

# Impact of Zn Nanoparticles on Growth, Survival and Activity of Antioxidant Enzymes in *Eisenia Fetida*

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## Abstract

Biotesting of nanomaterials assumes greater significance and includes various biological models. The research objective is to study the influence of different concentrations of nanoparticles (NP) of Zinc (Zn) on propagation, survival and activity of antioxidative enzymes of metal in *Eisenia fetida* in artificial soil (AS) and microcrystalline cellulose (MCC). Zn nanoparticles in size of  $90.7 \pm 0.3$  nm, Z-potential  $25 \pm 0.5$  mV have been used for the analysis. Artificial soil and microcrystalline cellulose were used as substrates for the study. Zn nanoparticles in high concentrations (from 500 to 1000 mg/kg) cause immobilization of 60% of earthworms within 24 hours. The highest accumulation of metal in earthworms and high mortality were registered at a concentration of 500 mg/kg in microcrystalline cellulose and at 1000 mg/kg in artificial soil.

It was established that after the increase in concentration from 50 to 500 mg/kg the activity of GPx and SOD in two substrates increases. The highest activity occurs when Zn nanoparticles have concentration of 500 mg/kg. The activity of catalase in worms reduced at the exposition in MCC, but it increased after increasing dosage of Zn NPs in the artificial soil.

The study demonstrates that Zn nanoparticles in dose of 500 mg/kg induced the highest toxic effect. It was proven by behavioral reactions, growth characteristics and indices of enzymatic activity in organism of redworm. Based on the obtained data, substrate from microcrystalline cellulose can be used as experimental.

**Keywords:** nanoparticles Zn, earthworm *E. fetida*, microcrystalline cellulose, activity of antioxidative enzymes, artificial soil, accumulation, development

## 1. Introduction

Biotesting of nanomaterials takes on special significance and includes various biological models: bacteria (*Photobacterium phosphoreum*), plants (*Lemna minor*, *Elodea Canadensis*, *Lipidium sativum*), protozoa (*Tetrahimena pyriformis*, *Paramecium putrinum*, *Stylonychia*) etc. Introduction of nanomaterials in soil defines the perspective of the earthworm model *Eisenia fetida* and allied species according to the estimation of new produced materials (Scott-Fordsmand et al., 2008; Hu et al., 2010; Unrine et al., 2010; Lapiet, 2010).

A high descriptiveness of this model refers to the biological characteristics of *Eisenia fetida* that is permeability the body surface for pollutants (Wallwork, 1983; Jajer, 2003; Vijver, 2003, 2004).

Earthworms process a lot of soil that is why they are always under the influence of materials adsorbed on the solids of the soil (Morgan et al., 1999). They are more sensitive to soiling with metals than other soil invertebrates, and the toxicity assessment on earthworms is an important link in defining the safe levels of metals and other pollutants in soil (Žaltauskaitė, 2010).

Because of its unique optical, catalytic, semi-conductor, piezoelectric and magnetic characteristics, Zn nanoparticles are produced in large quantities and used in various fields of production (Nowack and Bucheli, 2007). That is why bioavailability and potential ecological toxicity of nanomaterials in earthworm *Eisenia fetida* is estimated in terms of environment, including an assessment of the environmental effects of modifying certain matrices (base cations and anions). All parameters must fall within the ranges common in natural soils. In this study, the bioavailability and potential ecotoxicity of Zn nanoparticles (NPs) *Eisenia fetida* was estimated on

growth, survival, behavioral reactions and activity of antioxidant enzymes.

## 2. Methods

### 2.1 Experimental Animals

The toxicity tests were conducted according to the requirements of the Organization for Economic Co-operation and Development (OECD, 1984, 2004) on lab earthworms *Eisenia fetida* Andrei Bouche.

Earthworms 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». Mature worms weighing 500-600 mg naturalized during 7 days on clean substrates A and B at a constant temperature of 25°C. After acclimatization, three worms were selected, cleaned on wet filter paper, weighed and analyzed the concentration of Zn, which was considered the initial concentration in the worm.

