recently published. The LC 50 value for *Eisenia fetida* was above molybdenum contamination 450 mg/kg of dry soil (Van Gestel et al. 2012). Unfortunately, there is little information about the extent of the impact of molybdenum nanoparticles on the adaptive reaction of the worms. It is impossible to predict the direct and long-term effects of metals in nanoform. High bioavailability, the degree of penetration, and the prolonged effect of metals determine the significance of research.

In order to establish the toxic effects of metals, they should be bioavailable. Bioavailability of metals in soil comprises at least three dynamic processes, including desorption, soil absorption in living organisms, and toxodynamics of redistribution within an organism (Hamelink et al. 1994, Conder and Lanno 2000, Conder et al. 2002). All three processes have their own kinetics, and consequently, toxicity can be determined without taking into consideration the exposure time.

Perhaps, in our study, short-term exposure does not reveal all aspects of the impact of nanoparticles on the body, given the bioavailability of metals by soil components; it is necessary to take into account changes in the enzymatic activity of the soil.

Soil enzymes are involved in degradation of plant, animal, and microbial residues and synthesis of humus. They are distinguished by an outstanding activity, strict specificity of action, and a great dependence on different environmental conditions. The latter feature is of great importance in the prospects of their use for bioindication (Kazeev 1990).

In order to determine the response of *E. fetida* to the introduction of molybdenum oxide nanoparticles (MoO_3) into the substrate, the following tasks have been solved: (1) to establish the influence of MoO_3 nanoparticles on the growth and survival of *E. fetida*, (2) to determine the activity of antioxidant enzymes and changes in molybdenum concentration in the body of *E. fetida*, (3) to examine the bacterial bioluminescence inhibition of extracts of substrates and worm using luminescent strain *Escherichia coli* K12 TGI, and (4) to assess the enzymatic activity of substrates before and after the exposure of molybdenum oxide nanoparticles and worms.

Materials and methods

Chemicals and substrates

Two substrates were used within this study.

Substrate A: microcrystalline cellulose (MCC, Ankir-b, Evalar, Russia).

Substrate B: normal artificial soil (OECD 207 OECD 1984) was produced by mixing 70 % of quartz (particle size, 0.1–0.3 mm, LLC “Inesko,” Hydrotorf, Russia), 20 % kaolin (JSC «Novokaolinovsky» GOK, Chelyabinsk, Russia), and 10 % sphagnum peat (organic nitrogen—5.8 %, LLC «Lma» Torf, Volokolamsk, Russia). pH was adjusted to 6.0 ± 0.5 with powdered calcium carbonate (CaCO_3 , LLC «Mineralprom», Syzran, Russia).

We used oxidized nanoparticles MoO_3 (sized 92 ± 0.3 nm, Z-potential 42 ± 0.52 mV, molybdenum content 99.8 mass%,

specific area 12 m²/g; Plazmoterm, Moscow, Russia), obtained by plasma chemical synthesis.

The materials were assessed (particle size, polydispersity, volume, quantitative content of fractions, surface area) by electron scanning, transmission, and atomic force microscopy using the following equipment: a LEX T OLS4100, a JSM 7401 F, and a JEM-2000 FX, respectively (“JEOL,” Japan). The size distribution of particles was investigated using a Brookhaven 90Plus/BIMAS and ZetaPALS Photocor Compact (Photocor, Russia) in lysols after dispersing nanoparticles with an ultrasonic disperser (UZDN-2T, Russia) at f-35 kHz, N 300 W, and A-10 µa for 30 min.

Test organisms

Worms used in the study were grown in the nursery in the Laboratory for Agroecology of Technogeneous Nanomaterials, State Educational Institution “All-Russian Research Institute of Beef Cattle Breeding RAAS.”

E. fetida were cultured in horse manure without drugs at the temperature of 20 °C. Worms with body weight from 350 to 450 mg were used in the study.

Testing

The test was conducted according to the guidelines for the testing of chemicals and bioaccumulation in terrestrial oligochaetes OECD 207 OECD (1984). Doses of molybdenum nanoparticles were chosen with due regard to load: 10 mg/kg—background, 40 mg/kg—increased, and 500 mg/kg threshold. Before the experiment, the worms were washed with distilled water and placed on filter paper in a Petri dish for 24 h with 15 ml of distilled water to clear the digestive tract.

For each concentration, we used plastic containers with dimensions 5 × 4 × 4 cm (length, width, height) and filled with 500 g of substrate.

Lysols of molybdenum oxide were prepared by the method proposed by Scott-Fordsmand et al. (2008) by means of adding test metal (dry powder) at concentrations of 10, 40, and 500 mg/kg in deionized water (10 ml), followed by dispersing with an ultrasonic disperser (UZDN, f-35 kHz, N-300W, Russia) for 30 min. Subsequently, the prepared lysols for each replication and concentration were mixed with wet (40–45 %) substrates A and B (500 g); then, they were adjusted with distilled water to a moisture content of 65–70 % and stirred with a mixer.

Ten healthy worms were added to each container. All containers were closed with a perforated cap to prevent moisture loss and were incubated with continuous light. The experiment was performed within 14 days at the temperature of 20 °C in five repetitions. Every day, the containers were inspected in order to remove dead animals.

On days 7 and 14, worms were selected from each replicate for laboratory tests; they were washed with distilled water.

