

Influence of zinc nanoparticles on survival of worms *Eisenia fetida* and taxonomic diversity of the gut microflora

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Abstract The study was conducted to examine the effect of zinc nanoparticles on survival of worms *Eisenia fetida* and composition of the gut microflora. Analysis of the survival data has shown that the introduction of high doses of the nanoparticles causes death of worms in the second group with 35 % mortality rate and activates protective mechanisms realized as mucous film. DNA from the worm guts was extracted and 16S metagenomic sequencing was fulfilled using MiSeq (Illumina). Regarding the gut microflora of worms in the control group, high diversity of microorganisms (303 OTUs) was noted. Most of those belong to the taxa *Firmicutes* (51.9 % of the total high-quality united reads), *Proteobacteria* (24.1 % of the total), and *Actinobacteria* (13.3 % of the total), which were represented by numerous species of gen. *Clostridium* (*C. saccharobutylicum*, *C. saccharoperbutylacetonicum*, *C. beijerinckii*), gen. *Pseudomonas* (*P. hydrogenovora*, *P. aeruginosa*, and *P. putida*), gen. *Bacillus* (*B. megaterium*, *B. silvestris*), gen. *Cellulomonas* (*B. megaterium*, *B. silvestris*), and other numerically smaller genera. Adding of zinc nanoparticles to the substrate decreased the diversity of bacteria (78 OTUs) as well as percentage of bacteria belonging to the

taxon *Firmicutes* (−41.6 %) and increased the proportion of *Proteobacteria* due to growth in abundance of gen. *Verminephrobacter* (+46 %) and gen. *Ochrobactrum* (+19.5 %).

Keywords Nanoparticles · *Eisenia fetida* · Zinc · Microflora · Taxa · Microcrystalline cellulose · High-throughput sequencing · 16S metagenome

Introduction

Metal nanoparticles are widely used in modern industry. Due to their small size (less than 100 nm), nanoparticles exhibit a high reactivity and properties that differ from the characteristics of the element (Lin and Xing 2007; Moore 2006; Hoet et al. 2004). The introduction of nanotechnology in various fields of human activity inevitably leads to nanoparticles getting into the environment, mainly in soil (Tourinho et al. 2012). Nanoparticles enter soil because they are actively used in agrochemistry as nanoherbicides, nanopesticides, etc., in particular, zinc nanoparticles (Milani et al. 2012). The fungicide activity of zinc oxide nanoparticles contributing to combat diseases of tomatoes, as well as their antibacterial action and the ability to stimulate growth of plants are known (He et al. 2011; El-Kereti et al. 2013; Vijayakumar et al. 2015). At the same time, phytotoxicity of zinc oxide nanoparticles for some plants and ecotoxicity in respect of certain invertebrates are registered. It raises a question of more detailed studies of nanoparticles impact on soil mesofauna, particularly on earthworms (Hu et al. 2010; Fahmy et al. 2014; Song and Lee 2016; Wang et al. 2016). A single work on relative toxicity of titanium nanoparticles with respect to certain soil nematodes is known nowadays (Rocheleau et al. 2015).

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Earthworms are a major participant in the soil processing, which determines its chemical properties and actively regulates life of the soil microbial community. Among the main categories, an environmental group of epigeic earthworms inhabiting topsoil is of particular interest. *Eisenia fetida* is one of the most famous species of these worms. It is known as the California red worm and widely used in vermicomposting (Edwards 2004; Drake et al. 2006).

The soil processing depends mainly on a pattern of interaction between earthworms and microorganisms, which is important for the flow of soil processes such as decomposition and transformation of plant residues, formation of humus representing pool of nutrients, and microbial communities (Aira et al. 2009; Ferreira et al. 2015; Grumiaux et al. 2015). While feeding, earthworms regulate the growth of soil microorganisms by means of consumption of some microfungi taxa and create optimal growth conditions for others in their digestive tract and in casts formed by the earthworms during feeding (Tiunov and Scheu 2000). The gut of earthworm is an anoxic biotope the soil passes through. This point determines properties of soil microbial communities being in contact with earthworms, including their structure, functions, and population dynamics (Aira et al. 2006; Drake and Horn 2007). For instance, presence of *E. fetida* enhances biomass of some fungi in soil (Tiunov and Scheu 2000), especially taxa capable to degradation of cellulose because of their cellulase and beta-glucosidase enzyme activity (Aira et al. 2006). This is useful for intense and efficient cellulolysis during vermicomposting of organic wastes (Aira et al. 2006). Besides, earthworms are involved in processes of soil denitrification providing favorable conditions for activation of ingested soil bacterial denitrifiers and emission of the greenhouse gas N₂O (Drake et al. 2006). Relationships between earthworms and microorganisms depend on the ecological category and on the earthworm species. For example, Aira et al. (2009) studied effects of gut transit on the number of bacteria and the microbial activity in pig slurry, using three epigeic (*E. fetida*, *E. andrei*, *Eudrilus eugeniae*) and one anecic (*Octodrilus complanatus*) species of earthworm. As a result, they did not find any changes in the number of bacteria in the gut contents of *E. fetida* and *E. eugeniae*, whereas large decreases were recorded in those of *O. complanatus* and *E. andrei*. The authors suggested that unlike in the epigeic earthworms, microorganisms are preferentially utilized by anecic *O. complanatus* to meet its nutrient requirements, because of its limited digestive capacity (Aira et al. 2009). A wide range of interactions (earthworm–microorganisms) (Aira et al. 2006, 2009; Byzov et al. 2009; Davidson et al. 2014; Drake et al. 2006; 2007) suggests the close connection of the earthworms and the microorganisms in the process of soil formation as well as their effect on soil fertility. Microorganisms play a role of the main food source for the earthworms. In addition, due to these interactions, the soil microorganisms accelerate the turnover of organic matter in the soil (Edwards and Fletcher 1988). At the same time, in spite of

mentioned advanced investigations of earthworm–microorganism interactions as well as toxicity of nanoparticles for earthworms, we have not found in available literature data on ability of nanoparticles to affect gut microbial community of earthworms.

According to all the mentioned facts, the following hypothesis has been formulated. Zinc nanoparticles may influence both the viability of earthworms and microbiocenosis of worms' gut, particularly its composition and diversity. We further suppose that the obtained results contribute to evaluation of protective mechanisms efficiency and adaptability of earthworms regarding to their intestinal microflora exposed to nanoparticles.

