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EFFECT OF Al_2O_3 NANOPARTICLES ON SOIL MICROBIOCENOSIS, ANTIOXIDANT STATUS AND INTESTINAL MICROFLORA OF RED CALIFORNIAN WORM (*Eisenia foetida*)

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Abstract

With the accumulation of experimental data it is evident that nanomaterials, which are widely used in human activity, look promising for agronomy. However, available publications on a comprehensive assessment of biological risks arising from nanoherbicides, nanofertilizers, etc., in particular on how the metal nanoparticles affect geobionts, are very limited. In the model experiments with California red worms *Eisenia foetida* as test organisms we studied the influence of aluminum oxide nanoparticles on soil biocenosis and their biodegradation. We found an increase in mortality of worms up to 20 % at the maximum dosage of aluminum oxide nanoparticles introduced into the soil. Assay of antioxidant defense enzyme activity in the *E. foetida* revealed an increased superoxide dismutase and catalase level as influenced by the studied nanoparticles. The positive effect of their vermicomposting was shown. At increasing content of aluminum oxide nanoparticles (the nanoparticle dosage of 50, 100, 300, and 3000 mg/kg in the groups 1, 2, 3, and 4, respectively), the 61.7-67.6 % reduction in soil microorganism counts was found without vermicomposting vs. 55.6-61.3 % under vermicomposting. The number of microorganisms in the soil decreased in the groups 1, 2, 3 and 4 by 42.8, 52.4, 61.9 and 76.2 % for fungi, by 64.3, 77.9, 78.6 and 85.7 % for nitrogen-fixing bacteria, and by 22.7, 38.6, 84.1 and 86.4 % for bacteria cultured on starch-and-ammonia agar. The number of cellulolytic bacteria increased by 6.9 % in the group 1 and decreased by 16.7, 12.5 and 25.0 % in the groups 2, 3 and 4, respectively. A similar trend was observed under the influence of aluminum oxide nanoparticles on the *E. foetida* intestinal microflora. As the soil content of aluminum oxide nanoparticles increased from 50 to 3000 g/kg, the total number of microorganisms in the *E. foetida* intestine decreased by 9.7 to 43.2 %. In this, fungi decreased in the groups 1, 2, 3 and 4 by 18.0, 20.0, 39.0 and 40.0 % as compared to control. The number of nitrogen-fixing bacteria was insignificant in the control samples and decreased in the *E. foetida* intestine in the groups 1, 2, 3 and 4 by 16.0, 60.0, 78.8 and 80.0 %. The cellulolytic bacteria counts increased in the intestine of *E. foetida* (by 16.0 %) at minimum nanoparticle dosages, whereas in the groups 2, 3 and 4 this index was lower by 8.0, 32.0, 25.0 and 40.0 %. The number of bacteria cultured on starch-and-ammonia agar decreased in the *E. foetida* intestine in groups 1, 2, 3 and 4 by 13.3, 46.7, 60.0 and 73.3 %. Therefore, our data indicate dose-dependent effects of aluminum oxide nanoparticles and gradual development of their toxicity toward soil and intestinal microflora at increasing levels in the soil. The negative impact of the aluminum oxide nanoparticles on soil biocenosis was shown that was manifested in its depletion, leading to soil degradation and decreased fertility. We confirmed the necessity for complex assessment of nanoparticle biotoxicity in a variety of habitats. The antioxidant system activity in the presence of Al_2O_3 nanoparticles is indicative of *E. foetida* adaptability to stress caused by these agents.

Keywords: nanoparticles, *Eisenia foetida*, catalase, superoxide dismutase, microorganisms

In recent years, a sufficient number of studies have been accumulated to

substantiate the prospects for the use of nano-materials in microelectronics [1], energy physics [2], chemical [3], food [4], pharmaceutical [5], medical industry [6-8]. Nanoparticles (NPs) of metals are also of great practical importance in agriculture (in particular, in plant growing and for increasing soil fertility), where they can be used as nano-herbicides [9], nano-pesticides [10], as plant growth stimulants [11] and nano-fertilizers [12].