Weighing was performed before the design and on days 7 and 14 after exposure of the substrates to different concentrations of molybdenum oxide nanoparticles. Then, the total and average weight of worms in each replicate was measured. Survival was determined every day; dead species were removed from the substrate.

Test of bacterial bioluminescence inhibition

On days 7 and 14, worms were taken from substrates A and B; they were washed with distilled water and placed for 3 days in plastic containers with moist substrate from filter paper to clear the digestive tract (Dalby, 1996). Extracts of worm were prepared according to Li et al. (2011).

The sample substrate weighing 20 g, weighed with an accuracy of less than 0.1 g, was placed in a conical flask. One hundred milliliters of distilled water was poured on the samples. Soil and water were mixed for 3 min using a propeller mixer and left for 5 min for sedimentation, and then, the mixture was filtered through filter paper (filter paper of “F” brand with average carrying capacity, MiniMed, Bryansk, Russia). The use of this filter paper helps to avoid filtering of nanoparticles weighed in substrates.

The bacterial bioluminescence inhibition test was carried out with due regard to the recommendation of Deryabin and Aleshina (2010). The genetically engineered luminescent strain *E. coli K12 TG1* was used; this strain was engineered to constitutively express the *lux CDABE* genes of the natural marine microorganism *Photobacterium leiognathi 54D10* and was produced by Immunotech (Moscow, Russia) in lyophilized condition under the commercial name “Ecolum” (Danilov et al. 2002).

Immediately prior, the study strain *E. coli K12 TG1* was restored by the addition of chilled distilled water. The suspension of bacteria was maintained at +2–4 °C for 30 min, after which the temperature of the bacterial suspension was brought to 15–25 °C.

The test of inhibition of bacterial luminescence was carried out by making the cells in the 96-well plates of opaque plastic of the test substance and the suspension of luminescent bacteria in a 1:1 ratio; after that, the tray was placed in the measuring unit of the microplate analyzer Infinite PROF200 (TECAN, Austria), dynamically registering the luminescence intensity of the obtained mixtures for 180 min with an interval of 5 min.

The intensity of bacterial bioluminescence of extracts of substrates A and B and *E. fetida* was assessed using the formula

$$I = \frac{Ik_{0\min} \times Io_{n\min}}{Ik_{n\min} \times Io_{0\min}},$$

where I_k and I_0 are the illumination intensities of the control and experimental samples, respectively, from the 0th and n th minutes of measurement. Three threshold levels of toxicity are taken into account:

1. Less than 20—the sample is “non-toxic” (luminescence quenching $\leq 20\%$).
2. From 20 to 50—the sample is relatively toxic (luminescence quenching 50%).
3. Equal to or greater than 50—the sample is toxic (luminescence quenching $\geq 50\%$).

Molybdenum concentration in *E. fetida*

The concentration of molybdenum in worm was measured at the beginning of the test and on days 7 and 14. Five worms from five replicates were selected; their digestive tracts were cleared according to the above-described procedure.

The samples of worms were placed in a crucible and burned in a muffle furnace at $450\text{ }^\circ\text{C}$ to analyze the chemical elements. Dry ashing or acid extraction was used to prepare the desired solution. The resulting ash was dissolved in a crucible, heated in nitric acid (1:1) based on a volume of $1\text{--}5\text{ cm}^3$ of acid for the sample weight depending on the ash content of the product. The solution was evaporated to moist salts. The resulting precipitate was dissolved in $15\text{--}20\text{ cm}^3$ of nitric acid, mass fraction of 1% , and then, it was quantitatively transferred to a volumetric flask of 25 cm^3 and was adjusted to the mark with the same acid. The resulting solutions were placed in the instrument and measured.

The element composition of the biosubstrates was analyzed using atomic emission and mass spectroscopy in the test laboratory of ANO “Centre for Biotic Medicine,” Moscow, Russia (Registration Certificate of ISO 9001: 2000, Number 4017–5.04.06). The biosubstrates were ashed in the microwave decomposition system MD-2000 (USA). The content of elements in the resulting ash was measured on the mass spectrometer Elan 9000 and atomic emission spectrometer Optima 2000 V (Perkin Elmer, USA).

Determination of antioxidant enzyme activity in *E. fetida*

After the digestive tracts were cleared, the worms were placed in a glass tube to study the activity of antioxidant enzymes. Homogenization was performed in the buffer solution (Tris 50 mmol/l , DTT 1.0 mmol/l , EDTA 1.0 mmol/l (Tris, DTT, EDTA; Sigma, Russia); 250 mM sucrose/l, pH 7.5 (LenReaktiv, Saint Petersburg, Russia)), which was added in a ratio of 1:9. The worms were homogenized using the TissueLyser LT, QIAGEN (manufacturer—QIAGEN, Germany). The homogenate was centrifuged for 10 min at

$15,000\text{ rev/min}$. The resulting supernatant was diluted with buffer to a mixture of 10% homogenate (Li et al. 2011). The supernatant was used to determine glutathione reductase (GSR) and catalase (CAT).

The level of enzymes was determined using the semi-automatic biochemical analyzer 1904 Stat Fax Plus (manufacturer—Awareness Technology Inc, USA) and commercial kits by Randox (USA).