Materials and methods

Experimental animals and the cultivation substrate

Toxicity investigation was conducted following the guidelines of the Organisation for Economic Cooperation and Development for the testing of chemicals on laboratory cultures of earthworms *E. fetida* (OECD 2004). This worm species is industrial vermiculture and one of widely used for vermicomposting (Edwards 2004). Also, the worm *E. fetida* is the most often used test organism for monitoring of soil contamination (Hirano and Tamae 2011).

All selected worms were sexually mature. Acclimatization was conducted within 7 days on the soil substrate at the constant temperature of 25 °C. Body weight of worms varied from 500 to 750 mg. The substrate was microcrystalline cellulose (MCC), manufactured by Ankir-B, a pharmaceutical company (Evalar, TU 9100-026-21428156-14, State Reg. Cert. RU. 77.99.11.003.E.005713.05.14 as of May 14, 2014). To prepare the soil substrate, microcrystalline cellulose was pounded with distilled water for a day and was stirred into a thick mass without free water with a moisture content of 70–75 %. The substrate was left for a day. Samples of soil substrate were pre-tested on a model of *E. fetida* within 30 days; survival was 100 % with the appropriate development of the worms. The required amount of soil substrate was calculated on the basis of worm ability to process the amount of substrate equal to the weight of the worm per day according to the formula:

$$\text{Weight of substrate} = \text{weight of worms} \times \text{number of worms} \times \text{duration of experiment (days)}$$

Certification and sample preparation of nanoparticles

Zinc nanoparticles were used in the study (Table 1). Certification of the preparations included the following

Table 1 Characteristics of the powder of zinc nanoparticles

Kind of nanoparticles	Size (nm)	Chemical and phase composition	Method of obtaining
Zn	90	90 %, the rest is adsorbed gases, zinc oxide, and H ₂ O.	conductor electrical explosion in argon

procedures: scanning and transmission electron microscopy using JSM 7401F and JEM-2000FX instruments (JEOL, Japan) and X-ray diffraction analysis using DRON-7 diffractometer. Water lysols of the zinc nanoparticulate, before being incorporated into the substrate, were treated ultrasonically at 35 kHz (power 300 (450) W, oscillation amplitude 10 μm) for 30 min.

Study design

For the study, worms of equal mass were selected, of which two groups were formed (*n*=10). Group I was cultured on MCC without introducing nanoparticles into the substrate; group II was cultured with adding zinc nanoparticles in the concentration 1000 mg/kg soil. The concentration was chosen to provoke toxic effect (Li et al. 2011). The zinc content in natural soils ranges from 10 to 300 mg/kg. Zinc content reaches to 100 mg/kg in average S horizon (Voropaev and Pashkov 2009).

The experiment was performed in triplicate. Zinc nanoparticles were added to the substrate prior to introduction of worms, stirred using a household mixer and adjusted to humidity of about 85 %. Worms were cultivated in containers with the size of 0.4 × 0.15 × 0.02 m (sizes were selected based on the data of Krivolutskii (1994) on the distribution of earthworms in soil profile) that have been installed in a climatic chamber KK1200 (manufacturer—Pol-Eko-Aparatura, Poland) with humidity control, protected from light, at 25 °C. All containers were closed with perforated lid.

Before beginning of the experiment, the worms were washed with distilled water and kept in plastic containers for 3 days with wet filter paper for complete cleansing of the digestive tract (Reznichenko 2013). Results were registered on the 7th day after adding of the nanoparticles. Time period of 7 days was chosen based on Organisation for Economic Cooperation and Development (OECD) recommendations (OECD 2004). In addition, we took into consideration higher mortality of the earthworms during late period of the experiment and paucity of individuals in active state processing the substrate after this point of time.

The effect of zinc nanoparticles on the survival and the gut microflora of worms were considered. Analysis of the gut microflora was carried out by the method of metagenomic high-throughput sequencing using Illumina MiSeq sequencer (Illumina, USA).

Isolation of the guts

The guts were cleansed by one-day incubation of worms in a plastic container with wet filter paper. To isolate the clean guts, the worms were dissected using a sterile scalpel; the guts were separated with sterile tweezers, placed into a sterile vial, frozen at −70 °C and thus stored with no repeated thawing and freezing. In the experiment, all parts of earthworm guts were taken and used for the analysis.

Isolation of DNA

Total genomic DNA was isolated from gut of the worms. Every specimen contained gut of three individual worms from an experimental replica of single group. Then, we combined specimens from three replicates of the same group. DNA was isolated by a method of chemical extraction (Liu et al. 2009) modified according to Bel’kova et al. (2008). The modification included an additional step of incubation with lysozyme to improve destruction of gram-positive bacteria. Every sample was incubated in 300 μl of the sterile lysis buffer (20 mmol/L EDTA, 1400 mmol/L NaCl, 100 mmol/L Tris–HCl, pH 7.5) with addition 50 μl of lysozyme (100 mg/ml) at 37 °C for 30 min, followed by addition of both 10 μl proteinase K (10 mg/ml) and sodium dodecyl sulfate to 1.0 %. The mixture was incubated for 30 min at 60 °C. After extraction with phenol–chloroform–isoamyl alcohol (25:24:1) and chloroform–isoamyl alcohol (24:1), DNA in the aqueous phase was precipitated with an ammonium acetate (10 mol/L up to 10 % v/v) and threefold volume of anhydrous ethanol overnight at −20 °C. After centrifuging and double washing with 80 % ethanol, the DNA was dried and dissolved in TE buffer. To assess if contamination was introduced during DNA extraction, a negative parallel control was established by treatment of 100 μl of autoclaved deionized water using the same method as described above. Purity of the DNA was checked with electrophoresis in 1.5 % agarose gel. Concentration of the DNA was quantified using the Qubit 2.0 Fluorometer with the dsDNA High Sensitivity Assay (Life Technologies).

Preparing of DNA library and sequencing

Preparing of DNA libraries as well as sequencing was carried out in the Center of Shared Equipment (Persistence of microorganisms) of Institute for Cellular and Intracellular Symbiosis UrB RAS (Orenburg, Russia). 16S DNA libraries

were prepared in according to Illumina workflow (http://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf) with primers targeting the V3 and V4 regions of the SSU ribosomal RNA (rRNA) gene such as forward S-D-Bact-0341-b-S-17 and reverse S-D-Bact-0785-a-A-21 (Klindworth et al., 2013). The libraries were sequenced in MiSeq (Illumina) using 2 × 300 bp paired-end MiSeq v3 reagent kit.