### 2.2 Chemicals and Substrates

Zn nanoparticles in size of  $90.7 \pm 0.3$  nM, Z-potential  $25 \pm 0.5$  mV have been used for the analysis. NPs were purchased from LLC “Advanced Powder Technologies” (Russia, Tomsk). Nanoparticles were produced by electrical explosion of wires in argon.

These 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 7401F and a JEM-2000FX, respectively («JEOL», Tokyo, Japan).

The size distribution of particles was investigated using a Brookhaven 90Plus /BIMAS and ZetaPALS Photocor Compact (Russia) in lysols after dispersing the nanoparticles using an ultrasonic disperser UZDN-2T (Russia) at f-35 kHz, N 300 W, and A-10 µm for 30 min.

Toxicological assessment was performed on 2 substrates:

Substrate A was microcrystalline cellulose (MCC) purchased from company "Ankir-b" and pharmaceutical company "Evalar" (Russia).

Substrate B was artificial soil. The toxicity tests were conducted according to OECD requirements (OECD 1984). The artificial soil of the following composition (by dry weight) was prepared: 70% of quartz sand (more than 50% of the 0.05-0.2 mm particles), 20% of clay and 10% of Sphagnum peat. The soil pH was adjusted to  $7.0 \pm 0.5$  with powdered calcium carbonate ( $\text{CaCO}_3$ ) (Conder 2000, 2002).

Samples of substrates A and B have been tested on models *Eisenia fetida* within 30 days. The survival rate was 100% with appropriate development of worms. Filter paper used as a substrate for purifying the digestive tract was purchased from MiniMed Inc. Company (Russia).

### 2.3 Preparation Substrate and Incubation of *Eisenia Fetida*

Preparation of the substrate A. MCC was ground and watered distilled water for 1 day, stirred until a dense weight without free water. Humidity was 70-75%. The substrate was left for 1 day.

Preparation of substrate B. Artificial soil was carefully mixed, placed in tanks and humidified with distilled water to 45% humidity. The required number of substrates A and B was calculated on the basis of worm ability to digest substrate equal to the unladen mass of the substrate per day. The following formula was used: weight of substrate = mass worm x number of worms x number of days of the experiment.

Zn NPs were prepared in isotonic solution using the ultrasonic disperser (f-35 kHz, N-300 W) by dispersing for 30 minutes.

Nanoparticles were added to the prepared substrates A and B and thoroughly stirred using a mixer. Humidity was 85%.

The final concentration of Zn in substrates: 50 mg/kg, 200 mg/kg, 500 mg/kg and 1000 mg/kg. Substrates A and B without Zn NPs were considered as a control. Before the experiment, worms were washed with distilled water, divided into groups and placed in plastic containers with moist substrate of filter paper for 3 days to clear the digestive tract Reznichenko (2013).

After 3 nights of exposure on filter paper worms were washed, weighed and placed by 10 species in plastic containers (0,4x0,15x0,02 m) (dimensions were selected by the recommendations for distribution of worms in soil profile (Krivolucky, 1994)). Concentrations of Zn NPs in substrates A and B varied. All containers were sealed with perforated lid to prevent moisture loss. The experiment was conducted within 14 days at a temperature of 25°C (Dalby, 1996). Containers with animals were kept in a dark place (Taylor et al., 2004). Each

series of experiments had 3 repetitions. Containers were examined every day to remove dead animals.

#### 2.4 The Studied Parameters

To determine behavioral responses speed of worms getting below the substrates A and B were assessed. It was calculated by counting time from the moment of start-up of the worms in the substrate until they get below it. Worms were weighted on the 7 and 14 day of experiment. 3 worms from substrates A and B were placed in sterile test tubes and the content of Zn was analyzed by atomic absorption spectrophotometer Formula FM 400 («Labist», Russia).