Enzymatic activity of soil

The catalase activity of substrates A and B was investigated 7 and 14 days after exposure in the presence of molybdenum oxide nanoparticles and worms *E. fetida*. The catalase activity in soil was determined by the method of Galstyan (Kazeev 2003). A sample of substrate (1 g) was taken for the study. A 3% solution of H_2O_2 was used. It was required to check perhydrol concentration periodically; reaction buffer was prepared immediately prior to analysis. In order to determine the concentration of perhydrol, 1 g of H_2O_2 was weighed on an analytic balance in a volumetric flask (100 ml); the amount was made up to the mark and shaken. Twenty milliliters of the obtained solution was poured into a conical flask of 250 ml (three replicates); 50 ml of distilled water and 2 ml of 20% H_2SO_4 were added. Then, 0.1 n of KMnO_4 titrated. One milliliter KMnO_4 corresponds to $0.0017008\text{ g H}_2\text{O}_2$. After the concentration of perhydrol had been determined, 3% solution was prepared by diluting the solution with distilled water. Titration solution of KMnO_4 was prepared from fixanal and incubated for several days to determine the titer. Catalase activity was expressed in milliliters of O_2 that was emitted from 1 g of soil for a minute.

Statistical analysis

Statistical processing of the data was performed using the software package Statistica 6.0. The calculated values included the arithmetic mean value (M) and the standard error of the mean (m). Results with $p \leq 0.05$ were considered significant.

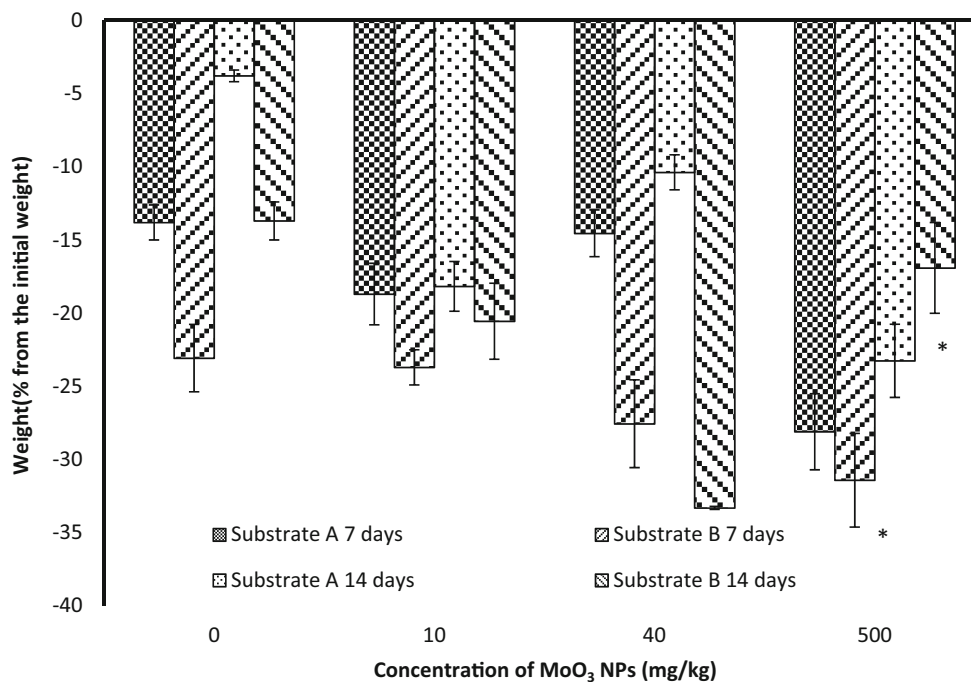
Results

Influence of MoO_3 nanoparticles on growth of worms

The weight of worms decreased throughout the experiment, regardless of the qualitative composition of the substrate.

On day 7, the live weight of worms decreased in comparison with the initial weight in substrate A (MoO_3 NP concentration was 0, 10, 40, and 500 mg/kg) by 13.8, 18.7, 14.5, and 28.1 %, respectively (Fig. 1). At the end of the experiment (14 days), the weight continued to decrease against the introduction of molybdenum nanoparticles to the substrate. The

Fig. 1 Change of weight of *Eisenia fetida* at different concentrations of MoO₃ NPs in substrates A and B (% from the initial weight)



only difference was that at the dose of 500 mg/kg, the effect was maximal—23.3 % ($p \leq 0.05$).

After the increase of the dose of MoO₃ NPs from 10 to 40 to 500 mg/kg in artificial soil, the weight of the worms decreased in comparison with the initial weight on day 7 of the experiment by 23.8, 27.1, and 31.4 %, respectively (Fig. 1).

A significant decrease in weight was registered when the dose of MoO₃ NPs was 40 mg/kg (33.3 %) and 500 mg/kg (16.9 %) ($p \leq 0.05$) on day 14 of the experiment.

Survival of worms

Assessing the mortality of the worms, it was found that the worms in the control substrates A and B were alive until the end of the testing period. The greatest toxic effect was demonstrated in substrate A at the concentration of MoO₃ NPs 500 mg/kg; the mortality on day 7 was 43 % and on day 14 73 % ($p \leq 0.05$) (Fig. 2).

Mortality indices were from 33 to 53 % ($p \leq 0.05$) on the 14th day of exposure in substrate B; the dose of MoO₃ was 40 and 500 mg/kg, respectively.