Sequence analysis

Data analyses were conducted using USEARCH v8.0.1623_win32 (Edgar 2010) and included merging of paired reads, quality filtering, and amplicon size selection (415 bp minimal size). During the filtering reads with Ns or an overall mean, Q-score of <15 were discarded. Evaluation of the filtering quality was carried out with FastQC v 0.11.3. As a result of dereplication and clustering with USEARCH, operational taxonomic unit (OTUs) were formed while singletons and doubletons were removed. OTUs were determined using similarity levels between sequences of at least 97 % for classifying a microorganism at the species level. Chimera detection was conducted via UCHIME (Edgar 2010) using USEARCH, and chimeric sequences were removed. Contaminating OTUs were found and removed via command—ublast USEARCH matching sequences from samples with negative control. Taxonomic classification of sequences using VAMPS and the SILVA reference database were conducted (Huse et al. 2014). Rarefaction curves were calculated for OTUs with an evolutionary distance of 0.03 using VAMPS as well as visualization of the OTUs taxonomy.

Results and discussion

Analysis of the obtained data has shown that in the control group survival rate was 100 %, while in the second group under the action of zinc nanoparticles mortality was 35 %. Similar results on reduction of earthworm survival were registered after the exposure of toxicants in high doses by Hirano and Tamae (2011).

The worms stopped substrate processing, fell into a passive state, and formed mucous film by cutaneous secretion. The film was composed of secreted mucus of skin and represented a clear structureless mucous layer less than 1 mm thick. Previously, the phenomenon of dermal uptake of dissolved metals in earthworms was investigated by Vijver et al. (2003). The authors suggested that mucus formation might be a way to eliminate metals from the worm body. The observation of mucous film formed by the earthworms treated with zinc nanoparticles suggests that the film represents a

protective adaptive reaction directed to prevent the dermal uptake of the toxicant.

The 16S metagenomic sequencing of the DNA from worm guts has resulted in 206,894 pair-end reads in combined specimen of the group I (GI) and 110,258 reads of the group II (GII). After merging and filtration, 110,673 and 54,785 high-quality united reads remained in GI and GII, respectively. Of those chimeras (GI—296, GII—31) and singleton reads (GI—26,261, GII—10,903) were recognized and removed. After clustering at the level, 97 % 374 OTUs (GI) and 107 OTUs (GII) were produced. From GI, 28 contaminating OTUs as well as 43 singletons and doubletons were removed. With the same procedure, 15 contaminating OTUs as well as 14 singletons and doubletons were discarded. At last, a total of 303 OTUs (GI) and 78 OTUs (GII) remained for downstream analysis.

Cultivation of worms on MCC without zinc nanoparticles allowed to consider the chosen substrate as the main factor affecting the gut microbiota of the test object, as described earlier (Parthasarathi et al. 2007).

As a result of the 16S metagenomic sequencing, the gut microbiota of group I worms was classified in 303 OTUs. Comparison with the SILVA reference database revealed 3 OTUs of organelles and 1 of unknown phylum, most of others belong to 15 phyla, 27 classes, 51 orders, 105 families, 172 genera, and 176 species. The rarefaction curve obtained with an evolutionary distance of 0.03 indicates that only a part of the prokaryotic diversity was detected in the sample, but expected diversity may be much higher (Fig. 1).

Evaluation of microbial diversity in the control specimen (GI) of worms' gut revealed that bacteria was the most abundant prokaryotic domain, constituting 99.3 % (297 OTUs), whereas archaeal reads were rather occasional (0.7 %, 2 OTUs) (Fig. 2).

The majority of the high-quality united reads from identified OTUs belongs to the taxa *Firmicutes* (51.9 % of the total), *Proteobacteria* (33.8 % of the total), and *Actinobacteria* (13.3 % of the total), while microorganisms belonging to other taxa (*Bacteroidetes*, *Planctomycetes* etc.) together contributed 10.7 % of the total abundance (Table 2).

The taxon *Firmicutes* was represented mostly by classes *Clostridia* and *Bacilli*, 44.9 and 7.06 % of the total number of high-quality united reads. Most *Proteobacteria* were of the taxa *Gammaproteobacteria* and *Alphaproteobacteria*, occupying 15.3 and 6.96 % of the total abundance. The taxon *Actinobacteria* was usually represented by the class *Actinobacteria*. Other classes (*Betaproteobacteria*, *Bacteroidetes*, *Sphingobacteria* etc.), whose abundance did not exceed 3.5 % each, accounted for 12.5 % of the total amount of reads.

Among the identified families, *Clostridiaceae* occupied the dominant position (47.7 % of the total abundance), fewer there were presented *Pseudomonadaceae* (6.6 % of the total),