The activity of antioxidant enzymes. Homogenization buffer was applied in liquid (50 mmol/L Tris, DTT 1.0 mmol/L EDTA, 1.0 mmol/L sucrose 250 mmol/l, pH 7.5), which added in a ratio of 1:9. Worms were homogenized using the TissueLyser LT, QIAGEN (manufacturer-QIAGEN, Germany). The resulting homogenate was centrifuged 10 minutes at 15000 rpm. The resulting supernatant diluted with buffer mixture up to 10% of homogenate.

Supernatant fluid was used to determine Superoxide dismutases (SOD), catalase (CAT), glutathione peroxidase (GPx). Determination of the level of enzymes were semi-automatic biochemical Analyzer Stat fax 1904 Plus (producer-Awareness Technology Inc., United States) using commercial sets firm Randox (United States).

#### 2.5 Statistic analysis

All experiments were performed in at least three repetitions and were processed by variation statistics using the software package "Statistica" V8 ("StatSoft Inc., United States).

### 3. Results

#### 3.1 Motor Activity of *Eisenia Fetida* in Substrates A and B

Motor activity of worms in substrates A and B with the same concentrations of Zn was the same. Concentration of Zn NPs from 50 to 200 mg/kg did not cause immobilization of enzyme. After placing on the surface of the substrate worms within from 3.3 to 4.9 min (Table 1) burrowed into the ground and remained in the substrate until the end of the experiment. Putting worms on the surface of the substrate with a concentration of 500 and 1000 mg NP/kg has been accompanied by an increase in the time of worms burrowing to from 20.1 to 45.5 min in the substrate A and from 15.8 to 32.7 min in the substrate B. Worms thereafter were exposed to the surface.

Table 1. The speed of the burrowing of the red Californian worm in the substrates A and B with different concentration of Zn NPs.

Concentration of Zn NPs, mg/kg	The time of burrowing, min	
	Substrate A	Substrate B
0 (control)	4,2±0,5	3,3±0,6
50 mg/kg	4,9±0,3	3,9±0,3
200 mg/kg	7,5±0,6*	6,5±0,6*
500 mg/kg	20,1±1,4*	15,8±1,2*
1000 mg/kg	45,5±3,8*	32,7±2,6*

Note: \* The difference in comparison with control is significant at  $p \leq 0.05$ .

#### 3.2 The Change in Mass

Mass of *Eisenia fetida* depended on the concentration of Zn NPs in substrate. Concentration increases Zn NPs from 50 to 200, 500 and 1000 mg/kg, the mass of worms decreased from 1.2 to 9% ( $p \geq 0.05$ ) after 7 days and 4.2 -7.4 ( $p \leq 0.05$ ) after 14 days (see Figure 1). A similar difference for the substrate was in from 4.4 to 6.9 % ( $p \leq 0.05$ ) after 7 days and from 10.2 to 13.6 % ( $p \leq 0.05$ ) after 14 days (see Figure 2).

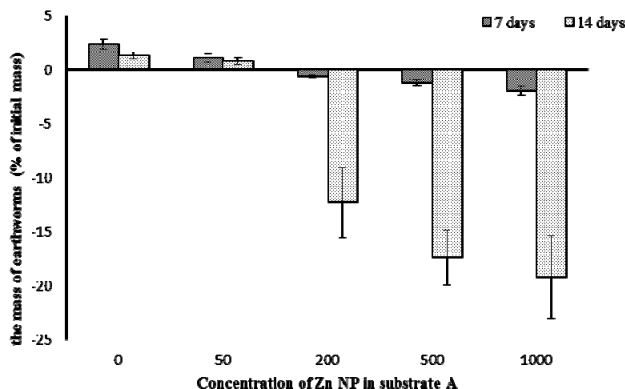


Figure 1. Change in mass *Eisenia fetida* at different concentrations of Zn NPs in substrate A (% of initial mass)