It should be noted that at concentrations of 40 and 500 mg/kg the decreased survival of worms attests to different exposure modes or molybdenum toxodynamics in two substrates A and B. Therefore, a high release of molybdenum from nanoparticles can be the possible cause of death at the dose of 40 mg/kg, while at the dose of 500 mg/kg, reductions of motor activity and trophic activity of the worms are the causes of death.

Toxicity of soil extracts in relation to *E. coli* K12 TGI with cloned *luxCDABE* genes of *P. leiognathi* 54D10 before and after the introduction of *E. fetida*

The inhibition of luminescence at the doses of 40 and 500 mg/kg was established after assessment of bacterial bioluminescence inhibition in substrates A and B (extracts) and before worms were put; it indicated relative and acute toxicity (Table 1).

Comparatively, on days 7 and 14, after exposure in the presence of *E. fetida*, no inhibition of bioluminescence was registered in extracts of substrates A and B; it indicated the reduction in toxicity of the substrates.

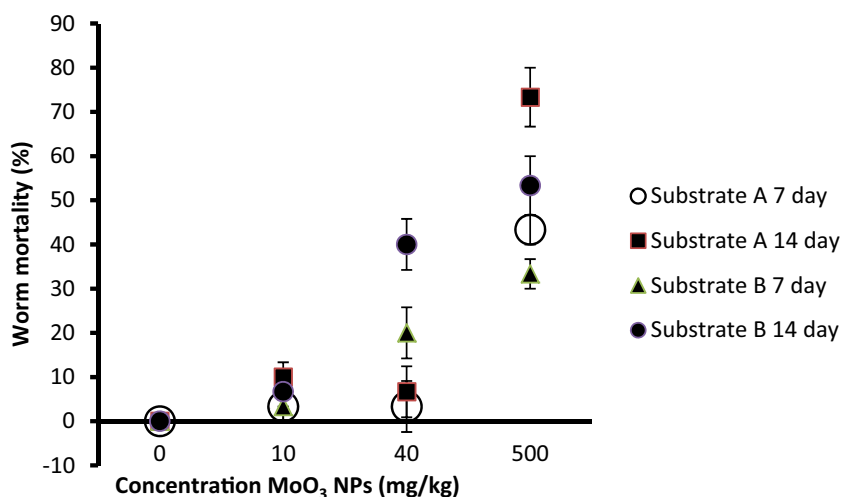
Toxicity studies of worm extracts towards *E. coli* K12 TGI demonstrated that increasing concentration had no significant effect on bacterial bioluminescence compared with the control. There was no quenching of bioluminescence irrespectively of concentration (Table 2).

Molybdenum concentration in *E. fetida*

The initial molybdenum concentration in worms was 0.9 ± 0.018 mg/kg dry matter (DM). The molybdenum concentration in substrate A and substrate B was 1.89 and 1.83 mg/kg DM, respectively.

On day 7 of exposure, the molybdenum concentration in worms increased from 2.43 to 2.78 mg/kg, with the highest value at a dose of 40 mg/kg. On day 14, the accumulation of molybdenum in the test samples of worms increased 62 times on average and was at a concentration of 10 mg/kg—

Fig. 2 Mortality of enzyme of *E. fetida* under the influence of nanoparticles of MoO₃ in 7 and 14 days. Bars represent the standard deviation of the mean value of the five repetitions in substrates A and B



7.53 mg/kg, 40 mg/kg—7.21 mg/kg, and 500 mg/kg—7.11 mg/kg (Fig. 3).

Molybdenum accumulation was high in conventional substrate made of substrate B (Fig. 4). So, molybdenum concentration in worm was 0.5 mg/kg (10 mg/kg), 15.2 (40 mg/kg), and 13.45 mg/kg (500 mg/kg) on day 7 of exposure in substrate B. On day 14, molybdenum concentration decreased by 64.8 and 47 % at doses of 40 and 500 mg/kg, respectively, compared with the previous period.

Influence of MoO₃ nanoparticles on antioxidant enzymatic activity in *E. fetida*

The influence of MoO₃ nanoparticles in various concentrations within 14 days is shown in Fig. 6. A slight increase in GSR was observed at concentrations of 10 and 40 mg/kg in substrate A with a subsequent decrease by 42.0 % ($p \leq 0.05$) at a dose of 500 mg/kg of MoO₃ nanoparticles. A similar dynamics was established in CAT activity; moreover, statistically significant differences by 37.5 % were established at a dose of 500 mg/kg of MoO₃.

Table 1 Toxicity of soil extracts in relation to *E. coli* K12 TG1 with cloned *luxCDABE* genes of *P. leiognathi* 54D10 before and after the introduction of *Eisenia fetida*

Concentration MoO ₃ NPs, mg/kg	Background ^a		Incubation period, days			
			7		14	
	Substrate A	Substrate B	Substrate A	Substrate B	Substrate A	Substrate B
0	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC
10	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC
40	IC 30	IC 30	NOEC	NOEC	NOEC	NOEC
500	IC 50	IC 50	NOEC	NOEC	NOEC	NOEC

IC 50 and IC 30 correspond to concentration values for 50 and 30 % inhibition of biosensor luminescence in comparison to control

NOEC no observed effect concentration

^a Background is soil before worms were put into substrates

GSR activity with increasing concentrations of MoO₃ nanoparticles from 10 to 500 mg/kg in substrate B compared with the untreated soil decreased by 46.7, 29.6 ($p \leq 0.05$), and 33.5 %, respectively. Catalase activity also decreased with insignificant differences (Fig. 5).

