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BIOACCUMULATION AND EFFECTS OF Zn AND Mn CONTAINED BY DITHANE M45 ON *EISENIA FOETIDA (OLIGOCHAETA-LUMBRICIDAE)* SPECIES

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Abstract
The study assesses the effects of Zn and Mn contained by Dithane M45 fungicide on Eisenia foetida epigenous species. There were five experimental variants of five repetitions each. Dithane M45 concentrations were: V1:250mgkg^{-1}/dry soil, V2:200mgkg^{-1}/dry soil, V3:150mgkg^{-1}/ dry soil, V4:100mgkg^{-1}/dry soil, with the fifth (V5) as control variant. There were analyzed: 1. survival rate (%) after applying toxic and correlations between survival rate and toxic concentrations; 2. initial biomass (g) and biomass resulting from (g) intoxication; 3. Zn and Mn bioaccumulation (ppm) in earthworms. Conclusions: the survival rate decreased to the greatest concentrations compared to control variant; individual biomass decreased after applying toxic; Mn and Zn bioaccumulation in earthworms increased with increasing concentration of Dithane M45.

Keywords: Dithane M45, Eisenia foetida, survival, biomass, bioaccumulation.

1. INTRODUCTION

Metal contamination of topsoil is usually the result of human activities such as waste disposal, agricultural inputs and industrial emissions (Onianwa et al., 2003).
The side effects of high metal concentrations on the soil functions and soil-dwelling organisms have been widely recognized (Lock & Janssen, 2001; Santorufo et al., 2012). Earthworms play a unique role in the formation and soil fertility (Blouin et al., 2013). They are particularly active in the topsoil and are continuously exposed to metal contamination through direct contact, providing information on contamination of ecosystems (Nachman et al., 2007; Sizmur and Hodson, 2009). Metal contamination is also a serious threat to the soil functions and resistance of ecosystems (De Boer et al., 2011).
Earthworms have a permeable surface for water through the epithelium. Although direct absorption through the intestinal wall cannot be completely excluded, several findings have suggested that penetration of metals occurs predominantly through an absorption flow path, which is connected to the absorption of water from the pores (e.g. porewater assumption) (Saxe et al., 2001; Jager et al., 2003; Van Gestel & Koolhaas, 2004). Vijver et al. (2003) also investigated a method to distinguish different ways of absorbing metals in earthworms and found that the main route is dermal absorption.

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Eisenia fetida is now used as standard species in terrestrial ecotoxicology tests (Langdon et al., 2005; Peijnenburg & Vijver, 2009). It is cultivated for vermicompost or fishing and is rarely found in natural soils. It lives only in rich organic matter soils (e.g. compost and manure) (Edwards & Arancón, 2004). Ecological differences of species have consequences on exposure, accumulation and sensitivity to metals. For example, various features of earthworms such as feeding or habitat (soil depth, soil type) can affect the contact degree with contaminated soil particles and, therefore, can lead to differences in metal exposure.

Zinc occurs naturally in air, water and soil. It is an essential mineral and its deficiency leads to harmful effects on plants (Broadley et al., 2007), animals (Prasad, 2008), and people. After iron, it is the only metal that occurs in all classes of enzymes (Broadley et al., 2007). Although zinc is an essential element, too much zinc can be harmful to organisms (plants and animals). When agricultural soils are polluted with zinc, animals can absorb concentrations that are harmful to their health. Water-soluble, zinc can contaminate the groundwater which, in turn, affects the bodies that inhabit the soil by absorption. Zinc toxicity for terrestrial organisms is dependent on bioavailability, which in turn is determined by various factors such as speciation of zinc and the physical, chemical and biological properties of the soil. Bioavailable fraction of zinc in the soil has been calculated to range from <1% to 10% of the total concentration of zinc.

Among terrestrial invertebrates, earthworms (*Eisenia andrei*) and woodlice (*Porcellio scaber*) showed negative effects on reproduction from total zinc concentrations of 560-1600 mg / kg soil.

Manganese (Mn) is the most abundant metal in natural environments and includes approximately 0.1% of the Earth's crust (Cicad, 2004). It is actively absorbed and used by plants and animals (ATSDR, 2008) being essential in ecosystem functioning. As a constituent of the soil, its concentrations range between 40 and 900 mg/kg^{-1} pure Mn. It does not occur in the environment in a pure form but combined with other elements such as oxygen, sulfur, carbon, silicon and chlorine. These types of Mn are solid and some of them may be dissolved in water or can exist as small particles in the air. Small particles of dust in the air usually lay on the ground, depending on their size, weight, density and weather conditions. Mn exists in soils, both as organic and inorganic forms in several oxidation states, i.e., 0, +2, +3, +4, +6, +7 (Post JE, 1999).