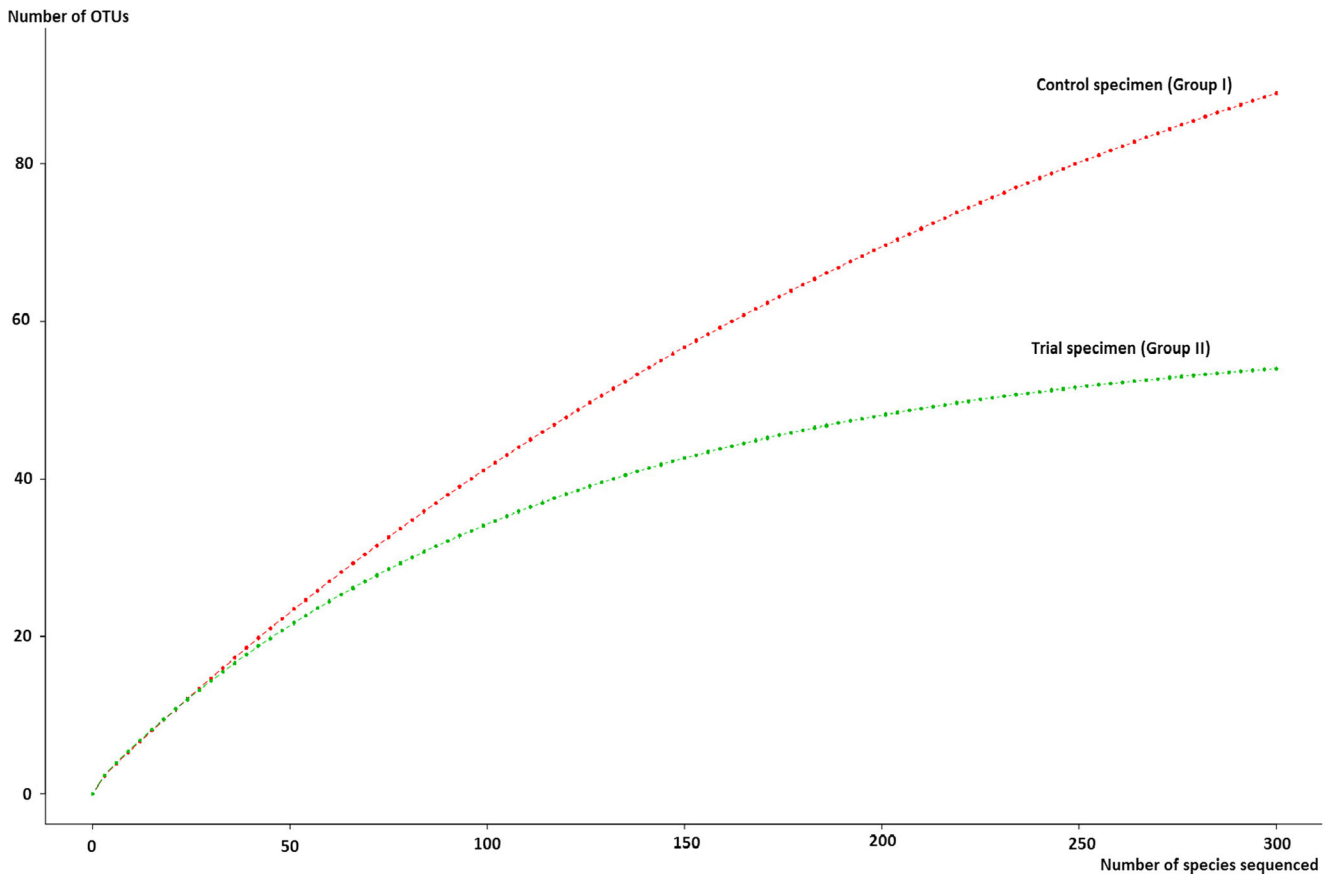


Fig. 1 Rarefaction curve of OTUs number in specimens observed with an evolutionary distance of 0.03 (3 %) in control specimen (group I, red line) and in trial one (group II, green line)

Bacillaceae (6 % of the total), *Enterobacteriaceae* (4.5 % of the total), *Methylobacteriaceae* (3.5 % of the total), *Cellulomonadaceae* (5.28 % of the total), and *Aeromonadaceae* (4.2 % of the total). Other families such as *Sphingobacteriaceae*, *Micrococcaceae*, *Nocardiaceae*, *Caulobacteraceae*, *Xanthobacteraceae*, *Comamonadaceae*, *Peptococcaceae*, *Rhodospirillaceae*, *Chitinophagaceae* etc. were rather rare, contributing together 25.2 % of the total abundance.

Species composition of gut microbiota in worms of the first group was almost by half represented by bacterial species of the genus *Clostridium*.

The main species belonging to this genus were *C. saccharobutylicum*, *C. saccharoperbutylacetonicum*, *C. beijerinckii*, and *C. cellulovorans*. The separated OTUs demonstrated high similarity of the 16S rRNA gene fragments with the following species and strains (similarity at 99 % or higher): *C. saccharoperbutylacetonicum* N1-4 (HMT), *C. saccharobutylicum* DSM 13864, *C. beijerinckii* NCIMB 8052, and *C. cellulovorans* 743B.

The second most abundant taxon was gen. *Pseudomonas* (*P. hydrogenvora*, *P. aeruginosa*, and *P. putida*), which contributed 6.6 % of the total. Next most abundant genera were found gen. *Bacillus* (*B. megaterium*, *B. silvestris* etc.), gen.

Aeromonas (*A. hydrophila*), and gen. *Cellulomonas* (*C. flavigena* and *C. fimi*), contributing 5.1, 4.2, and 5.28 % of the total, respectively. Among bacterial community of the gut microbiota in *Eisenia fetida Andrei Bouche* of the group I, there were also revealed some representatives of the genera *Nocardioides*, *Arthrobacter*, *Rhodococcus*, *Flexibacter*, *Novosphingobium*, *Protochlamydia*, *Corynebacterium*, *Rothia*, *Brevundimonas*, *Rhodobacter*, *Rhizobium*, *Klebsiella*, *Citrobacter*, *Enterococcus*, *Desulfovibrio*, *Verminephrobacter* etc.

The united reads from the gut microflora of group II worms were composed of 78 OTUs including 3 OTUs of unknown phyla and other 75 OTUs of the bacteria domain (Fig. 3). Comparison with the SILVA reference database demonstrated that microbial diversity in the trial specimen (GII) was significantly lower than in the control specimen (GI). So gut bacterial microflora in *E. fetida* of the group II belongs to 8 phyla, 14 classes, 23 orders, 51 families, 67 genera, and 68 species. The rarefaction curve obtained with an evolutionary distance of 0.03 indicates that most of the prokaryotic diversity was detected in the sample; i.e., in fact, the observed diversity is quiet equal to expected diversity (Fig. 1).

Most of the identified OTUs were representatives of the phylum *Proteobacteria*, contributed 73.1 % of the total



Fig. 2 Pie chart of the taxonomic abundance of the gut bacterial consortium in control specimen (group I) of worm *Eisenia fetida* based on comparison of the 16S rDNA sequences with the SILVA reference database

amount of the high-quality united reads from identified OTUs. Phyla *Firmicutes* and *Actinobacteria* contributed 10.3 and 8.9 % of the total. Bacterial OTUs of other phyla (*Bacteroidetes*, *Planctomycetes*, *Chloroflexi* etc.) contributed 7.7 % of the total.

Dominant classes were *Betaproteobacteria* and *Alphaproteobacteria*, contributed 51.5 and 21.2 % of the total. Among the classes characterized by numerous

representatives, there were also noted *Clostridia* (5.69 % of the total), *Bacilli* (4.49 % of the total), and *Actinobacteria* (8.9 % of the total). Other identified classes (*Chlamydiae*, *Caldilineae*, *Sphingobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria* etc.) whose abundance did not exceed 3.5 % each, in the sum contributed 8.22 % of the total.