The enzymatic activity of catalase in substrates A and B on days 7 and 14

The study of this indicator in the experiment with substrate A helped us to identify stimulation of enzyme activity on the 7th and 14th days of exposure. If the catalase exposure level on day 7 was 0.6 cm³ O₂/g per 1 min in the control, the enzymatic activity at the doses of 10 and 40 mg/kg was higher by 25.0 and 14.3 %, respectively, and at the dose of 500 mg/kg, a decrease by 50 % ($p < 0.05$) was registered. On day 14, the catalase level in the substrate was higher by 5.2 % of the control level (1.8 cm³ O₂/g per 1 min) at the dose of 10 mg/kg, while at 40 and 500 mg/kg its decrease by 5.5 and 11.1 % ($p < 0.05$), respectively, was noted (Fig. 6). This is due to the weakening of the metabolic effect of *E. fetida* on the substrate:

Table 2 Toxicity of worm extracts in relation to *E. coli* K12 TGI with cloned luxCDABE genes of *P. leiognathi* 54D10 before and after the introduction of nanoparticles

Concentration MoO ₃ NPs, mg/kg	Background ^a	Incubation period, days			
		7		14	
		Substrate A	Substrate B	Substrate A	Substrate B
0	NOEC	NOEC	NOEC	NOEC	NOEC
10		NOEC	NOEC	NOEC	NOEC
40		NOEC	NOEC	NOEC	NOEC
500		NOEC	NOEC	NOEC	NOEC

NOEC no quenching of luminescence registered

^a Background is worm extract before the beginning of experiment

the enrichment of its microorganisms, organic matter, and enzymes.

In the experiment with substrate B with MoO₃ nanoparticle concentrations 10, 40, and 500 mg/kg, enzyme activity increased by 16.6, 33.3, and 9.1 %, respectively, on day 7 of exposure compared with the control (0.2 cm³ O₂/g per 1 min) (Fig. 7). But by day 14 of the test, the stimulating influence of nanoparticles stopped and further dynamics of catalase activity became dose dependent with the smallest value in the test with the MoO₃ concentration of 500 mg/kg (1.25 cm³ O₂/g for 1 min), which is below the level of control by 20.0 % (*p* < 0.05).

Discussion

Little attention is paid to the determination of molybdenum toxicity for earthworms compared to other metals, especially in nanoform.

That is why compost worms *E. fetida* were used in our studies of acute toxicity of MoO₃ nanoparticles in various concentrations with a duration of 14 days in compliance with the eligibility criteria established by the OECD 207 OECD (1984) with the following parameters: temperature—20 °C and continuous light. Molybdenum nanoparticle doses were chosen with due regard to the load: 10 mg/kg—background, 40 mg/kg—increased, and 500 mg/kg—threshold. The doses of molybdenum used for biotesting are not accidental; they are based on studies of Landa (1984) and Wichard et al. (2009), where molybdenum content in natural soils ranges from 1 to 2 mg/g of dry weight, while concentration in contaminated soil may reach 100–210 mg/g of dry weight (Landa 1984; Wichard et al. 2009).

Two substrates were used when modeling the toxic load on soil organisms; one was substrate B and the other was substrate A. The use of this substrate is based on advanced methods of cleansing of bowels contained in the manual for the study of the structure, functioning, and diversity of detritus food chains edited by Pokarzhevsky et al. (2003), and based

Fig. 3 Molybdenum concentration in *Eisenia fetida* at different concentrations of MoO₃ nanoparticles in substrate A. Bars represent standard deviation from the mean of three replicates. Asterisks significant difference compared with the control (0 mg/kg) (*p* ≤ 0.05)