Research has focused on the toxic effects of inorganic compounds containing Mn^{2+}, Mn^{3+}, and Mn^{4+} ions because they are the most prevalent forms in the biological systems (Millaleo et al., 2010).

Most studies have approached Mn bioaccumulation in animals by food ingestion and direct absorption from the soil by skin contact and/or ingestion of soil generally used by earthworms, nematodes and springtails (Hartenstein, 1981; Kuperman, 2004).

The study aims at zinc and manganese effects of Dithane M45 content, on *Eisenia foetida* species. The specific objectives were: 1. analysis of survival rates (%) after applying toxic and correlations between survival rate and toxic concentrations; 2. analysis of initial biomass (g) and biomass (g) after intoxication; 3. analysis of Zn and Mn bioaccumulation (ppm) by earthworms.

2. MATERIALS AND METHODS

Eisenia foetida individuals used in the experimental variants were mature and obtained from a farm in Romania practicing vermiculture.

Test underlayer. Preparation of OECD underlayer (OECD 207/1984, 222/2004) was conducted as follows: 10% peat moss; 20% kaolinite clay; 69% industrial quartz sand with a particle size from 0.05mm up to 0.2mm; 1% calcium carbonate ($CaCO_3$). All amounts were mixed, while plant debris, dirt and possible pebbles were removed. The water content of the underlayer was determined by bringing it to a constant weight while drying it in an oven at 105^0C for 8 hours. After determining humidity, the basic underlayer was filled with 1.5 liters of distilled water in order to bring it to the

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optimal humidity (approx. 25-42% dry weight of the basic underlayer). Calcium carbonate was added to bring the underlayer to a pH of 6.0.

Acclimatization. Experimental samples for acclimatization were prepared before applying toxic. The underlayer (soil) sampled from the maize cultivated area was introduced in 800 g glass jars. There were five experimental variants V1, V2, V3, V4, V5. The 5th was the control variant. There were 5 repetitions for each concentration. Before inoculation, earthworms were immersed into distilled water to remove excess soil on their body surface, then they were placed on filter paper to remove the water. Individual biomass (g) on the analytical balance was also determined. 10 individuals for each variant were introduced in concentrations. After all individuals had entered the soil, samples were covered with previously drilled lids to let the air penetrate and to prevent water evaporation. Then they were brought to the climate chamber for 7 days at a temperature of 20°C and constant humidity.

After acclimatization, the samples were removed to check the soil humidity, the survival rate was analyzed, and they were placed again in the pots with underlayer / soil. The fine powder toxic was applied over the soil surface. The following toxic concentrations were used: V1= 250mgkg^{-1}/dry soil, V2= 200mgkg^{-1} dry soil, V3= 150mgkg^{-1} dry soil, V4=100mgkg^{-1} dry soil, with V5 as the control variant. Concentrations were determined according to specialized studies starting from LC_{50} for lumbricidae. After applying toxic, the samples were placed again in the climate chamber at a temperature of 20°C with constant humidity for 30 days. Earthworms were not fed throughout the test. After 30 days, earthworms were removed from the climate chamber and there were analyzed: survival rate, individual biomass and bioaccumulation of zinc and manganese.

Dithane M45. According to Regulation (EU) no.1907/2006, as amended by Regulation (EU) No. 453/2010 and Regulation (EU) nr.1272/2008, Dithane M45 is contact fungicide containing 80% mancozeb. It is part of the 4th group of toxicity, dithiocarbamate and thiuram derivatives with broad-spectrum action. It is the most used worldwide fungicide approved to combat more than 400 diseases in more than 70 crops. By the multi-site action model (interrupts enzymatic activity in 6 different points), it prevents resistance to pathogen.

Bioaccumulation. After completion of the test it was determined bioaccumulation of Zn and Mn Dithane M45 content by atomic emission spectrometry with inductively coupled plasma (ICP-AES). Variant Liberty 110 spectrometer was used for the quantitative determination of zinc and manganese. The instrument had a 40.68 MHz radio frequency generator and 0.75m Czerny-Turner monochromator. The instrument operating parameters were: plasma flow 12L / min, V-Groove nebulizer, rotation pump 15 rpm, 10 sec integration time and automatic background. The reagents used for the mineralization of the samples were nitric acid (67% -75 ml), Merck hydrogen peroxide (15ml) and distilled water to bring the 10 ml flasks to their volume, after the mineralization of the samples on the sand bath. To calibrate the spectrometer there were used five reference solutions of various concentrations obtained by the dilution of a multi-element standard solution (ICP-AES Etalon multi-element Merck IV solution) with a concentration of 1000mg / l.