Among the various nano-materials, nanoparticles of aluminum and its oxides attract attention. About 250 minerals containing aluminum are known and used in various fields, including in the agricultural sector, the possibility of using them in the nanoform is being actively studied at the present time [13, 14]. However, there is only a limited number of works on the integrated assessment of biological risks associated with aluminum nanoparticles [15-19].

One of the bioindicator species in determining the potential toxicity of chemicals in soils [20, 21] is a red Californian worm (*Eisenia fetida*). Worms play an important role in the circulation of substances and the formation of soil structure, improving the degradation of organic substrates, mixing soil layers and enhancing aeration [22, 23].

In the presented study, using the red Californian worm as a test object, we first demonstrated the dose-dependent action of aluminum oxide nanoparticles, the development of a bactericidal effect on the soil microflora and intestinal microbiocenosis of *E. foetida* with an increase in Al_2O_3 nanoparticle content in the soil, as well as the manifestation of adaptation reactions in *E. fetida*.

The aim of our work was to study the influence of Al_2O_3 nanoparticles on soil microbiocenosis, vermicomposting and the state of the antioxidant system and intestinal microflora in a red Californian worm.

Techniques. The study used a preparation of aluminum oxide nanoparticles (NPs of Al_2O_3 , Advanced Powder Technologies, Ltd, Russia), obtained by electric explosion of a conductor in an argon atmosphere. The size of nanoparticles is 54 nm , the Z-potential is $30 \pm 0.1 \text{ mV}$. The nanoparticle powder composition included: Al_2O_3 (95 %), sorbed nitrogen and hydrocarbons (3 %), water (2 %). Material certification of the preparations included electronic scanning and transmission microscopy on instruments JSM 7401F and JEM-2000FX (JEOL, Japan). X-ray phase analysis was performed on a DRON-7 diffractometer (NPO Burevestnik, Russia). The zeta-potential of the particles was determined on a Photocor Compact-Z analyzer (Photocor, Russia).

A preliminary assessment of the biological activity of NPs Al_2O_3 in vitro was carried out in the inhibition test of bacterial bioluminescence. Suspension samples of nanoparticles were prepared in the concentration range of $0.00625\text{-}4.0 \text{ M}$ and sonicated for 30 min. The genetically engineered luminescent strain *Echerichia coli* K12 TG1, constitutively expressing the genes of *luxCDABE* of the sea microorganism *Photobacterium leiongnathi* 54D10 (lyophilized preparation Ecolum, NVO Immunotech, Russia) was used. Immediately prior to the beginning of the *E. coli* K12 TG1 experiments, the bacteria were revived with chilled distilled water, kept at $2\text{-}4 \text{ }^\circ\text{C}$ for 30 min, after which the temperature of the bacterial suspension was adjusted to $15\text{-}25 \text{ }^\circ\text{C}$. The bacterial luminescence inhibition test was carried out using an Infinite PROF200 microplate analyzer (Tecan Group, Ltd, Switzerland), dynamically recording the luminescence intensity of the resulting mixtures for 180 min with an interval of 5 min. The results of the influence of nano-materials on the intensity of bacterial bioluminescence (I) were estimated by the formula:

$$I = \frac{I_{k0 \text{ min}} \times I_{o n \text{ min}}}{I_{k n \text{ min}} \times I_{o 0 \text{ min}}},$$

where I_k and I_o are the intensity of the luminescence of the control and test sam-

ples at the 0 and n -th minutes of the measurement.