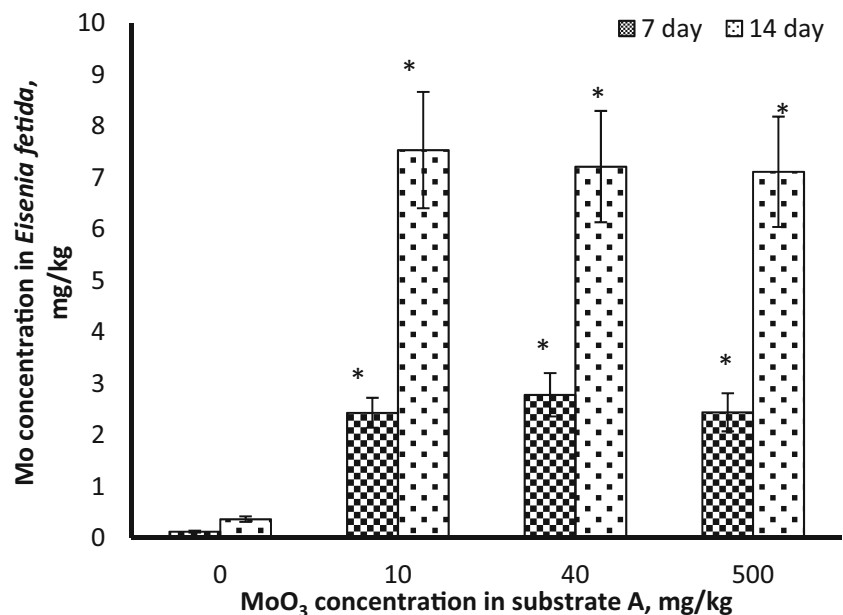
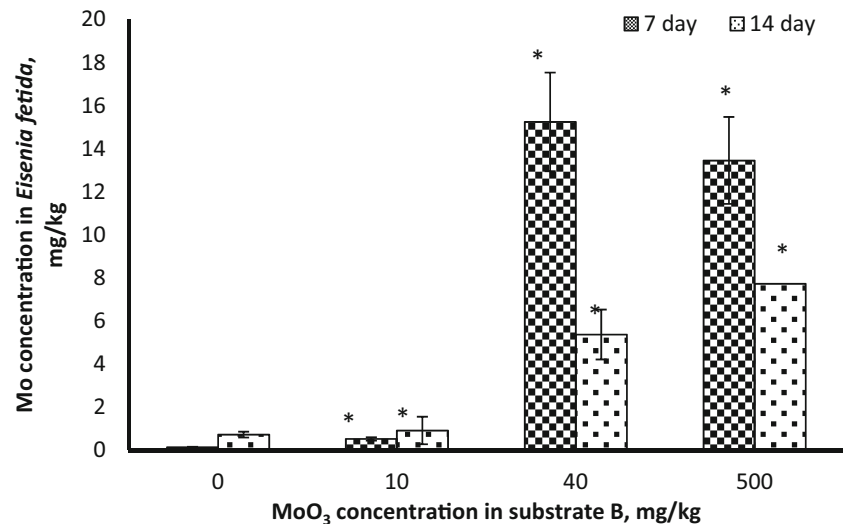


Fig. 4 Molybdenum concentration in *Eisenia fetida* at different concentrations of MoO_3 nanoparticles in substrate B. Bars represent standard deviation from the mean of three replicates. Asterisks significant difference compared with the control (0 mg/kg) ($p \leq 0.05$)



on the method of evacuation of the stomach contents of enzyme using pulp crumb (Reznichenko 2013). This method has been transformed by us to assess the toxicity or stimulatory effect of nanoparticles by replacing the contents of the digestive tract to the chemically pure substrate with nanoparticles.

It was established by studies of Diez-Ortiz et al. (2010) that the absorption of molybdenum increases with its increasing concentration in soil, but factors of biological accumulation are lower at higher levels of exposure.

In this regard, we set an objective to determine changes in the biochemical processes of the worm upon exposure in substrates with different levels of nanoparticles of molybdenum.

Determination of growth as an integral index includes a set of biochemical and physiological effects, demonstrating changes in energy metabolism in the detoxification of pollutants. Additional energy need leads to the decrease in growth. The growth and reproduction of earthworms under the

influence of the metal negatively correlated with reproduction, i.e., if worms do not grow, they do not produce cocoons, and vice versa, if they do not produce cocoons, they continue to grow (Burgos et al. 2005).

The weight of the worms linearly decreased throughout the experiment with increasing concentration of MoO_3 nanoparticles. Toxic effects of MoO_3 nanoparticles on the weight of the worms depended on the composition of the substrate. The worms adapted to substrate A faster; it was expressed in less weight loss compared with the initial weight; the most significant decrease was recorded at the dose of 500 mg/kg (23.3 %, $p \leq 0.05$), whereas when substrate B was used, the weight continued to decrease until the end of the registered period, with the maximum decrease by 33.3 and 16.9 % ($p \leq 0.05$) at the doses of 40 and 500 mg/kg, respectively. A possible reason for the greater weight decrease at the dose of 40 mg/kg after exposure in substrate B in our opinion was the high

Fig. 5 Action of MoO_3 nanoparticles on the activity of glutathione reductase (GSR) and catalase (CAT) in the earthworm *Eisenia fetida* after 14-day exposure in substrates A and B. Bars represent standard deviation from the mean of three replicates. Asterisks significant difference compared with the control (0 mg/kg) ($p \leq 0.05$)

