



ACTIVATING THE GLUTATHIONE SYSTEM OF *EISENIA FETIDA* DURING EXPOSURE TO CONTAMINATED SILT SLUDGE

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Received 24th October 2016, accepted 23rd December 2016

Abstract. In order to identify effective and inexpensive method of purifying wastewater sludge, we tested the hypothesis about the ability of earthworm *Eisenia fetida* to detoxify the silt complex of different chemical components through the activation of glutathione system. In the laboratory conditions, earthworms were exposed to silt from the sedimentation tanks during 112 days in the boxes, while the control earthworms were on a sunflower husk substrate. Glutathione S-transferase (GST) activity and reduced glutathione (GSH) content in coelomic fluid of earthworms were measured spectrophotometrically. The results revealed biomass of the experimental *E. fetida* worms was declined by 69% compared to control after the exposure. Heavy metals content in the silt decreased in the range from 13% (for zinc and lead) to almost 100% in the case of cadmium compared to beginning of the experiment. The GST activity increased significantly (87% above the control level), as well as GSH content (36% above the control). Induction of 4 new proteins biosynthesis was found by gel-electrophoresis exploration of coelomic fluid proteins of the worms exposed to contaminated silt. Study results indicate a high level of toxicant-induced activation of the earthworm glutathione-dependent system, and confirmed the efficiency of *E. fetida* using for detoxification of silt from the sedimentation tanks in the case of complex contamination.

Key words: earthworm, wastewater sludge, heavy metals, detoxification, glutathione S-transferase, glutathione.

1. Introduction

In Ukraine, the annual accumulation of compacted sewage sludge (CSS) in the sedimentation tanks reaches 40 million tons, implying a need to occupy 120 hectares of natural land for storage silt on the drying beds and burning [1]. Such actions ultimately lead to pollution of atmosphere, surface and ground water, soil, and vegetation, and thereby deteriorate the quality of human life. Silt wastewater sludge utilization is also actual problem in many countries [2], [3], where the CSS application includes the addition to agricultural and other soils. Silt wastewater sludge contains a

high amount of various organic compounds [4], heavy metals and trace elements [5], so the pretreatment is required for the successful application of CSS [3]. One of the affordable and effective methods of silt sludge cleaning might be ensured by vermiculture, in particular *Eisenia fetida* culture. The final product of processing is the vermicompost (or biohumus), which can be used as fertilizer, and for the soils restoration and cleaning from radionuclides, heavy metals and pesticide residues as well. It has been found [6] that earthworms can bio-

accumulate, biodegrade or bio-transform the heavy metals and a wider range of other xenobiotics, such as herbicides [7] and veterinary drugs [8]. It was established [9], [10], that resistance of *E. fetida* to the toxins action has always been associated with activation of the metabolic defense including the glutathione-dependent system. In particular, the increase in glutathione-S-transferase (GST) activity was found towards the pesticides [7], [11] and heavy metals [12], [13]. On the other hand, the high inhibition of *E. fetida* GST activity was observed due to the action of carbamate pesticide carbaryl [14]. The goals of our work were (1) to evaluate the detoxifying ability of *E. fetida* in the case of integrated substrate contamination and (2) to identify the role of earthworm glutathione-dependent system in the substrate cleaning processes.

2. Materials and methods

The object of the study (vermiculture of earthworm *Eisenia fetida*) was provided by the Research Institute "Biotechnology" State University, Dnipropetrovsk; specification 3336406.00 2–95.

Substrates for vermiculture were prepared on the basis of modified sunflower husk (SH, control); mixed husk of sunflower, rice and buckwheat (MH), and compacted sewage sludge (CSS) from the sedimentation tanks. Each substrate was ground, and the fraction 200 – 500 microns was subjected to fermentation in the clamps 50 – 60 cm high, while maintaining 70 – 80% humidity. Process of substrates fermentation provided by microflora activity, lasted 14 days at a daily stirring. The fermented substrates were used for vermiculture, and the *E. fetida* worms were placed in substrate with

layer thickness 30 cm at a density of 5 – 10 thousand of the earthworms per square meter. The following conditions been complied during the experiment: pH level of substrate was in the range 6.5 – 7.5, humidity reached 70 – 80%, and the temperature fluctuation was within 20 – 25° C. For each substrate, three replicates were prepared. The worms was selected at 112 day and placed on wet filter paper to remove the gut content. After 3 days with daily replacement of filter paper coelomic fluid was extracted, frozen, and stored at – 20° C.

The average biomass weight of *E. fetida* from each substrate was determined by weighing a group on analytical scales. Earthworm's weight measurement was carried out during the whole experiment, selecting samples every 14 days. Parameters of growth inhibition for the various exposure periods were computed according to [15].