Statistical analysis. Statistics of the results was made using SPSS 16 for Windows. For the analyzed parameters Duncan test was applied for analysis of variance, and significance threshold $p<0.05$. It has been drawn the trend line and the coefficient of determination calculated (R Square) to illustrate the correlation between toxic concentrations in the experimental variants and survival rate, the significant correlations between toxic concentrations and the amount of Zn and Mn bioaccumulated by earthworms.

3. RESULTS AND DISCUSSIONS

Effects of Dithane M45 on survival rate.

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Eisenia fetida is the standard test species used in terrestrial ecotoxicology, because it can be easily grown on organic waste and has a short generation cycle (ISO 1993, OECD, 2004).
Tests of the sensitivity of several species of earthworms have revealed that *Eisenia fetida* is relatively less sensitive to chemicals (Owojori end Reinecke, 2009).
Although their tolerance varies from species to species, research has shown a decline in populations of earthworms, in response to large amounts of chemicals. Experiments on *Eisenia foetida* are consistent with results published in the literature on mortality of this species after applying toxic (Abbiramy et al., 2014).
Figure 1 shows the experimental variants and survival rate after applying different concentrations of Dithane M45. The lowest survival rate with significant differences was observed in V1 for a concentration of 250mgKg^{-1} (76%) compared to control variant (V5 = 100%), although decreases in the survival rate were recorded in all experimental variants V2 (200mgKg^{-1}) = 82%; V3(150mgkg^{-1})=88%; V4 (100mgkg^{-1})=98%) compared to control variant (V5).
The average difference was significant for a confidence interval of 0.05. Analysis of the correlation between survival rate and toxic concentrations (Fig. 2) showed a significant decrease in the survival rate of individuals with increased toxic concentration (R^2=0.624; p<0.0001).
Mortality was the most commonly used parameter for evaluating chemical toxicity to earthworms (Van Gestel end Van Dis, 1988, Robidoux et al., 1999). However, research has shown that survival is less sensitive to an ecotoxicological point (Moriarty, 1983) and tests of acute mortality provide risk estimation in 95% of cases (Frampton et al., 2006, Eftekhari et al., 2013).

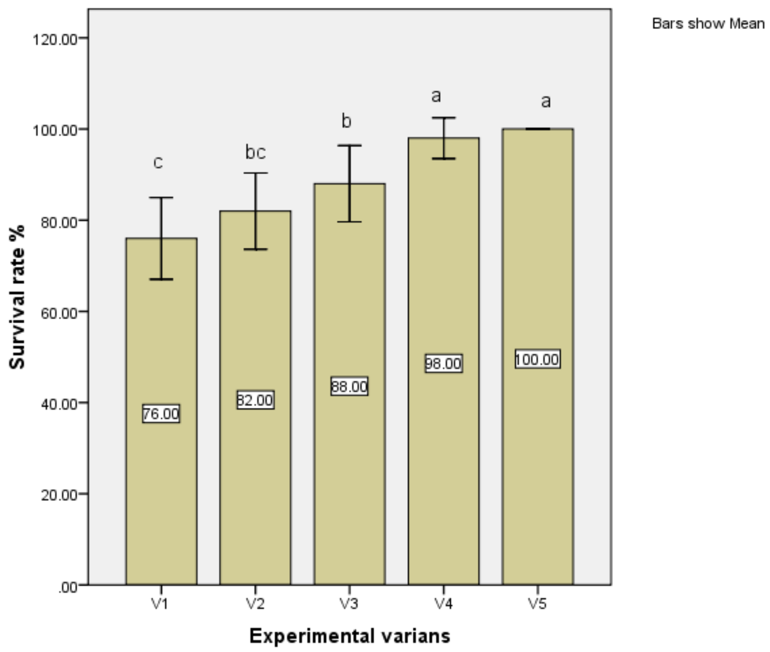

Figure 1. Survival rate (%) of individuals exposed to intoxication with Dithane M45 in the 5 experimental variants V1 (250 mg·Kg^{-1}); V2 (200 mg·Kg^{-1}); V3 (150 mg·Kg^{-1}); V4 (100 mg·Kg^{-1}); V5 (control variant). Bars with the same letters are not significantly different at 5% level, according to Duncan's multiple range test