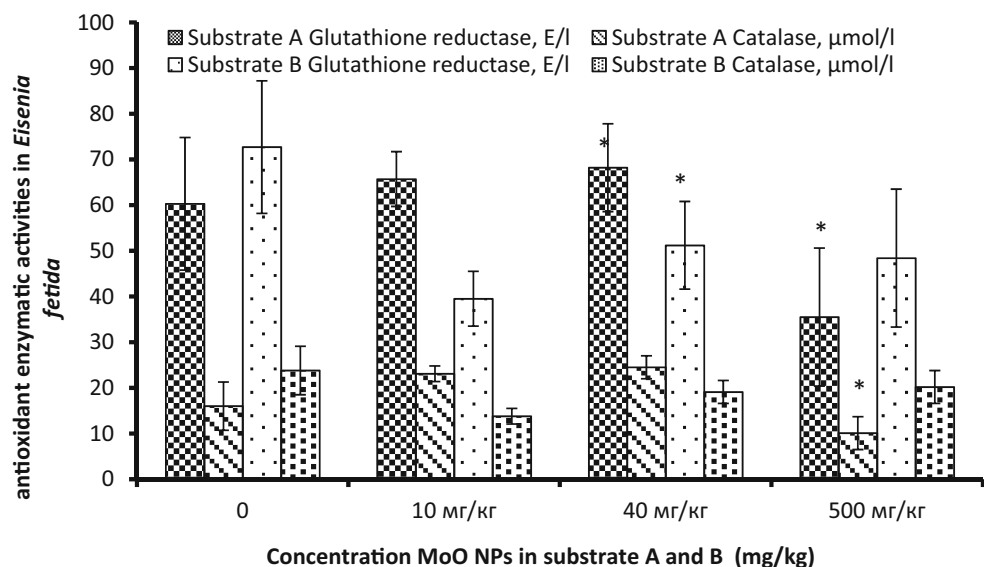
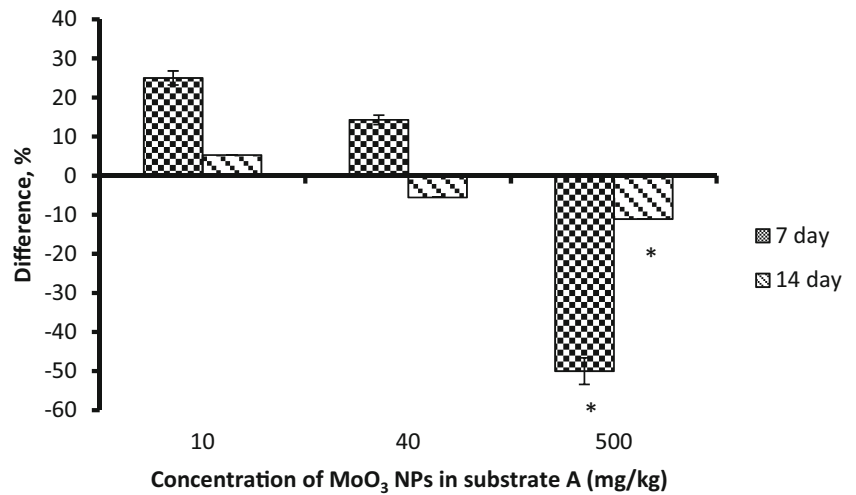


Fig. 6 Activity of catalase in substrate A. Bars represent standard deviation from the mean of three replicates. Asterisks significant difference compared with the control (0 mg/kg) ($p \leq 0.05$)



degree of metal ion dissolution (Li et al. 2011) and, as a consequence, a high penetrating ability of metal through the digestive system, whereas at the dose of 500 mg/kg, there was a possible agglomeration of nanoparticles and, as a consequence, a decrease of metal penetration through the digestive system and dermal route. The weight decrease of worms is associated both with nutrient deficiencies and the degree of influence of metal on the body (Vijver et al. 2003).

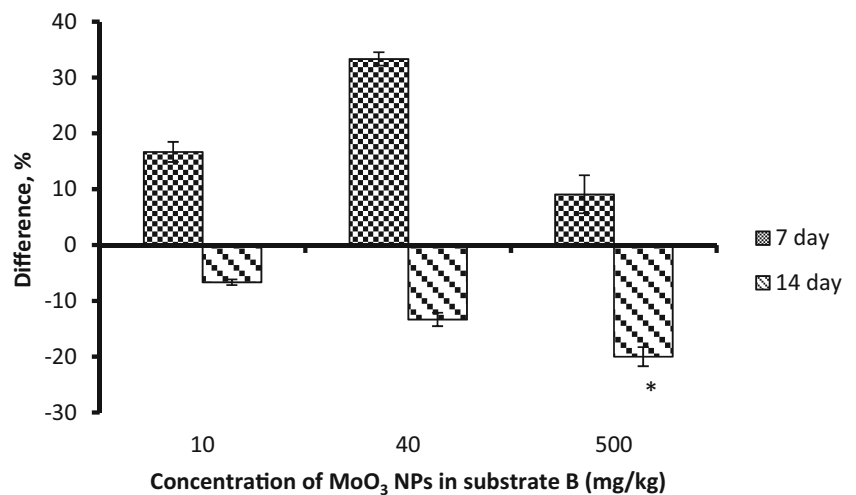
Assessing the mortality of the worms in the test on the toxicity of MoO₃ nanoparticles, it was found that the worms in the control substrates A and B were alive until the end of the testing period. The greatest toxic effect was demonstrated at the MoO₃ concentration of 500 mg/kg in substrate A; the mortality rate on day 14 was 73 % ($p \leq 0.05$). Significant mortality indices were established in substrate B at doses of 40 and 500 mg/kg; mortality was 33 and 53 %, respectively, on day 14 of exposure, although according to Van Gestel et al. (2011a, 2011b) the death rate has not been established even at high exposure levels of dry soil (3200 mg/kg). These data were obtained for soil under natural conditions. Identifying

potential risks of ecotoxicity using test substrates, we can agree with the conclusions of Van Gestel et al. (2011a, 2011b) that worms are not very well able to regulate the concentration of molybdenum in the body; however, risks of negative impact on soil organisms grow after soil contamination by molybdenum nanoparticles. In the results obtained by us, the toxicity of molybdenum nanoparticles increased with increasing concentrations of nanoparticles at statistically significant values with the control.