The content of heavy metals was determined by atomic absorption method in the substrates before placing of earthworms and after their removal at the end of 112 days.

Activity of glutathione-S-transferase (GST, EC 2.5.1.18) in the coelomic fluid was measured according to the method described by Habig et al. [16] and Saint-Denis et al. [9] with 1-chloro-2,4-dinitro-benzene (CDNB) as a substrate. The assay mixture (100 μ l of 0.1 M/L Tris buffer, pH 8.0, 100 μ l GSH, and 200 μ l sample) was incubated during 10 min at 30° C, and the reaction was initiated by addition of 100 μ l CDNB. Optical density change was detected at 340 nm during four minutes, and GST activity was expressed in nMol CDNB $\text{sec}^{-1}\text{ml}^{-1}$ (nkatal ml^{-1}).

The reduced glutathione (GSH) content determination in the coelomic fluid was conducted by the method of Anderson [17], based on spectrophotometric

registration of the glutathione reaction with the Ellman's reagent (dithiobis-2-nitrodenzoic acid). Optical density of assay mixture (110 μl of 0.1 m/L K-phosphate buffer contained EDTA, and 300 μl of sample) was detected at 412 nm after the addition of Ellman's reagent. GSH content was calculated by using calibration graph, and expressed in nMol GSH ml^{-1} .

Protein spectrum study of *E. fetida* coelomic fluid was carried out by a modified SDS-PAGE method of Laemmly [18] in 7.5 – 17.5% gradient acrylamide gel. Protein samples (50 $\mu\text{g ml}^{-1}$) were boiled for 5 min at 95° C and applied to the respective wells in the gel. Analysis was run at 20 mA in the electrophoresis unit VE-20 (Russia). Protein standards were run in parallel

with the samples. Bands were stained with 0.25% Coomassie brilliant R-250. All measurements were performed in triplicate at least. Data present mean values and standard deviation ($M \pm SD$). Significance of the differences between control and treated samples was estimated with Student's t-test ($P < 0.05$). Fisher's test was used for analysis of variances, $F_{\text{crit.}}(0.05; 4; 4) = 6.39$.

3. Results and discussion

The biomass growth of *E. fetida* exposed to the sunflower husk, as well as to the mixed husk, was a positive trend during the experiment, whereas decrease in weight observed in the case of worms exposed to the silt wastewater sludge (Figure 1).

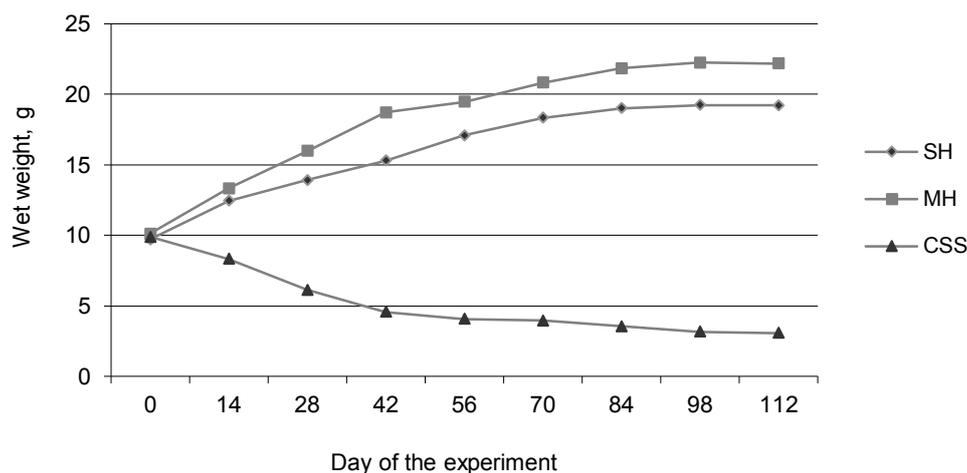


Fig 1. Wet weight of *E. fetida* earthworms exposed to different substrates: sunflower husk (SH, control), mixed husk (MH), and compacted sewage sludge (CSS)

The increase in *E. fetida* biomass at both substrates SH and MH occurred 98 days until the experiment and reached, respectively 198% and 220% compared to initial weight of the worms. At the same time, the wet weight of the earthworms exposed to substrate CSS diminished up to 112 days; weight loss was 16% of control already at 14 days of the experiment, and the final weight

loss was 69% of baseline. The results obtained indicated, that growth of *E. fetida* worms exposed to compacted sewage sludge was inhibited drastically. Decrease in the wet weight was observed during whole experiment, however, the most significant decrease was observed up to 42 days, then becoming a less pronounced. A similar non-linear decrease in growth rate of *E.*

fetida was occurred under the action of herbicide acetochlor [19]. Likewise, the growth of earthworms *Aporrectodea caliginosa* was decreased noticeably after 7 and 14 days of exposure to insect growth regulators flufenoxuron and pyriproxyfen [20]. In general, growth inhibition is a good indicator of

chemical stress, which may link effects to energy dynamics and ultimately inhibit growth of the organisms [15]. In our study, the content of heavy metals discovered in the substrate CSS before the earthworm's introduction, was strongly reduced at the end of the experiment (Table 1).