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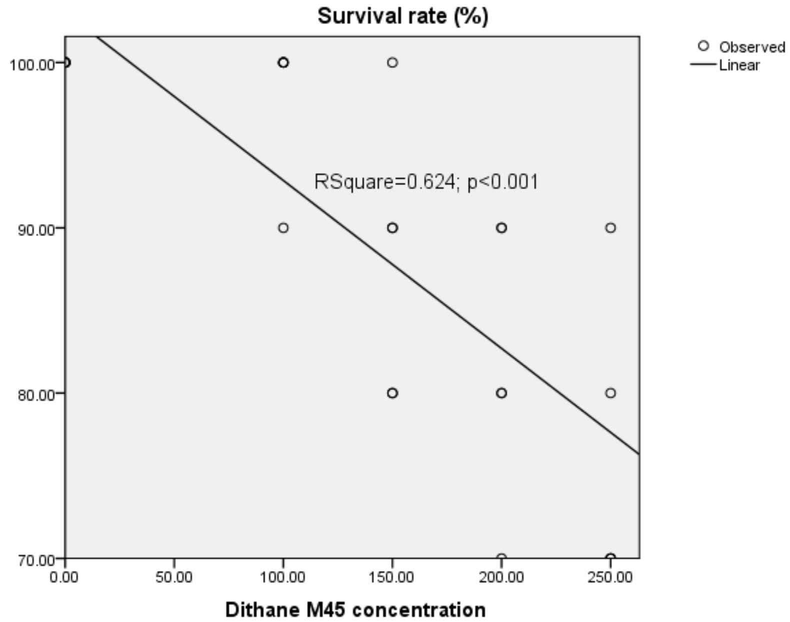

Figure 2. Correlation between survival rate of individuals (%), represented by the trend line in the 5 experimental variants exposed to toxic action (Dithane M45): V1 (250 mg·Kg^{-1}); V2 (200 mg·Kg^{-1}); V3 (150 mg·Kg^{-1}); V4 (100 mg·Kg^{-1}); V5 (control variant).

Biomass. A series of studies on *Eisenia fetida* species highlighted the response of earthworms under the action of pesticides. Figure 3 shows biomass of experimental variants prior to application of toxic. At the end of toxicity test, statistical analysis showed that individual biomass of V1, V2, V3 and V4 has not changed significantly compared to control variant (V5) ($p > 0.05$). Average values of biomass before applying toxic ranged from 3.110g - 2.698g. Biomass determined after intoxication (Figure 4) with Dithane M45 in concentrations corresponding to variants V1 and V2 is significantly lower ($p < 0.05$) compared to control variant (V5). There were no significant decreases in biomass for V3 and V4 compared to control variant. The decrease in biomass was likely due to lack of food during the experiment and the test underlayer used. Decreases in earthworms' biomass after applying fungicides were also observed by Römbke et al. (2004) and Iglesias et al., (2003) in their research.
Other studies have shown that earthworms' response to exposure to pesticides in laboratory conditions could be influenced by the type of underlayer, temperature, light, humidity and the length of exposure (Butt, 2007, Lowe and Butt 2007, Zhang et al . 2013).
Bioaccumulation. Limited mobility of earthworms makes them appropriate for monitoring the potential impact of soil contaminants (Hsu et al., 2006, Suthar et al., 2008).
According to Suthar et al., (2008), earthworms accumulate a considerable content of metals in their tissues. Figures 5, 6 show correlations between concentrations of Dithane M45 in experimental variants and the amount of Mn and Zn (contained by Dithane M45), bioaccumulated by earthworms. In case of Mn bioaccumulation there was a significant positive correlation (R^2=0.540; $p<0.0001$) between concentration of toxic and bioaccumulation compared to control variant (V5). The highest amount of bioaccumulated Mn was recorded in concentrations of 250mgKg^{-1} (10.3480 ppm) and 200 mgKg^{-1} (10.7580 ppm) for V1 and V2.