The most prevalent families of *Betaproteobacteria* and *Alphaproteobacteria* were *Comamonadaceae* (51.3 % of the

Table 2 Taxonomic composition of gut microflora of *Eisenia fetida* worms from groups I and II

Group	Taxon			
	Phylum	Class	Family	Genus
I	<i>Firmicutes</i> (51.9 %)	<i>Clostridia</i> (44.9 %)	<i>Clostridiaceae</i> (44.7 %)	<i>Clostridium</i> (44.7 %)
		<i>Bacilli</i> (7.09 %)	<i>Bacillaceae</i> (6 %)	<i>Bacillus</i> (5.1 %)
	<i>Proteobacteria</i> (33.8 %)	<i>Gammaproteobacteria</i> (15.3 %)	<i>Enterobacteriaceae</i> (4.5 %)	<i>Enterobacter</i> (3.5 %)
			<i>Aeromonadaceae</i> (4.2 %)	<i>Aeromonas</i> (4.2 %)
			<i>Pseudomonadaceae</i> (6.6 %)	<i>Pseudomonas</i> (6.6 %)
	<i>Actinobacteria</i> (13.3 %)	<i>Alphaproteobacteria</i> (6.96 %)	<i>Methylobacteriaceae</i> (3.5 %)	–
			<i>Cellulomonadaceae</i> (5.28 %)	<i>Cellulomonas</i> (5.28 %)
<i>Others^a</i> (12.5 %)			<i>Others^a</i> (30.6 %)	
<i>Others^a</i> (1 %)	<i>Others^a</i> (12.5 %)	<i>Others^a</i> (25.2 %)	<i>Others^a</i> (30.6 %)	
II	<i>Firmicutes</i> (10.3 %)	<i>Clostridia</i> (5.69 %)	<i>Clostridiaceae</i> (5.6 %)	<i>Clostridium</i> (5.6 %)
		<i>Bacilli</i> (4.49 %)	<i>Bacillaceae</i> (4.2 %)	<i>Bacillus</i> (4.2 %)
	<i>Proteobacteria</i> (73.1 %)	<i>Betaproteobacteria</i> (51.5 %)	<i>Comamonadaceae</i> (51.3 %)	<i>Verminephrobacter</i> (46.8 %)
			<i>Brucellaceae</i> (19.6 %)	<i>Ochrobactrum</i> (19.5 %)
			<i>Nocardiaceae</i> (3.5 %)	<i>Nocardia</i> (3.5 %)
	<i>Actinobacteria</i> (8.9 %)	<i>Actinobacteria</i> (8.9 %)	<i>Cellulomonadaceae</i> (5.2 %)	<i>Cellulomonas</i> (5.2 %)
	<i>Others^a</i> (7.7 %)	<i>Others^a</i> (8.22 %)	<i>Others^a</i> (10.6 %)	<i>Others^a</i> (18.6 %)

^a This group unites taxa whose abundance (each) does not exceed 3.5 % of the total

total) and *Brucellaceae* (19.6 % of the total), respectively. *Clostridia* and *Bacilli* were mainly represented by the families *Clostridiaceae* (5.6 % of the total) and *Bacillaceae* (4.2 % of the total), respectively. Class *Actinobacteria* was represented mostly by the families *Cellulomonadaceae* and *Nocardiaceae*, 5.2 and 3.5 % of the total. Other identified families (*Hyphomicrobiaceae*, *Xanthomonadaceae*, *Rhodobacteraceae*, *Cytophagaceae*, *Sphingobacteriaceae*, *Planctomycetaceae* etc.) were rather rare, in the sum contributing 10.6 % of the total.

The high-quality united reads of all OTUs in gut community of the group II worms by 46.8 % were represented by bacteria of gen. *Verminephrobacter* and by 19.6 %—of gen. *Ochrobactrum*.

Identified OTUs belonging to gen. *Verminephrobacter* and gen. *Ochrobactrum* demonstrated high similarity of the 16S rRNA gene fragments with the following species and strains (similarity at 99 % or higher): *V. eiseniae* strain EfG3-23, *V. eiseniae* strain EF05-2, *O. anthropi* strain GPK 3, and *O. pituitosum* strain SPT1-119a. Next in number among the identified OTUs were the genera *Clostridium* (5.6 % of the total) and *Bacillus* (4.2 % of the total), whose main representatives were *B. niacini*, *B. megaterium*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum*. The rest of identified bacteria were representatives of the genera *Stenotrophomonas*, *Arthrobacter*, *Pseudonocardia*, *Corynebacterium*, *Flexibacter*, *Planococcus*, *Parachlamydia*, *Zavarzinella*, *Sphingomonas*, *Sphingopyxis*, *Ralstonia* etc., in the sum amounted 18.6 % of the total. These taxa were rare and were represented by not numerous reads.

As a result, the analysis of the gut bacterial diversity in *E. fetida* of the group I has shown the presence of common species of soil microflora, mainly representing a complex of cellulose destructors with genera *Clostridium* and *Pseudomonas* being prevalent. In addition, there have been found numerous species associated with nitrogen exchange. It is known that activity of cellulolytic microorganisms is connected with the presence of mineral nitrogen in the substrate (Nazarko and Lobanov 2007). So, the prevalence of cellulolytic bacteria is due to the substrate chosen for cultivation.

According to the literature, typical symbionts of earthworms are bacteria of the genus *Verminephrobacter* (*V. eiseniae*); also, the symbiosis with worms was reported for the genera *Ochrobactrum*, *Aeromonas*, and *Acinetobacter* (Lund et al. 2010; Byzov et al. 2009; Toyoto and Kimura 2000; Davidson et al. 2014). Poor knowledge about gut microbiota of earthworms and the lack of a unified approach to its investigation leads to the presence of contradictory data on the dominant taxa in the literature (Fajardo et al. 2012). However, most researchers point to a leading role of a chosen cultivation substrate in the formation of worms' gut microbiocenosis (Parthasarathi et al. 2007).