Toxicity was studied according to the OECD Guidelines for the Testing of Chemicals (OECD, 1984, 2004) on laboratory earthworm cultures (red Californian hybrid) *Eisenia foetida* Andrei Bouche. All the selected individuals were reproductive. As the substrate, artificial soil was used (70 % quartz sand, 20 % clay, 10 % peat). Groups ($n = 10$ each) were formed from the individuals of the same weight. In groups I, II, III and IV, NPs of Al_2O_3 were added to the soil at doses of 50, 100, 300, 3000 mg/kg, respectively; V group served as a control (without NPs). NPs were introduced into the substrate before the worms were placed into, mixed with a household mixer and adjust to a humidity of about 85 %. At the same time, the ground soil was incubated with NPs Al_2O_3 in the same dosages without vermicomposting. The experiment was carried out in 3 replicates.

The antioxidant activity of catalase (CAT, EC 1.11.1.6) and superoxide dismutase (SOD, EC 1.15.1.1) in worms were determined on day 14 of the experiment on an automatic biochemical analyzer CS-T240 (Dirui Industrial Co., Ltd, China) with commercial Randox biochemical sets (Randox, USA).

Influence of NPs on the worm intestinal microflora and soil microflora was assessed on day 14. For purification of intestines, worms were kept in a plastic container on wet filter paper for 1 day, then, using a sterile scalpel, the intestine taken and placed in a sterile tube. The soil samples were also taken into sterile test tubes. Microbiological studies were carried out within one day after sampling. Meat-peptone agar (MPA) was used to determine the total count of microorganisms. Microorganisms metabolizing mineral nitrogen were isolated on starch-ammonia agar (SAA), microscopic fungi on Chapek medium, nitrogen fixators on Ashby medium, cellulolytic microorganisms on Getchinson medium [24, 25]. Nystatin (40 $\mu\text{g}/\text{ml}$) was added to prevent fungal growth on SAA, and penicillin (50 $\mu\text{g}/\text{ml}$) was added to prevent bacterial growth on Chapek medium. Inoculations were conducted in triplicate.

Fig. 1. Dynamics of luminescence (I) of *Escherichia coli* K12 TG1 with cloned genes of *luxCDABE* *Photobacterium leiongnathi* 54D10 upon contact with Al_2O_3 nanoparticles in different concentrations: 1 – 4.0 M, 2 – 2.0 M, 3 – 1.0 M, 4 – 0.5 M, 5 – 0.25 M, 6 – 0.1 M, 7 – 0.05 M, 8 – 0.025 M, 9 – 0.0125 M, 10 – 0.00625 M, c – control.

Statistical analysis of the data was carried out using the Statistica 10.0 software package (StatSoft Inc., USA). The arithmetic mean (M) with the standard error of the mean (m) are presented.

Results. The contact of *E. coli* K12 TG1 with increasing concentrations (0.00625–4.0 M) NPs Al_2O_3 did not cause suppression of the luminescence of bacteria for 60 min, which made it possible to characterize NPs Al_2O_3 as non-toxic (Fig. 1). After 180 min, the bioluminescence was reduced by 20–30 % compared to the control values, which characterized the studied NPs as being slightly toxic for living cells. The absence of cytotoxic and genotoxic affection of nano-

particles of aluminum compounds on a living cell in in vitro experiments has been earlier reported [26, 27]. Reducing the luminescence of bacteria with the prolongation of contact up to 180 min suggests the presence of a chronic toxic effect of NPs which requires time for development. Nanoparticles of other metals demonstrate similar chronic biotoxicity [28–30].

Fig. 2. The difference between the activity of superoxide dismutase (a) and catalase (b) in *Eisenia fetida* in control and experiment after 14 days of cultivation in artificial soil with different content of Al_2O_3 nanoparticles.

only at 3000 mg/kg of Al_2O_3 NPs, an increase in CAT activity as compared to the control, was significantly different from that in the other groups. For SOD, the increment of activity was approximately the same for different concentrations of NPs of Al_2O_3 , that is, we did not observe a dose-dependent effect (Fig. 2).