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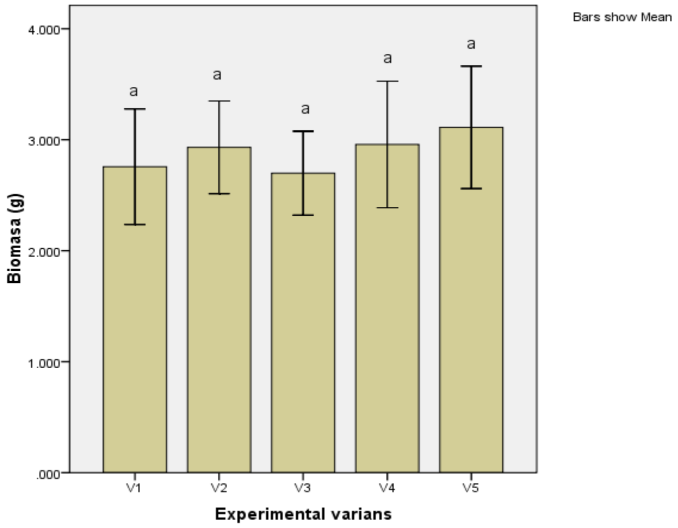

Figure3. Biomass of individuals (g) in the 5 experimental variants before applying toxic (Dithane M45). Bars with the same letters are not significantly different at the 5% level, according to Duncan's multiple range test.

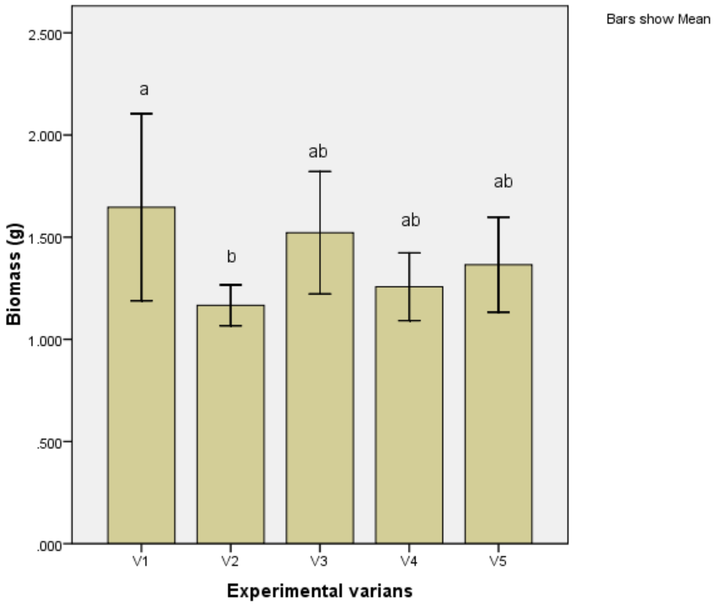

Figure 4. Biomass of individuals (g) in the 5 experimental variants after applying toxic (Dithane M45). Bars with the same letters are not significantly different at the 5% level, according to Duncan's multiple range test.

Suther (2007) stated that earthworms accumulate metals in their tissues by linking with some metalloproteins called metallothioneins. Generally, earthworms need to consume a large amount of soil whereas the digestive process releases heavy metals in their free forms in the lumen. Metals are then absorbed by the intestinal epithelial mucosa. In this way, earthworms accumulate heavy metals in cells of the digestive canal (Suther, 2008).

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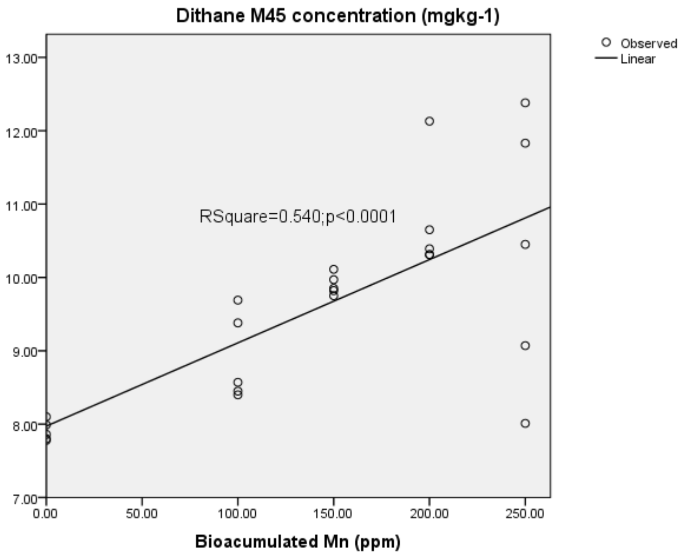

Figure 5. Trend line of Mn amount (ppm) bioaccumulated by earthworms depending on Dithane M45 concentration (V1 =250 mg·Kg^{-1}; V2 =200mg·Kg^{-1}; V3 =150 mg·Kg^{-1}; V= 100mg·Kg^{-1}; V5 = control variant).