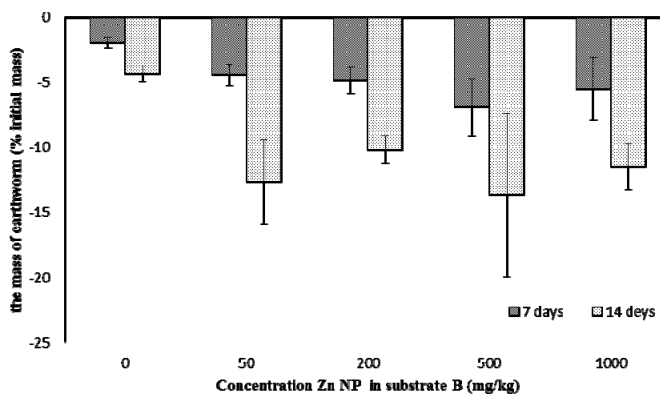


Figure 2. The change in mass *Eisenia fetida* at different concentrations of Zn NPs in substrate B (% of initial mass)

Toxic effects of nanoparticles, the estimated mass of worms, depended on the composition of the substrate. On a substrate A a significant reduction in the mass of worms under the influence of nanoparticles was shown on the 2nd week of the experiment, on a substrate B on the first weeks at a concentration from 200 to 1000 mg/kg.

### 3.3 Surviving of *Eisenia Fetida*

In the control substrate, survival was 100%, while mortality at a concentration of 500 mg/kg Zn NPs was higher than at a concentration of 1000 mg/kg. During the first week at a concentration of 500 mg/kg 30% of lethality was observed, whereas in the end of the research it was 80% ( $p \leq 0.05$ ) (see Figure 3).

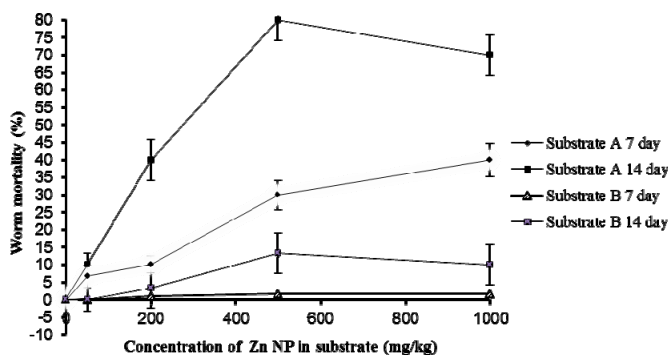


Figure 3. Mortality of enzyme *Eisenia Fetida* under the influence of nanoparticles of Zn NPs on the 7 and 14 day. Bars represent the standard deviation of the mean value of the three repetitions

If at a concentration Zn NPs of 500 mg/kg the worms were moving on the substrate up to 7 days, at a

concentration of 1000 mg/kg on day 4 they already stopped processing substrate, fell into a passive state, have shrunk into a ball, with mucous discharge their skin glands formed a film. Such a protective-adaptive reaction leveled toxic effect of substrate. The death-rate didn't exceed 2% on the 7<sup>th</sup> day. On the 14<sup>th</sup> day the maximum death-rate of the worms was recorded at the dose of 500 mg/kg and was 13%.

### 3.4 Accumulation of Zn in the Earthworm and Substrates

Initial content of Zn in the worms amounted to  $53.3 \pm 3.8$  mg/kg. The 7 day upon exposure to the substrate and the concentration of Zn in worm control reliably increased, while in comparison with the initial concentration, amount of Zn was decreased when statistically significant difference of 70%, 60.6%, 52.4, 36.3% and 30.1% of the dose is 0, 50, 200, 500 and 1000 mg/kg Zn NPs respectively. The 14 day compared to the previous period, increased concentration, with a maximum value of Zn 40.0 mg/kg dose Zn NPs 500 mg/kg, with a slight decrease in the dose of 1000 mg/kg. (see Figure 4).