It is known that aluminum compounds are capable of activating the expression of SOD and CAT genes and, as a consequence, the enzymes themselves [32]. The uncontrolled increase in the concentration of the oxidant active forms under the action of a toxic agent in the cells promoted the enhancement of the functions of the defense mechanisms, which serves as an important element of the body reaction to the toxic dosages of the substance in the environment [33].

In studies of W. Sun et al. [34], the SOD activity in earthworms increased with moderate ecological stress and decreased with severe ecological stress. That is, NPs of aluminum has a prolonged effect, and the process of relaxation of the system (return to normal) can take a certain time after the elimination of the toxicant. Acting as a primary antioxidant defense system, CAT and SOD catalyze the conversion of reactive oxygen species to less active or inert forms [35, 36]. In our experiments, the effect of Al_2O_3 NPs on SOD in *E. foetida*, as in the case of CAT, was accompanied by an increase in activity with an increment in the Al_2O_3 content from 0 to 3000 mg/kg.

Fig. 3. Total counts of microorganisms in the soil after 14 days of incubation with Al_2O_3 nanoparticles in different dosages without vermicomposting (a) and under vermicomposting (b).

Counts of both soil microorganisms and intestinal microorganisms of worms varied after 14 days of incubation with increasing amounts of Al_2O_3 NPs. As the Al_2O_3 NPs dose increased from 50 to 3000 mg/kg, the total number of microorganisms consistently decreased in the soil by 61.7-67.6 % without vermicomposting and by 55.6-61.3 under vermicomposting, and in the *E. foetida* intestine by 9.7-43.2 % (Fig. 3, 4). At the same time, the minimum dosage

To assess the biotoxicity of NPs of Al_2O_3 to *E. foetida*, the activity of antioxidant defense enzymes was studied. Low and medium doses (50-300 mg/kg) of nanoparticles caused a manifest induction of catalase activity with maximum at 300 mg/kg. Further increase in the load of nanoparticles on soil (up to 3000 mg/kg) led to CAT inhibition. A similar result was obtained by H.Y. Liang et al. [31]. At the same time,

A decrease in enzyme activity at dosage of 3000 mg/kg was accompanied with the increase in mortality of the test object reaching 20 % compared to 100 % of the survival in other groups.

The ability of soil components to interact with NPs metals, bind them and reduce bioavailability levels the toxic effect of NPs on *E. foetida*. Dissolved or solid particles of some organic substances can agglomerate and sorb NPs in the soil matrix and thereby reduce their bioactivity [37, 38].

(50 mg/kg) of Al_2O_3 NPs reduced the total number of soil microorganisms, but did not affect the intestinal microbiocenosis of *E. betida*, which was due to the action of barrier and adaptive mechanisms in this soil habitant. A dose dependent decrease in the amount of soil microflora, due to changes in metabolic processes in the cell, is specifically of other types of nanoparticles [39].

Fig. 4. The total number of microorganisms in the intestine of *Eisenia fetida* after 14 days of cultivation with Al_2O_3 nanoparticles in different dosages.

There were differences in the toxic effect of Al_2O_3 NPs on microorganisms in vitro and in vivo: at comparable doses bactericidal properties in vivo were shown, while in vitro absent. The development of the toxic effect was determined by the time factor and characteristics of growth medium.

In control samples of soil with vermicomposting and in the intestine quantitative analysis revealed the dominance of bacteria, while the number of microscopic fungi was

several orders lower.

When incubating with Al_2O_3 NPs, the number of all groups of microorganisms was changing. The number of fungi in the soil decreased by 42.8, 52.4, 61.9 and 76.2 % in I, II, III and IV groups, respectively, compared to control, and in the intestines of *E. foetida* — by 18.0 and 20.0 % in groups I and II, and by 39.0 and 40.0 % in groups III and IV (Table 2). That is, with the increase in the content of Al_2O_3 NPs, the number of microorganisms decreased steadily which indicates a bactericidal effect on the soil and intestinal microflora. There was a decrease in the resistance of fungi to the action of NPs, especially when studying the microbiocenosis of the intestine of *E. foetida*. Similar results on the effect on microscopic fungi are known for iron nanoparticles [40]. Soil fungi, as a rule, are more resistant to heavy metals than bacteria [41].