Table 1
Effect of *E. fetida* vermiculture on heavy metal content in compacted sewage sludge (CSS)

Metal	Content of metal in CSS, mg/kg	
	before the experiment	after the experiment
Cu	76.21±2.54	5.40±0.20
Zn	218.04±8.07	17.32±0.61
Mn	794.10±30.17	54.74±1.86
Ni	29.20±1.05	0.70±0.03
Cr	38.12±1.49	2.54±0.12
Pb	39.03±1.40	3.02±0.10
Cd	5.12±0.19	-

The smallest drop was found for the zinc and lead levels (respectively, 12.6 and 13 times compared to baseline). Decrease in the levels of copper, manganese and chromium was greater (14.1, 14.5 and 15.2 times, respectively). Nickel content was reduced to 41.4 times; however, the most striking was the almost 100% reduction in level of cadmium, which was not identified in the substrate after the experiment. That is, the worm's biomass reduction was accompanied by a sharp decrease in heavy metal content in contaminated substrate. It is obvious that inhibition of *E. fetida* growth could be due to enormous amplification of detoxification processes of heavy metals. Our results are consistent with data [6] about earthworm's ability to accumulate high concentrations of mercury, lead, copper, manganese, zinc, and the extreme cadmium content (up to 100 mg per kg dry weight). It was established [21], that accumulation of a single heavy metal does not affect the worms' physiology; while in our study, the sharp decline in biomass could be

caused by presence of several heavy metals and possibly other toxicants in the substrate.

Increase in activity of glutathione-S-transferase was revealed in coelomic fluids of *E. fetida* exposed to both substrate MH (insignificant difference with control) and CSS (187% compared to control, $P < 0.05$), as it shown in Figure 2. F-test indicated the difference of data dispersions for two different substrates MH and CSS.

Effective metabolic degradation of different xenobiotics by the earthworms based on the functioning of glutathione-S-transferase, which can catalyze the conjugation of toxic compounds with reduced glutathione, and is involved in antioxidant defense [22]. In our study, almost 2-fold activation of GST was found in coelomic fluid of *E. fetida* after 112 days of exposition to complex contaminated substrate. This result confirms the involvement of *E. fetida* glutathione-S-transferase in detoxification of various components as well as the capacity for long-term

functioning under the influence of substrate with an integrated pollution.

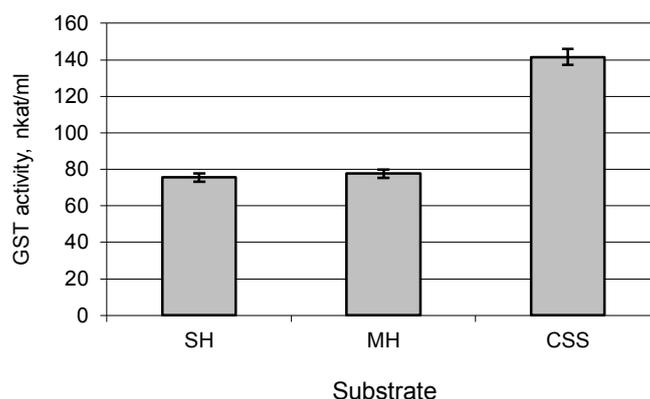


Fig. 2. GST activity (nkat/ml) in coelomic fluid of *E. fetida* exposed to SH (sunflower husk), MH (mixed husk), and CSS (compacted sewage sludge)

Outcomes are comparable with the data [13] about significant increasing GST activity of *E. fetida* in soils with copper after a period of contaminants accumulation in body tissues, as well, as about GST activation with longer duration and increasing concentrations of herbicide acetochlor [23].

In our study, the control level of reduced glutathione content in the *E. fetida* coelomic fluid was exceeded in the worms exposed to substrate MH (10 % above control, $P < 0.05$), and to substrate CSS (36% above control, $P < 0.05$) as well (Figure 3). F-test revealed the difference of data dispersions for substrates MH and CSS.