Investigation of gut microbial diversity in *E. fetida* of the group II with the 16S metagenomic sequencing has shown a decrease in the amount of gram-positive microflora (the phyla *Firmicutes* and *Actinobacteria*) and increase of gram-negative bacteria (the phylum *Proteobacteria*). Our data demonstrated an increase in abundance of the *Proteobacteria* by 51.7 % as compared to the control group, and a decrease in abundance of

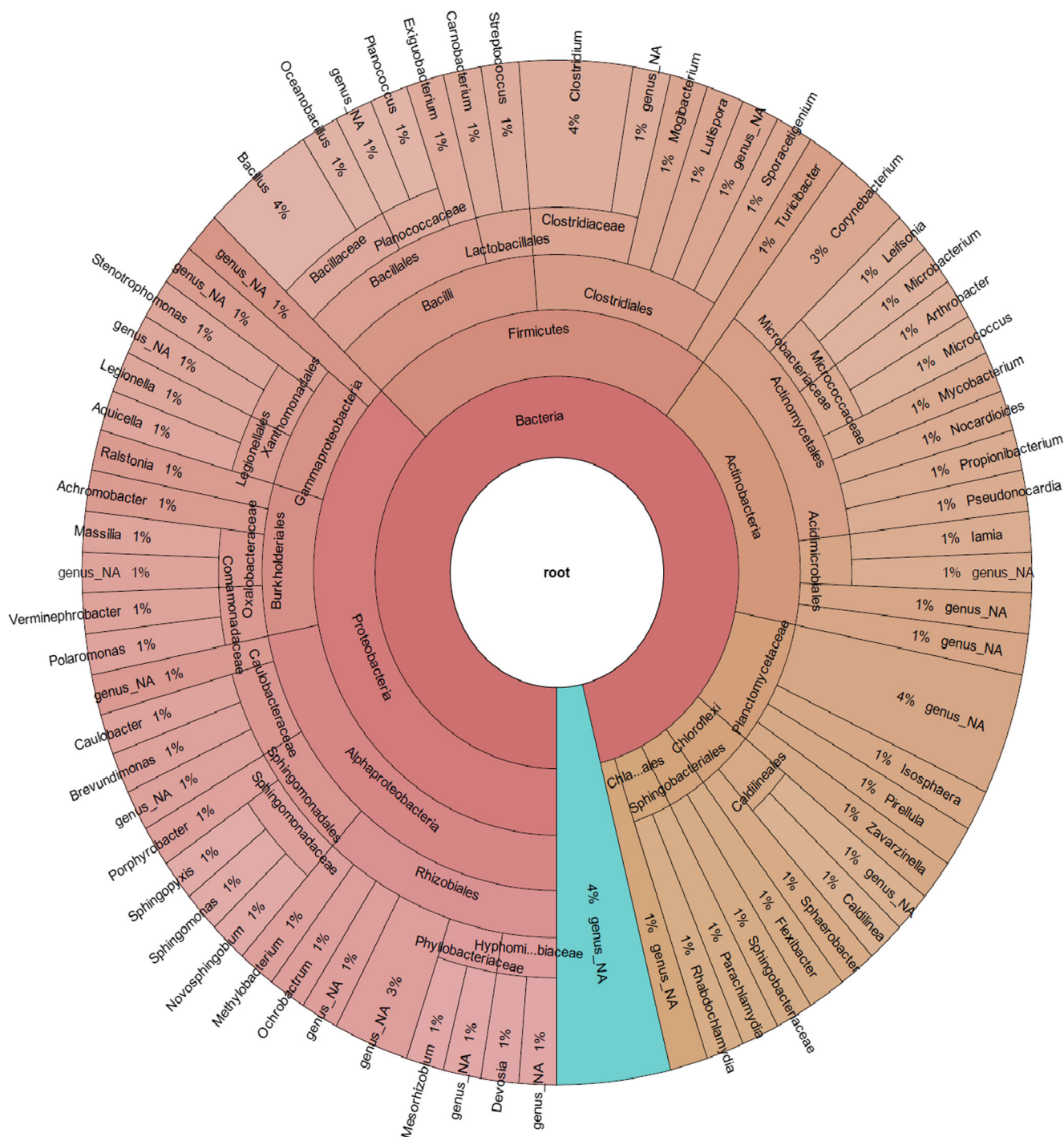


Fig. 3 Pie chart of the taxonomic abundance of the gut bacterial consortium in trial specimen (group II) of worm *Eisenia fetida* based on comparison of the 16S rDNA sequences with the SILVA reference database

bacteria belonging to the *Firmicutes* and *Actinobacteria* by 41.6 and 4.4 %, respectively. There was a significant decrease in abundance of such taxa as *Clostridium* (−39.1 %) and *Pseudomonas* (−6.2 %), which were the main representatives of the gut microflora of *E. fetida* in the control group, and an increase in abundance of the genera *Verminephrobacter* (+46 %) and *Ochrobactrum* (+19.5 %).

Similar results on the impact of nanoparticles on bacterial cultures were presented in a study of Yakout and Mostafa (2015). In their work, the authors showed greater level of antimicrobial activity of silver nanoparticles against gram-positive bacteria as compared to the effect on gram-negative microorganisms. Similar data are also known for zinc oxide nanoparticles. The paper of Premanathan M et al. (2010)

reported a more pronounced cytotoxic effect of zinc oxide nanoparticles when contacting with *Staphylococcus aureus* than with *Escherichia coli* and *P. aeruginosa*. Many researchers suggest that such action is related to structure of the cell wall; its thickness and structural changes under the influence of nanoparticles (Lok et al. 2006; Rahisuddin et al. 2015). However, some authors point out that high concentrations of nanoparticles, as a rule, have an equal effect on gram-positive and gram-negative microbiota (Ananpattarachai et al. 2015).

Also, when analyzing data, one should take into account that the population dynamics of the gut microbiota in *E. fetida* of the group II is significantly determined by the changes in the soil microbial composition. An addition of high concentrations of zinc nanoparticles to the substrate first of all affected the soil microflora, whose dominant representative in our case was *Clostridium* spp. This led to a decrease in its abundance, as was found in another work (García-Gómez et al. 2015). At direct contact, metal nanoparticles can exert bactericidal and bacteriostatic effect by violating the integrity of the bacterial cell membrane and disturbing the functioning of intracellular systems (Li et al. 2015; Saccà et al. 2014).

In turn, active reproduction of worms' symbionts such as *Verminephrobacter* sp. and *Ochrobactrum* sp. is conditioned by protective effect of the worms' antioxidant system. Exposure to toxicants does not lead to blocking of the antioxidant system; at certain concentrations, it increases production of such enzymes as catalase, glutathione peroxidase, and superoxide dismutase (Li et al. 2011; Xu et al. 2013; Zhang et al. 2013). These enzymes are main biologically active substances involved in neutralizing the action of toxicants. The work of these enzymes ensures maintenance of the intracellular redox balance (Rosa et al. 2008).

Thus, this study has allowed us to estimate gut microbial diversity in earthworms and demonstrated the presence of protective mechanisms in the worms related to activity of their symbiotic gut bacteria against high doses of the toxic nanoparticles, which contributed to the survival of both the worm and its symbionts.