The number of nitrogen fixing bacteria was insignificant in control samples and under the influence of Al_2O_3 NPs decreased in the soil in I, II, III and IV groups, respectively, by 64.3, 77.9, 78,6 and 85,7 %, in the intestine of *E. foetida* — by 16.0, 60.0, 78.8 and 80.0 %. The amount of cellulolytic bacteria increased in the soil and intestine of *E. foetida* with minimal dosages of nanoparticles by 6.9 and 16.0 %, respectively. At the same time, the number of these bacteria in group II decreased by 16.7 and 8.0 %, in III — by 12.5 and 32.0 %, in IV — by 25.0 and 40.0 %.

2. The number of different microorganisms in the soil and intestine of *Eisenia fetida* during vermicomposting after 14 days after the application of Al_2O_3 nanoparticles

Group	Microscopic fungi, thousand CFU/g	Bacteria		
		cellulose-destroying, million CFU/g	utilizing the mineral nitro- gen, million CFU/g	nitrogen fixing, million CFU/g
S o i l				
Control	21.0±3.20	72.0±9.70	44.0±6.50	14.0±4.20
I	12.0±2.60	77.0±4.30	34.0±2.10	5.0±0.70
II	10.0±2.10	60.0±6.40	27.0±3.10	3.1±0.20
III	8.0±0.65	63.0±8.20	7.0±0.80	3.0±0.13
IV	5.0±0.40	54.0±2.50	6.0±0.60	2.0±0.10
I n t e s t i n e				
Control	5.0±1.60	25.0±1.60	15.0±0.30	4.1±0.80
I	4.1±1.20	29.0±2.40	13.0±1.20	2.4±0.40
II	4.0±0.45	23.0±1.02	8.0±0.50	2.0±0.20
III	3.1±0.10	17.0±2.20	6.0±0.20	1.1±0.30
IV	3.0±0.20	15.0±1.50	4.0±0.90	0.8±0.04

According to the literature data, metal NPs in low doses are capable of stimulating the growth of individual bacteria groups, whereas in high doses they have a bactericidal and bacteriostatic effect, causing damage to the integrity of the cell membrane and changes in the functioning of intracellular systems [42]. It is also known that an influence of some NPs on the viability of nitrogen-fixing bacteria and the colonization of substrates by them is determined by the charge of NPs [43]. The number of microorganisms using mineral forms of nitrogen, especially actinomyces, is an important indicator, since some actinomyces are typical symbionts of invertebrates, including earthworms involved in the transformation of nutrient components of the soil [44].

Analysis of the soil microflora and intestinal microflora of *E. foetida* after 14-day incubation showed a decrease in the number of bacteria on SAA, as influenced by Al_2O_3 NPs: by 22.7, 38.6, 84.1 and 86.4 % for soil, and by 13.3, 46.7, 60.0 and 73.3 % for the intestine of worms in I, II, III and IV groups, respectively. As a result, there was a general tendency to the decrease of microorganism number in soil and intestines under the influence of Al_2O_3 NPs as the dosage of nanoparticles increased. The exception was the minimum dose of NPs: in this case there was an increase in the number of cellulose-destroying bacteria.

Thus, the model experiment confirmed the adverse effect of Al_2O_3 nanopreparations on soil biocenosis, manifested in its impoverishment which is accompanied by degradation and a decrease in fertility. The adaptive abilities of the red Californian worm (*Eisenia fetida*) under introduction of Al_2O_3 nanoparticles into the soil have been established based on antioxidant system parameters. The positive effect of vermicomposting is shown to reduce the toxicity of nanoparticles in the soil. In general, the obtained results confirm the need for a comprehensive assessment of the biotoxicity of nanoparticles in different habitats.

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