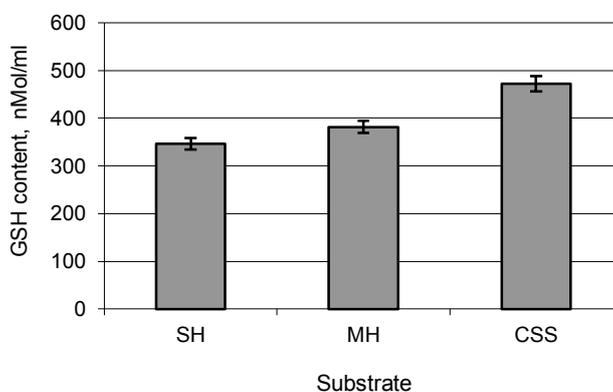


Fig. 3. Effect of substrates on GSH content (nMol/ml) in coelomic fluid of *E. fetida* exposed to SH (sunflower husk), MH (mixed husk), and CSS (compacted sewage sludge)

Thus, amount of reduced glutathione in the *E. fetida* coelomic fluid increased by 1.4 times, indicating enhancement the biosynthesis of glutathione and recovery of the oxidized glutathione during exposition to the contaminated

substrate. Reduced glutathione carries out many functions besides conjugation with xenobiotics; in particular, in the worms' cells, GSH was involved in the biosynthesis of metallothioneins due to the action of copper [13] and cadmium

[24]. In our experiment, GSH pool enhancement was less than GST activation level; this could be attributed to glutathione spending for the synthesis of metallothioneins. Anyway, the glutathione content was sufficient to ensure the growth of GST activity, which is consistent with the assertion [25] about the absence of GST

activation in all earthworm species with low GSH levels.

In our study, SDS-PAGE analysis of *E. fetida* coelomic fluids revealed increase in intensity of certain protein bands in the spectra of the worms exposed to both substrates MH and CSS, compared with the control spectrum (Figure 4).

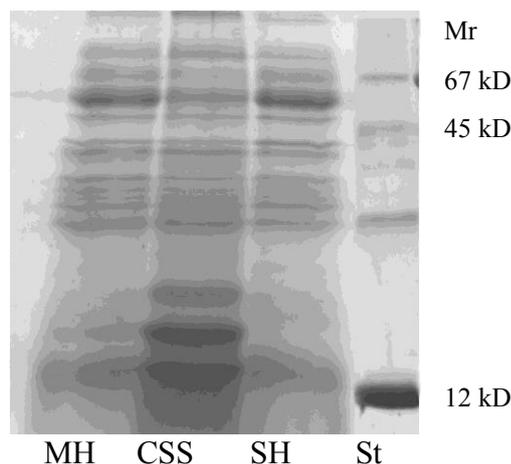


Fig. 4. Polypeptide content of coelomic fluid of the *E. fetida* worms exposed to different substrates: MH – mixed husk; CSS – compacted sewage sludge; SH – sunflower husk (control). St – standard proteins (cytochrome C, $M_r=12$ kD; egg albumin, $M_r=45$ kD; albumin bovine serum, $M_r=67$ kD).

E. fetida coelomic fluid proteins have been shown to have molecular masses ranging from 14.3 kDa to 97.8 kDa. Noteworthy, the components with $M_r=18.2$; $M_r=78.5$, and $M_r=81.3$ kDa were absent in the spectrum of the worms exposed to contaminated silt. At the same time, strengthening the bands corresponding to low-molecular polypeptides with $M_r=14.3$; $M_r=16.6$, $M_r=21.4$ kDa, and to high-molecular zone with $M_r=97.8$ kDa was observed in the spectrum of the worms exposed to silt compared to control spectrum. Beside them, proteins with $M_r=16.6$; $M_r=41.3$; $M_r=84.1$, and $M_r=87.1$ kDa were presented only in the samples exposed to SCC. Therefore, the appearance of these proteins in the worm's coelomic fluid reflects the changes in the protein metabolism including induction and / or

enhancement of biosynthesis of enzymes responsible for detoxification of contaminated substrate. So, it can be concluded the metabolic systems of *E. fetida* generally actively reacted to substrate content, providing a high adaptive capacity of worm organism to polluted environment by means of the xenobiotics detoxification.

4. Conclusion

Summing up, the studies confirmed the ability of *E. fetida* to simultaneously reduce the content of copper, zinc, manganese, nickel, chromium, lead and cadmium in contaminated substrate. Processes of the substrate cleaning against nickel and cadmium were the most effective. Induction of four new proteins biosynthesis along with activation of glutathione-S-transferase

was found in the coelomic fluid of earthworms exposed to polluted silt. Moreover, increasing pool of reduced glutathione was also observed in the coelomic fluid at the end of earthworms' exposition to contaminated substrate. Hence, vermiculture of *E. fetida* can be an effective way of sewage sludge purification in the case of integrated pollution by enhancing the

activity of glutathione-dependent system.

5. Acknowledgements

The present research was conducted under the grants of Ministry of Education and Science of Ukraine (0113U003034 and 0116U001723).

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