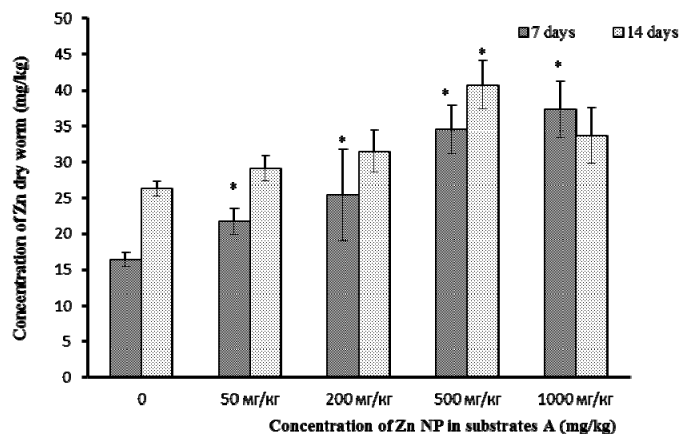


Figure 4. The accumulation of Zn in *Eisenia fetida* at different concentrations of Zn in substrate A. Bars represent the standard deviation of the average value of three povtornostej. (\*) a significant difference from control (0 mg/kg) ( $p \leq 0.05$ )

Concentration of Zn in *Eisenia fetida* on 7 day exposure with increasing concentration in artificial soil (substrate) Zn NPs up to 500 mg/kg zinc content decreased, while a dose of 1000 mg/kg Zn content in worm reached  $63.1 \pm 2.6$  when reliable difference from the original concentration of 15.6% ( $p \leq 0.05$ ) (see Figure 5).

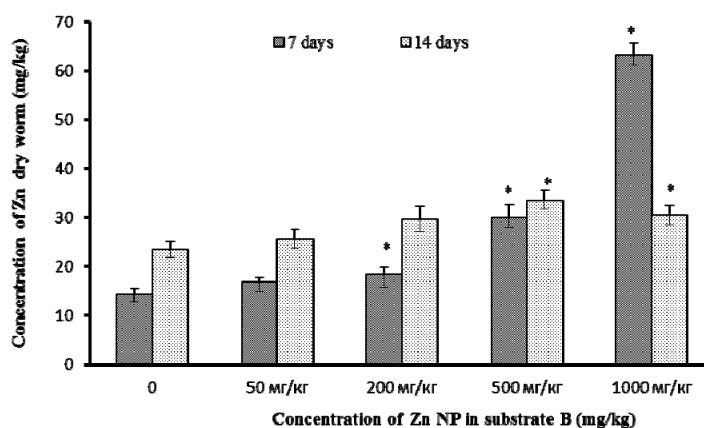


Figure 5. The accumulation of Zn in *Eisenia fetida* at different concentrations of Zn in the substrate B in the bars represent standard deviations of the mean value of the three povtornostej. (\*) a significant difference from control (0 mg/kg) ( $p \leq 0.05$ )

When comparing with the worms reside in the substrate without Zn NPs concentration in *Eisenia fetida* grew in response to the increase in the concentration of Zn in substrates.

### 3.5. Antioxidant Enzyme Activity in *Eisenia Fetida*

Glutathione peroxidase (GPx). The activity of activity of GPx varies depending on the concentration of Zn nanoparticles in the substrate (see Figure 6). In the substrate and increased activity of GPx at 29.1% ( $p \leq 0.05$ ) at a concentration of 500 mg/kg nanoparticles with a subsequent decrease of 23.1% ( $p \leq 0.05$ ) at 1000 mg/kg, relative to the control. Other concentrations of significant change has not been committed. In the substrate in a similar activity GPx is set at a concentration of 500 mg/kg. Other concentrations of activity slowed under false distinctions.