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References

- Aira M, Monroy F, Domínguez J (2006) *Eisenia fetida* (Oligochaeta, Lumbricidae) activates fungal growth, triggering cellulose decomposition during vermicomposting. *Microb Ecol* 52(4):738–747
- Aira M, Monroy F, Domínguez J (2009) Changes in bacterial numbers and microbial activity of pig slurry during gut transit of epigeic and anecic earthworms. *J Hazard Mater* 162(2–3):1404–1407. doi:10.1016/j.jhazmat.2008.06.031
- Ananpattarachai J, Boonto Y, Kajitvichyanukul P (2015) Visible light photocatalytic antibacterial activity of Ni-doped and N-doped TiO₂ on *Staphylococcus aureus* and *Escherichia coli* bacteria. *Environ Sci Pollut Res Int*. doi:10.1007/s11356-015-4775-1
- Bel'kova NL, Dzyuba EV, Sukhanova EV, Khanaeva TA (2008) Adaptation of molecular genetic methods to study microorganisms associated with fish. *Inland Water Biol* 1(2):192–195. doi:10.1134/S1995082908020120
- Byzov BA, Nechitailo TY, Bumazhkin BK, Kurakov AV, Golyshin PN, Zvyagintsev DG (2009) Culturable microorganisms from the earthworm digestive tract. *Mikrobiology* 78(3):360–368. doi:10.1134/S0026261709030151
- Davidson SK, Dulla GF, Go RA, Stahl DA, Pinel N (2014) Earthworm symbiont *Verminephrobacter eiseniae* mediates natural transformation within host egg capsules using type IV pili. *Front Microbiol* 5: 546.1–10. doi:10.3389/fmicb.2014.00546
- Drake HL, Horn MA (2007) As the worm turns: the earthworm gut as a transient habitat for soil microbial biomes. *Annu Rev Microbiol* 61: 169–189
- Drake HL, Schramm A, Horn MA (2006) Earthworm gut microbial biomes: their importance to soil microorganisms, denitrification, and the terrestrial production of the greenhouse gas N₂O. In: König H, Varma A (eds) *Intestinal microorganisms of termites and other invertebrates*. Springer, New York
- Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26(19):460–2461. doi:10.1093/bioinformatics/btq461
- Edwards CA (2004) The importance of earthworms as key representatives of the soil fauna. In: Edwards CA (ed) *Earthworm ecology*, 2nd edn. CRC Press, Boca Raton, pp 3–11
- Edwards CA, Fletcher KE (1988) Interactions between earthworms and microorganisms in organic matter breakdown. *Agric, Ecosyst Environ* 24(1–3):235–247. doi:10.1016/0167-8809(88)90069-2
- El-Kereti MA, El-feky SA, Khater MS, Osman YA, El-sherbini e-SA (2013) ZnO nanofertilizer and He Ne laser irradiation for promoting growth and yield of sweet basil plant. *Rec Pat Food Nutr Agric* 5(3):169–181
- Fahmy SR, Abdel-Ghaffar F, Bakry FA, Sayed DA (2014) Ecotoxicological effect of sublethal exposure to zinc oxide nanoparticles on freshwater snail *Biomphalaria alexandrina*. *Arch Environ Contam Toxicol* 67(2):192–202. doi:10.1007/s00244-014-0020-z
- Fajardo C, Ortíz LT, Rodríguez-Membibre ML, Nande M, Lobo MC et al (2012) Assessing the impact of zero-valent iron (ZVI) nanotechnology on soil microbial structure and functionality: a molecular approach. *Chemosphere* 86:802–808. doi:10.1016/j.chemosphere.2011.11.041
- Ferreira RC, Papini S, de Andréa MM (2015) Bioavailability and influence of C-carbofuran on *Eisenia andrei* avoidance, growth and reproduction in treated natural tropical soils. *J Environ Sci Health B* 50(4):266–274. doi:10.1080/03601234.2015.999599
- García-Gómez C, Babin M, Obrador A, Álvarez JM, Fernández MD (2015) Integrating ecotoxicity and chemical approaches to compare the effects of ZnO nanoparticles, ZnO bulk, and ZnCl₂ on plants and microorganisms in a natural soil. *Environ Sci Pollut Res* 22(21): 16803–16813. doi:10.1007/s11356-015-4867-y
- Grumiaux F, Demuynck S, Permin C, Leprière A (2015) Earthworm populations of highly metal-contaminated soils restored by fly ash-aided phytostabilisation. *Ecotoxicol Environ Saf* 113:183–190. doi:10.1016/j.ecoenv.2014.12.004
- He L, Liu Y, Mustapha A, Lin M (2011) Antifungal activity of zinc oxide nanoparticles against *Botrytis cinerea* and *Penicillium expansum*. *Microbiol Res* 166(3):207–215. doi:10.1016/j.micres.2010.03.003
- Hirano T, Tamae K (2011) Earthworms and soil pollutants. *Sensors (Basel)* 11(12):11157–11167. doi:10.3390/s111211157
- Hoet P, Bruske-Hohlfeld I, Salata O (2004) Nanoparticles—known and unknown health risks. *J Nanobiotechnol* 2:12. doi:10.1186/1477-3155-2-12