As regards the amount of Zn (fig.6), there was a significant positive correlation between the concentration of toxic and the amount of Zn bioaccumulated by earthworms (R^2=0.937; p<0.0001). For a concentration of 250mg·Kg^{-1} (V1), the amount of Zn bioaccumulated by earthworms was 238.9080 ppm and 193.7140 ppm for the concentration of 200mg·Kg^{-1}(V2). The lowest amounts of bio-accumulated Zn were recorded in the experimental variants V3 (163.8800 ppm) and V4 (120.6320 ppm). Results are in agreement with Enuneku, AA & Ayobahan, S.U., (2014) who stated that the highest bioaccumulation of metals in earthworms produced in the highest concentration of toxic.

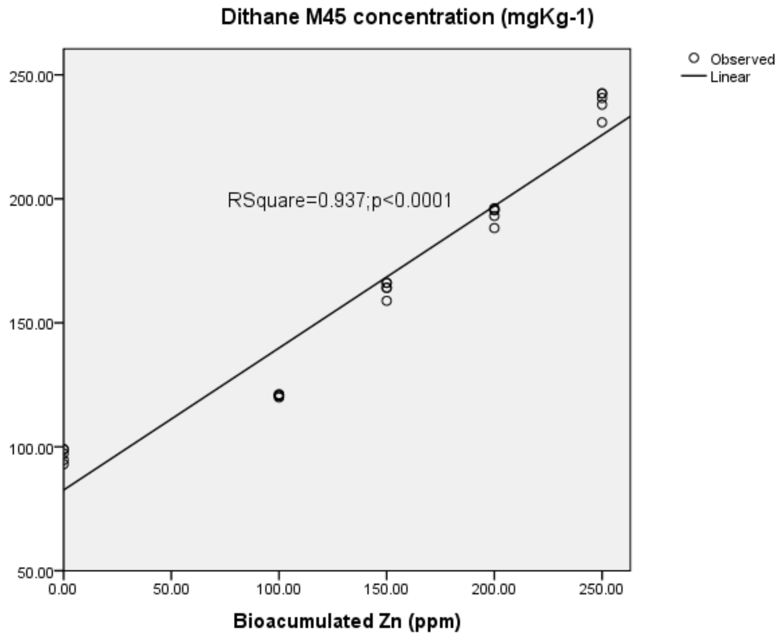

Figure 6. Trend line of Zn amount (ppm) bioaccumulated by earthworms depending on Dithane M45 concentration (V1=250mg·Kg^{-1}; V2 = 200mg·Kg^{-1}; V3=150mg·Kg^{-1}; V=100mg·Kg^{-1}; V5=control variant).

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4. CONCLUSIONS

Results of the present study show that survival rate decreased in higher concentrations of toxic (250mg·Kg^{-1}/dry soil, 200mg·Kg^{-1} /dry soil). Individual biomass after applying toxic was followed by a significant decrease in experimental variants. Bioaccumulation of metals in earthworms increases with increasing concentration of toxic.

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6. REFERENCES

Abbiramy, K.S., Ronald, R.P., Paramanandham, J. (2014). Validation of tropical artificial soil by chronic toxicity studies on Eisenia fetida against superphosphate, *International Letters of Natural Sciences*, 8(1), 31-40

ATSDR, (2008). Draft toxicological profile for manganese. Agency for toxic substances and disease registry. Division of toxicology and environmental medicine/applied toxicology branch, Atlanta, Georgia. 151p.

Blouin M, Hodson, M.E., Delgado, E.A, Baker, G., Brussaard, L., Butt, K.R., Dai, J., Dendooven, L., Peres, G., Tondoh, J.E., Cluzeau, D., Brun, J.J. (2013). A review of earthworm impact on soil function and ecosystem services, *Eur J Soil Sci 64*, 161-182.

Broadley M.R., White P,J., Hammond J.P., Zelko, I., Lux, A. (2007). Zinc in plants. *Journal compilation © New Phytologist*, *173*, 677–702

Cicad (2004). Manganese and its compounds: environmental aspects. Concise international chemical assessment document 63. WHO, Geneva, Switzerland. Retrieved august 25, 2013 from http://www.who.int/ipcs/publications/cicad/cicad63_rev_1.pdf.