Superoxide dismutase (SOD). Activity of SOD increased with increasing concentration of Zn NPs in the substrate (see Figure 6). Noted a significant increase of 40.8% ( $p \leq 0.05$ ) ODS at a concentration of 500 mg/kg, followed by a decline at 1000 mg/kg. Similar fluctuations observed in substrate of artificial soil (see Figure 6).

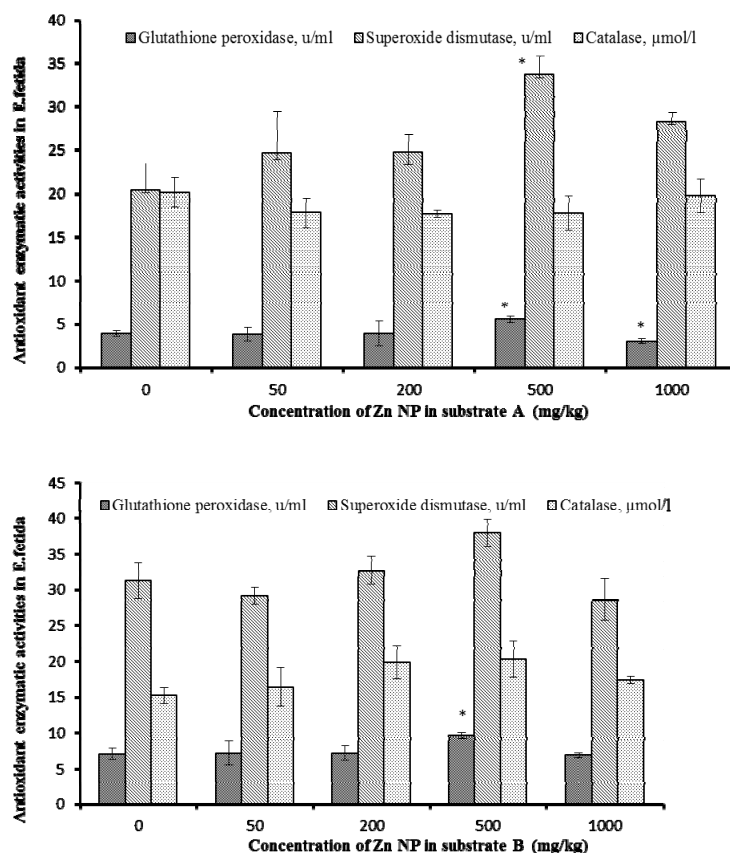


Figure 6. Effect of nanoparticles of zinc on the activity of Glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) in the rain worm *Eisenia fetida* after 14 daily excerpts in the substrate a and b. Bars represent the standard deviation of the average value of three povtornostej. Reliable differences from controls:  $*p < 0.05$

Catalase (CAT). The changes in catalase activity of the worms in the substrate A were not revealed. On artificial soil catalase activity in worm has grown in response to the increase in the concentration of Zn in the substrate A to 500 mg/kg, with inhibition of enzyme activity at 1000 mg/kg of Zn NPs.

## 4. Discussion

The widespread use of nanomaterials based on heavy metals defines the prospects of these substances into the environment. Traditional metals in soil are Zinc and Copper, and enzyme accumulate and tolerate high concentrations of zinc (Peijnenburg, 2002), which is reflected in their ability in the absorption and circulation of these heavy metals (J. Morgan & A. Morgan, 1999). One of the biological assessment of the impact of nanomaterials on the environment is *Eisenia fetida* (Unrine, 2010). Our studies found toxic effect of Zn nanoparticles on *Eisenia fetida* in various concentrations. The selected dosage of nanoparticles aligned with research results (Li et al., 2011).

In our experiment of acute toxicity, as well as in research on Li et al. (2011), the following doses were used 0, 50, 200, 500, 1000 mg/kg, because they simulate the background and the threshold concentration of zinc in natural soil. The range of test concentrations was selected for modelling levels that may occur in the environment under certain conditions. Depending on the degree of influence of metal on soil organisms (Neuhauser et al., 1985) we defined values 14-day LC50 value 662 mg/kg soil for *Eisenia fetida*, at the same time R. Panda et. al. working on the same kinds set the value of LC50 828-995 mg/kg soil, a similar range was obtained by Spurgeon (1994).