- Hu CW, Lia M, Cui YB, Li DS, Chen J, Yang LY (2010) Toxicological effects of TiO₂ and ZnO nanoparticles in soil on earthworm *Eisenia fetida*. *Soil Biol Biochem* 42:586–591. doi:10.1016/j.soilbio.2009.12.007
- Huse SM, Mark Welch DB, Voorhis A, Shipunova A, Morrison HG et al (2014) VAMPS: a website for visualization and analysis of microbial population structures. *BMC Bioinform* 15:41
- Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glöckner FO (2013) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research* 41(1):e1. doi:10.1093/nar/gks808
- Krivolutskii DA (1994) Soil fauna in environmental control. Nauka, Moscow. (in Russian)
- Li LZ, Zhou DM, Peijnenburg WJ, van Gestel CA, Jin SY, Wang YJ, Wang P (2011) Toxicity of zinc oxide nanoparticles in the earthworm, *Eisenia fetida* and subcellular fractionation of Zn. *Environ Int* 37(6):1098–1104. doi:10.1016/j.envint.2011.01.008
- Li H, Chen Q, Zhao J, Urmila K (2015) Enhancing the antimicrobial activity of natural extraction using the synthetic ultrasmall metal nanoparticles. *Sci Rep* 5(5):11033. doi:10.1038/srep11033
- Lin D, Xing B (2007) Phytotoxicity of nanoparticles: inhibition of seed germination and root growth. *Environ Pollut* 150:243–250. doi:10.1016/j.envpol.2007.01.016
- Liu Y, Yao T, Jiao N, Kang S, Xu B, Zeng Y, Huang S, Liu X (2009) Bacterial diversity in the snow over Tibetan Plateau Glaciers. *Extremophiles* 13:411–423. doi:10.1007/s00792-009-0227-5
- Lok CN, Ho CM, Chen R, He QY, Yu WY, Sun H, Tam PK, Chiu JF, Che CM (2006) Proteomic analysis of the mode of antibacterial action of silver nanoparticles. *J Proteome Res* 5:916–924. doi:10.1186/1477-3155-8-1
- Lund MB, Holmstrup M, Lomstein BA, Damgaard Ch Schramm A (2010) Beneficial effect of vermiphobacter nephridial symbionts on the fitness of the earthworm *Aporrectodea tuberculata*. *Appl Environ Microbiol* 76(14):4738–4743. doi:10.1128/AEM.00108-10
- Milani N, McLaughlin MJ, Stacey SP, Kirby JK, Hettiarachchi GM, Beak DG, Cornelis G (2012) Dissolution kinetics of macronutrient fertilizers coated with manufactured zinc oxide nanoparticles. *J Agric Food Chem* 60(16):3991–3998. doi:10.1021/jf205191y
- Moore MN (2006) Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? *Environ Int* 32:967–976. doi:10.1016/j.envint.2006.06.014
- Nazarko MD, Lobanov VG (2007) Influence of the microflora composition on ecological balance of soils in Kuban. *Izvestiya Vuzov. Pishchevaya Tekhnologiya* 1:92–93 (in Russian)
- OECD (2004) OECD guideline for the testing of chemicals: earthworm reproduction test (*Eisenia fetida*). OECD, Paris
- Parthasarathi K, Ranganathan LS, Anandi V, Zeyer J (2007) Diversity of microflora in the gut and casts of tropical composting earthworms reared on different substrates. *J Environ Biol* 28(1):87–97
- Premanathan M, Karthikeyan K, Jeyasubramanian K et al. (2010) Selective toxicity of ZnO nanoparticles toward gram-positive bacteria and cancer cells by apoptosis through lipid peroxidation. *7(2)*: 184–92. doi: 10.1016/j.nano.2010.10.001
- Rahisuddin A-TSA, Khan Z, Manzoor N (2015) Biosynthesis of silver nanoparticles and its antibacterial and antifungal activities towards Gram-positive, Gram-negative bacterial strains and different species of *Candida* fungus. *Bioprocess Biosyst Eng* 38(9):1773–1781. doi:10.1007/s00449-015-1418-3
- Reznichenko IS (2013) A comparative analysis of methods of cleansing the digestive system of earthworms for ecotoxicological studies on *Eisenia fetida* (Savigny, 1826). *Fundamentalnaya Nauka* 6:1156–1159 (in Russian)
- Rocheleau S, Arbour M, Elias M, Sunahara GI, Masson L (2015) Toxicogenomic effects of nano- and bulk-TiO₂ particles in the soil nematode *Caenorhabditis elegans*. *Nanotoxicology* 9(4):502–512. doi:10.3109/17435390.2014.948941
- Rosa CE, Bianchini A, Monserrat JM (2008) Antioxidant responses of *Laonereis acuta* (Polychaeta) after exposure to hydrogen peroxide. *Braz J Med Biol Res* 41(2):117–121
- Saccà ML, Fajardo C, Martínez-Gomariz M, Costa G, Nande M, Martín M (2014) Molecular stress responses to Nano-Sized Zero-Valent Iron (nZVI) particles in the soil bacterium *Pseudomonas stutzeri*. *PLoS One* 9(2):e89677. doi:10.1371/journal.pone.0089677
- Song U, Lee S (2016) Phytotoxicity and accumulation of zinc oxide nanoparticles on the aquatic plants *Hydrilla verticillata* and *Phragmites Australis*: leaf-type-dependent responses. *Environ Sci Pollut Res Int*
- Tiunov AV, Scheu S (2000) Microfungal communities in soil, litter and casts of *Lumbricus terrestris* L. (Lumbricidae): a laboratory experiment. *Appl Soil Ecol* 14(2):187–197. doi:10.1016/j.femsec.2004.01.007
- Tourinho PS, van Gestel CA, Lofis S et al. (2012) Metal-based nanoparticles in soil: fate, behavior, and effects on soil invertebrates. *Environ Toxicol Chem* 31(8):1679–92. doi: 10.1002
- Toyoto K, Kimura M (2000) Microbial community indigenous to the earthworm *Eisenia foetida*. *Biol Fertil Soils* 31(3):187–190
- Vijayakumar S, Vinoj G, Malaikozhundan B, Shanthi S, Vaseeharan B (2015) *Plectranthus amboinicus* leaf extract mediated synthesis of zinc oxide nanoparticles and its control of methicillin resistant *Staphylococcus aureus* biofilm and blood sucking mosquito larvae. *Spectrochim Acta A Mol Biomol Spectrosc* 137:886–891. doi:10.1016/j.saa.2014.08.064
- Vijver MG, Vink JPM, Miermans CJH, van Gestel CAM (2003) Oral sealing using glue; a new method to distinguish between intestinal and dermal uptake of metals in earthworms. *Soil Biol Biochem* 35(1):125–132. doi:10.1016/S0038-0717(02)00245-6
- Voropaev VN, Pashkov OM (2009) Zinc in soils and crop production steady experience. *Bull Bryansk State Agric Acad* 2:31–36 (in Russian)
- Wang F, Liu X, Shi Z, Tong R, Adams CA, Shi X (2016) Arbuscular mycorrhizae alleviate negative effects of zinc oxide nanoparticle and zinc accumulation in maize plants—a soil microcosm experiment. *Chemosphere* 147:88–97. doi:10.1016/j.chemosphere.2015.12.076
- Xu D, Li C, Wen Y, Liu W (2013) Antioxidant defense system responses and DNA damage of earthworms exposed to perfluorooctane sulfonate (PFOS). *Environ Pollut* 174:121–127. doi:10.1016/j.envpol.2012.10.030
- Yakout SM, Mostafa AA (2015) A novel green synthesis of silver nanoparticles using soluble starch and its antibacterial activity. *Int J Clin Exp Med* 8(3):3538–3544
- Zhang J, Yu J, Ouyang Y, Xu H (2013) Responses of earthworm to aluminum toxicity in latosol. *Environ Sci Pollut Res* 20(2):1135–1141. doi:10.1007/s11356-012-0969-y