De Boer, T.E., Taş, N., Braster, M., Temminghoff, E.J.M., Röling, W.F.M., Roelofs, D. (2011). The influence of long-term copper contaminated agricultural soil at different pH levels on microbial communities and springtail transcriptional regulation, *Environ Sci Technol 46*, 60-68.

Edwards, C.A., Arancon, N.Q. (2004). The use of earthworms in the breakdown of organic wastes to produce vermicomposts and animal feed protein. In: Edwards CA. (ed) Earthworm Ecology, 2nd edn. CRC Press LLC, Florida, pp 345-379.

Eftekhari, A., Valadabadi, A. , Shiranirad, A., Samdaliri, M., Mobasser, H., Daneshian, J. (2013). Role of earthworm on bioremeiation of heavy metals (lead and cadmium) in two varieties of maize (Zea MaysL.), *Annals of Biological Research*, *4(8)*, 42-45.

Enuneku, A.A., Ayobahan, S.U.(2014). Sublethal Toxic effects of spent Oil Based Drilling Mud and Cuttings to Earthworm Aporrectodea Longa. *J. Appl. Sci. Environ. Manage*, 18(4), 615-620.

Frampton, G.K., Jansch, S., J. J., Scott-Fordsmand, Rombke, J., & Van den Brink P.J. (2006). Effects of pesticides on soil invertebrates in laboratory studies: a review and analysis using species sensitivity distributions, *Environmental Toxicology and Chemistry*, 25(9), 2480–2489.

Hartenstein, R, Neuhauser, E.F., Narahara, A. (1981). Effects of heavy metal and other elemental additives to activated sludge on growth of Eisenia foetida, *J Environ Qual 10*, 372–376

Iglesias, J, Castillejo, J, Castro, R. (2003). The effects of repeated applications of the molluscicide metaldehyde and the biocontrol nematode, Phasmarhabditis hermaphrodita on molluscs, earthworms, nematodes, acarids and collembolans: a two-year study in northwest Spain. *Pest Manag Sci 59*, 1217–1224.

Jager, T., Fleuren R.H.L.J., Hogendoorn, E.A., De Korte, G. (2003). Elucidating the routes of exposure for organic chemicals in the earthworm, Eisenia andrei (Oligochaeta). *Environ Sci Technol 37*, 3399-3404.

Kuperman, R.G., Checkai, R.T., Simini, M., Phillips, C.T. (2004). Manganese toxicity in soil for Eisenia fetida, Enchytraeus crypticus (Oligochaeta), and Folsomia candida (Collembola). *Ecotoxicol Environ Saf 57*, 48–53.

Langdon, C.J., Hodson, M.E., Arnold, R.E., Black, S.(2005). Survival, Pb-uptake and behaviour of three species of earthworm in Pb treated soils determined using an OECD-style toxicity test and a soil avoidance test. *Environ Pollut 138*, 368-375.

Lock, K., Janssen, C.R. (2001). Zinc and cadmium body burdens in terrestrial Oligochaetes: Use and significance in environmental risk assessment. *Environ Toxicol Chem 20*, 2067-2072.

Millaleo, R, Reyes-Diaz, M., Ivanov, A.G., Mora, M.L., Alberdi, M. (2010). Manganese as essential and toxic element for plants: transport, accumulation and resistance mechanisms. *J Soil Sci Plant Nutr 10*, 470–481.

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Current Trends in Natural Sciences (CD-Rom)
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ISSN-L: 2284-9521

Moriarty, F. (1983). Ecotoxicology: The Study of Pollutants in Ecosystems, Academic Press, London, UK.

Nahmani, J., Hodson, M.E., Black, S. (2007). A review of studies performed to assess metal uptake by earthworms. *Environ Pollut 145*, 402-424.

OECD (2004). Guideline for testing of chemicals, no. 222, Earthworm Reproduction Test (Eisenia fetida/andrei). Organization for Economic Co-Operation and Development, Paris, France.

Onianwa, P.C., Jaiyeola, O.M., Egekenze, R.N. (2003). Heavy metals contamination of topsoil in the vicinities of auto-repair workshops, gas stations and motor-parks in a Nigerian city. *Toxicol Environ Chem 84*, 33-39.

Owojori, O.J., Reinecke, A.J. (2009). Avoidance behaviour of two eco-physiologically different earthworms (Eisenia fetida and Aporectodea caliginosa) in natural and artificial saline soil, *Chemosphere, 75*, 279-283.