Using the method of assessment of the inhibition of bacterial bioluminescence against *E. coli* K12 TGI allowed us to quantify the biological toxicity of test objects.

The mechanism of toxic action is a quenching of bioluminescence. The toxicity of substrates was established during the assessment of bacterial bioluminescence inhibition of extracts from substrates A and B; it was expressed in inhibitory concentration (IC) 30 and IC 50 values. While on days 7 and 14 after exposure in the presence of *E. fetida* no inhibition of bioluminescence was registered in extracts of substrates A and B, it indicated the reduction in toxicity of substrates.

Fig. 7 Activity of catalase in substrate B. Bars represent standard deviation from the mean of three replicates. Asterisk significant difference compared with the control (0 mg/kg) ($p \leq 0.05$)



These data let us suggest at first, as it was indicated by Sheppard et al. (1997) and Smolders et al. (2009), that the impact of some elements is slow and their accumulation may continue for a prolonged period (months) and, secondly, toxokinetics of exposure of molybdenum nanoparticles may involve adaptive mechanisms that were unknown earlier; it is mainly connected with the regulation of molybdenum in the body of worms.

The degree of molybdenum accumulation in worm tissue was dependent on the dose and substrate quality. In particular, 2–7 mg/kg of molybdenum accumulated from substrate A, while up to 15 mg/kg of molybdenum accumulated from substrate B (day 7). Molybdenum concentration decreased by 64.8 and 57.4 % at doses 40 and 500 mg/kg, respectively, on day 14.

On the one hand, these results make it possible to assume that a high degree of accumulation of molybdenum in the body of worms placed into substrate A is connected with the passive process, which is caused by available concentrations of molybdenum in the substrate and not the active processes, which are conditioned by earthworms. Cornelis et al. (2011) received similar conclusions in the course of experimental studies. On the other hand, some active excretion mechanisms were established on day 14 at exposure in substrate B, which may be due to a decrease of motor activity and trophic activities of worms.

The reaction of antioxidant enzymes was shown in an insignificant increase of GSR and CAT at concentrations of 10 and 40 mg/kg in substrate A, followed by the subsequent reduction of their activity at the dose of 500 mg/kg MoO₃. The activity of GSR in substrate B against the presence of MoO₃ nanoparticles decreased, with significant difference ($p \leq 0.05$) at a dose of 500 mg/kg compared with that of the untreated soil.

Similar dependence of biological activity of nanoparticles was described by Sun et al. (2007). He found that the activity of antioxidant enzymes in earthworms increases under moderate environmental stress and decreases with severe ecological stress.

In the course of study of catalase activity in substrate A, it was established that enzymatic activity is promoted at concentrations of molybdenum nanoparticles of 10 and 40 mg/kg, which is probably related to the role of molybdenum in maintaining and promoting soil microorganisms (Taran et al. 2014).

It should be noted that substrate A of microcrystalline cellulose has a component that is favorable for the development of soil microorganisms. Cellulose is a source of carbon and energy for bacteria and is also decomposed quickly and easily by them. Increasing the concentration of MoO₃ nanoparticles up to 500 mg/kg caused a suppression of microbiological processes, and catalase activity significantly decreased by more than 17 % as compared with the control. Similar dynamics is observed in the experiment with artificial soil. Lower

values of catalase activity in substrate B as compared with substrate A can be explained, primarily, by its nature. Substrate B as the nutrient medium for microorganisms has peat (10 %), which consists of bitumen, humic substances, and lignin which are resistant to microbial decomposition. Moreover, humic acids promote the aggregation of nanoparticles and are able to form complex compounds with them (Fatehah et al. 2014; Erhayem and Sohn 2014). The findings of our study that characterize a decrease of catalase activity by increasing the concentration of molybdenum nanoparticles are similar to the results on the effect of nanoparticles of titanium on the activity of soil enzymes (Wenchao et al. 2011).

Conclusion

A comprehensive study of the potential toxicity of molybdenum nanoparticles in relation to *E. fetida* and two substrates make it possible for us to conclude:

1. The quality composition of substrates influenced the toxodynamics of molybdenum in test objects.

In substrate A, the high penetration ability of molybdenum nanoparticles was established; it was expressed in high mortality, metal accumulation, and the activity of antioxidant enzymes in *E. fetida*. It was more clearly demonstrated at the dose of 40 mg/kg compared with the higher dose of 500 mg/kg. This proves that the dermal route of earthworms is an important intake channel of water ions of metals from soil, whereas absorption by food intake does not promote accumulation of metals.

The high aggregating capacity of components of substrate B leveled the effect of molybdenum oxide nanoparticles; it was expressed in a relatively low mortality, molybdenum excretion from the body of worms on day 14, a decrease of antioxidant enzymes in *E. fetida*, and reduction of the soil enzyme activity with increasing concentration of molybdenum oxide nanoparticles.

2. Mortality and weight to our mind are not sensitive indicators in ecotoxicological studies due to the low nutritional capacity of food substrates. Determination of metal accumulation in the body of worms is the most significant in the study; it allows the projection of data on natural ecosystems.
3. Substrate A can be used for testing soil components with high content of organic substances.

This research was conducted with financial support from the Russian Science Foundation (Grant No. 14-36-00023).

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