Modeling the toxic load on soil organisms there were used two substrates, one of artificial soil, another of microcrystalline cellulose. The use of this substrate is based on progressive method of intestinal cleansing, this method was listed in the manual for the study of the structure, functioning and diversity of detritus food chains edited by Pokarzhevsky et al. (2003) and based on the method of evacuation of the stomach contents of enzyme using pulp crumb (Reznichenko, 2013). This method was transformed to assess toxic/or stimulating activity of nanoparticles by replacing the contents of the digestive tract on chemically pure substrate with nanoparticles.

Regardless of the quality of the substrate, the reaction of worms on a high concentration of Zn NPs (500 and 1000 mg/kg) expressed in long-term denial to burrow into the ground, showing responses to pollution, they were moving throughout the volume of the container avoiding contact with a substrate which is probably associated with high penetrating power of metal ions in the body of the worm, which has been demonstrated in studies of Li et al. (2011).

Ecotoxicological screening of the influence of nanoparticles of zinc on representatives of soil organisms has shown that Zn nanoparticles are more toxic than ZnCl<sub>2</sub>, and according to the authors it is related to the influence of pH on dissolution of the zinc in the substrate (Heggelund et al., 2014).

Whereas the pH of the substrate during the experimental studies was at the same level (6.5 -7.5) and it was not the impact factor.

Growth is an integral indicator and it unites a set of biochemical and physiological effects. It represents a change in energy exchange with the detoxification of pollutants. Additional need for energy leads to a decrease in growth. Growth and reproduction of enzyme when exposed to metal negatively correlated with reproduction, i.e. if the worms do not grow they produce cocoons, and vice versa, if they do not produce cocoons, they continue to grow (Burgos et al., 2005).

When exposed to high concentrations of Zn significant reduction was observed in the body weight in a substrate B on the first week, and on the 14 the day in the substrate A. Toxic effects on growth of the worm has been demonstrated in studies of Berthelot et al. (2008), Van Gestel et al. (2009), whereas there is evidence indicating a stimulating effect of metal on the body mass (Spurgeon and Hopkin, 1996). At the same time worms' weight, loss on artificial soil was higher than on the microcrystalline cellulose and according to Spurgeon et al. (1994) worms living in metal-polluted soils need more time to reach maximum mass than in uncontaminated plots.

On the one hand, a substrate from microcrystalline cellulose contains smaller fractions than artificial soil substrate and toxic effect is mainly due to the high degree of dissolved Zinc ions (Li et al., 2011) in substrate A and high penetrating power of metal through the digestive system. On the other hand, this also contradicts the conclusions of Vijver et al. (2003) which showed that enzyme dermal route is a major channel for the water intake of metal ions from the soil, whereas absorption through the meal does not contribute to the accumulation of metals.

In the test for a survival, there was an established fact of death of worms at high concentration of LF of Zn that corresponds to data (Li et al., 2011). The relatively high survival rate could be explained by the binding of Zinc components of the substrate, as well as zinc-binding metallothioneins (Morgan et al., 1989; Sturzenbaum et al., 2004).

Low mortality in the soil substrate is explained in the studies Honsi et al. (2003), where it is shown, that high concentrations of metals in soil do not affect the survival of enzyme (Cu, Zn, Cd and Pb).

We agree with the conclusions of the Moriarty (1999) that mortality is a very sensitive indicator for predicting the effects of field populations, at the same time the tests of Zn NPs in microcrystalline cellulose prove a direct impact of the metal on the body of the worm and lethal and sublethal effects on metal impact.

Enzyme can reduce the toxic load on the body through the redeployment of the metal from the soil in the organs and tissues (Beyer, 1987).