Peijnenburg, W.J.G.M., Vijver, M.G. (2009). Earthworm and their use in eco(toxico)logical modelling. In: Devillers J.(ed.) Ecotoxicology Modelling. Springer, New York, pp 177- 204.

Post, J.E. (1999). Manganese oxide minerals: Crystal structures and economic and environmental significance. *Proc Natl Acad Sci USA 96*, 3447–3454.

Prasad, A.S., Frances W.J., Beck,B.B., Snell D., Fitzgerald J.T. (2008). Duration and Severity of Symptoms and Levels of Plasma Interleukin-1 Receptor Antagonist, Soluble Tumor Necrosis Factor Receptor, and Adhesion Molecules in Patients with Common Cold Treated with Zinc Acetate, *The Journal of Infectious Diseases*, 197, 795– 802

Robidoux, P.Y., Hawari, J.S., Thiboutot, G. A., & Sunahara, G. I. (1999). Acute toxicity of 2,4,6-trinitrotoluene in earthworm (Eisenia andrei), *Ecotoxicology and Environmental Safety, 44, 3*, 311–321.

Römbke, J., Van Gestel, C.A.M., Jones, S.E., Koolhaas, J.E., Rodrigues, J.M.L., Moser, T. (2004). Ring-testing and field-validation of a terrestrial model ecosystem (TME)—an instrument for testing potentiallyharmful substances: effects of carbendazim on earthworms. *Ecotox, 13*, 105–118.

Santorufo, L, Van Gestel C.A.M., Maisto, G. (2012). Ecotoxicological assessment of metalpolluted urban soils using bioassays with three soil invertebrates. *Chemosphere, 88,* 418- 425.

Saxe, J.K., Impellitteri, C.A., Peijnenburg, W.J.G.M., Allen, H.E. (2001). Novel model describing trace metal concentrations in the earthworm, Eisenia andrei. *Environ Sci Technol 35,* 4522- 4529.

Sizmur, T., Hodson, M.E. (2009). Do earthworms impact metal mobility and availability in soil? A review. *Environ Pollut, 157*, 1981-1989.

Suhane, R.K. (2007) .Vermicompost (In Hindi*);* Pub. Of Rajendra Agriculture University, Pusa, Bihar; pp: 88

Suthar, S., Singh, S., Dhawan, S. (2008). Earthworm as bioindicators of metals (Zn, Fe, Mn, Cu, Pb and Cd) in soils: Is metal bioaccumulation affected by their ecological categories, *Ecol Eng* 32, 99–107.

Van Gestel, C.A.M., Van Dis, W.A. (1988), The influence of soil characteristics on the toxicity of four chemicals to the earthworm Eisenia fetida andrei (Oligochaeta), *Biology and Fertility of Soils, 6, 3,* 262–265.

Van Gestel, C.A.M., Koolhaas, J.E. (2004). Water-extractability, free ion activity, and pH explain cadmium sorption and toxicity to Folsomia candida (Collembola) in seven soil-pH combinations. *Environ Toxicol Chem, 23,* 1822-1833.

Vijver, M.G., Vink, J.P.M., Miermans, C.J.H., Van Gestel, C.A.M. (2003). Oral sealing using glue: a new method to distinguish between intestinal and dermal uptake of metals in earthworms. *Soil Biol Biochem, 35,* 125-132.

***ISO (1993). Soil quality—effects of pollutants on earthworms (Eisenia fetida)—part 1: determination of acute toxicity using artificial soil substrate, ISO 11268-1, International Organization for Standardization, Geneva, Switzerland.

***Regulamentul (CE) nr. 1272/2008 al Parlamentului European şi al consiliului din 16 decembrie 2008 privind clasificarea, etichetarea şi ambalarea substanțelor şi a amestecurilor, de modificare şi de abrogare a Directivelor 67/548/CEE şi 1999/45/CE, precum şi de modificare a Regulamentului (CE) nr. 1907/2006

***Regulamentul (CE) nr. 1907/2006 al Parlamentului European şi al Consiliului din 18 decembrie 2006, privind înregistrarea, evaluarea, autorizarea şi restricționarea substanțelor chimice (REACH), de înființare a Agenției Europene pentru Produse Chimice, de modificare a Directivei 1999/45/CE şi de abrogare a Regulamentului (CEE) nr. 793/93 al Consiliului şi a Regulamentului (CE) nr. 1488/94 al Comisiei, precum şi a Directivei 76/769/CEE a Consiliului şi a directivelor 91/155/CEE, 93/67/CEE, 93/105/CE şi 2000/21/CE ale Comisiei.