Bioavailability and bioaccumulation of metals depend on factors such as the dimension and its concentration (Hobbelen et al., 2006 Heggelund et al., 2014).

Accumulation of Zn in *Eisenia fetida* was taking place depending on the increase of Zinc concentration in the substrate. The highest accumulation of zinc in the body of the worm was at a concentration of 500 and 1000 mg/kg in the substrate from microcrystalline cellulose, whereas the artificial soil indicator was 1000 mg/kg on the 7<sup>th</sup> day of exposure, the maximum toxic effect was set during these intervals.

Different Zn absorption on the 7<sup>th</sup> day, i.e., reducing its concentration in the worm in our opinion first connected with adaptation of the worm to the substrate, and secondly with the ability to regulate the content of Zn in the body.

Living organisms in particular enzyme according to Morgan and Morris (1982) are capable to bind zinc in tissues by participating metallothionines in this process, allowing the rapid excretion of Zn. Van Gestel et al. (1993) also reported that enzyme can regulate content in the body of the Zn concentration in soil up to 560 mg/kg and above this concentration occurs a significant accumulation of the metal. These findings can be explained by many of the results obtained, in particular high mortality at a concentration of 500 mg/kg.

As shown by the data obtained in this study, after 7 days of exposure, Zn NPs can significantly stimulate the activity of GPx in dose Zn NPs 500 mg/kg ( $p \leq 0.05$ ), and lower at 1000 mg/kg. As another key antioxidant, SOD enzyme is involved in the removal of reactive oxygen species. Any changes in SOD reflects changes of oxidative stress that can be caused by pollutants. Often these changes are discussed in the early stages of environmental pollution (Brown et al. 2004). In our study established trend of increasing activity of SOD with maximum effect at a concentration of 500 mg/kg Zn and lower dose of 1000 mg/kg.

Similar dependence of biological activity of nanoparticles described Sun et al. (2007), he argued that the activity of SOD in enzyme increases with moderate environmental stress and decreases with severe ecological tension. A similar trend was detected Calabrese and Baldwin (2002). Catalase is an important enzyme in the antioxidant defense system of organisms, therefore, protects the cells from damage. *Eisenia fetida* have CAT activity increased when reaching a concentration of nanoparticles in an artificial soil Zn 500 mg/kg and decreased at a dose of 1000 mg/kg. A similar effect of Zn NPs was registered by Liu et al. (2010).

This is possible due to the high activity of catalase in soil substrate and high turnover of metal between the substrate and the body of the worm (Chelikani et al., 2004). These studies illustrate that the largest toxic effect was obtained with a dose of Zn NPs 500 mg/kg, informed behavioral reactions, growth characteristics and enzyme activity in the body of the worm. Use the substrate of microcrystalline cellulose as a test allows you to evaluate the direct impact of NP through various paths.

## 5. Conclusion

Zn nanoparticles in size of  $90.7 \pm 0.3$  nm, Z-potential  $25 \pm 0.5$  mV have been used for the analysis. Artificial soil and microcrystalline cellulose were used as substrates for the study. Zn nanoparticles in high concentrations (from 500 to 1000 mg/kg) caused immobilization of 60% of earthworms within 24 hours. The highest accumulation of metal in earthworms and high mortality were registered at a concentration of 500 mg/kg in microcrystalline cellulose and at 1000 mg/kg in artificial soil.

It was established that after the increase in concentration from 50 to 500 mg/kg the activity of GPx and SOD in two substrates increases. The highest activity occurs when Zn nanoparticles have concentration of 500 mg/kg. The activity of catalase in worms reduced at the exposition in MCC, but it increased after increasing dosage of Zn NPs in the artificial soil.

The study demonstrates that Zn nanoparticles in dose of 500 mg/kg induced the highest toxic effect. It was proven by behavioral reactions, growth characteristics and indices of enzymatic activity in organism of redworm. Based on the obtained data, substrate from microcrystalline cellulose can be used as experimental.

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