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BIOACCUMULATION AND EFFECTS OF Zn, Mn AND DITHANE M45 ON CERTAIN LUMBRICIDAE SPECIES (OLIGOCHAETA-LUMBRICIDAE)

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Abstract. The importance of earthworms in the ecosystem functioning has led to many studies on the impact of metals on them. This study evaluates the effects of Zn, Mn and Dithane M45 fungicide on different species of earthworms sampled from maize cultivated soils. There were approached: 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. bioaccumulation (ppm) of Zn and Mn by earthworms. There were 5 experimental variants of 5 repetitions each. The concentrations of Zn and Mn were 250 mgkg^{-1}/dry soil (V1), 200 mgkg^{-1}/ dry soil (V2), 150 mgkg^{-1}/ dry soil (V3), 100 mgkg^{-1}/ dry soil (V4), with the fifth as control variant (V5). Statistics has revealed that survival rate for the highest concentration of Zn and Mn corresponding to V1, V2 and V3 concentrations, decreased significantly compared to V5. Initial biomass for the 5 experimental variants ranged between 2.84 g - 4.23 g and after exposure to toxic concentrations corresponding to V2, V3 and V4 variants it was significantly lower ($p < 0.05$) compared to control variant (V5). There was a significant positive correlation (Zn=R^2=0.793; $p<0.0001$), (Mn=R^2=0.971; $p<0.0001$) between Dithane M45 concentration used in the experimental variants and quantity of Zn and Mn bioaccumulated by earthworms. The study suggests that increasing the concentration of Zn and Mn in the soil can be toxic to lumbricidae.

Keywords: Dithane M45, earthworms, survival, biomass, bioaccumulation.

Rezumat. Bioacumularea și efectele Zn, Mn conținut de Dithane M45 asupra unor specii de lumbricide (Oligochaeta-Lumbricidae). Importanța râmelor în funcționarea ecosistemelor a dus la multe studii privind impactul metalelor asupra râmelor. Acest studiu evaluează efectele Zn şi Mn conţinut de fungicidul Dithane M45, pe diferite specii de râme prelevate dintr-un teren cultivat cu porumb. S-a analizat: 1. analiza ratei de supravieţuire (%) după aplicarea toxicului şi corelaţiile ce se stabilesc între rata de supravieţuire şi concentraţiile de toxic; 2. analiza biomasei iniţiale (g) şi biomasei rezultate (g) după intoxicare; 3. analiza bioacumulării (ppm) cantităţii de Zn şi Mn de către râme. S-a lucrat cu 5 variante experimentale a câte 5 repetiţii. Concentraţiile de Zn şi Mn au fost de 250 mgkg^{-1}/sol uscat (V1), 200 mgkg^{-1}/ sol uscat (V2), 150 mgkg^{-1}/ sol uscat (V3), 100 mgkg^{-1}/sol uscat (V4), a 5-a variantă reprezentând varianta martor (V5). Analiza statistică a datelor privind rata de supravieţuire a scos în evidenţă că, la concentraţia cea mai mare de Zn şi Mn corespunzătoare concentraţiilor din V1,V2 şi V3, se produce o scădere semnificativă a ratei de supravieţuire comparativ cu V5. Biomasa iniţială în cele 5 variante experimentale a fost cuprinsă între 2,84 g – 4,23 g, iar după expunerea la toxic în concentraţiile corespunzătoare variantelor V2, V3 şi V4 a fost semnificativ mai mică ($p<0,05$) faţă de varianta martor (V5). Între concentraţia de Dithane M45 folosită în variantele experimentale şi cantitatea de Zn şi Mn bioacumulat de către râme s-a stabilit o corelaţie pozitivă semnificativă (Zn=R^2=0,793; $p<0,0001$), (Mn=R^2=0,971; $p<0,0001$). Studiul sugerează că o creştere a concentraţiei de Zn şi Mn în sol, poate fi toxic pentru lumbricide.

Cuvinte cheie: Dithane M45, râme, supravieţuire, biomasă, bioacumulare.

INTRODUCTION

Soil is an important natural resource providing habitat and nutrients for plants, animals, soil organisms and humans. However, human activities, industry and the use of synthetic products (pesticides, industrial waste) can lead to urban and agricultural soil contamination with metals (McGRATH et al., 2001). Most pesticides are organic compounds, some are inorganic or mineral compounds, and other pesticides contain Hg, Ca, Cu, Zn or heavy metals (LAW et al., 1998).

Metals identified in the polluted environment include Cu, Cd, Pb, Cr, Ni, Mn, Hg and Zn. These metals come mainly from endogenous sources and human activities (NRIAGU & PACYNA, 1988), leading to excessive metal in the soil. Among these metals, Zn is an essential mineral for health. Zn deficiency can harm plants (BROADLEY et al., 2007), animals (PRASAD, 2008), even humans. After iron (Fe), it is the only metal present in all classes of enzymes (BROADLEY et al., 2007). Although zinc is essential for health, it can be harmful when excessive. Soils contaminated with Zn may contain up to several grams of Zn / kg of dry soil. MA (1982) found that the level of zinc in *Lumbricus rubellus* species was generally associated with zinc concentrations in the soil and correlated with zinc concentration in soils with low pH. At lower pH, the soil absorbs less zinc, making it bioavailable to earthworms. LC_{50}= 80 mg Zn kg^{-1} dry soil was reported for *Eisenia foetida* species (SHEPPARD et al., 1993).

Manganese (Mn) is the most abundant metal in the natural environment and an essential microelement for all living systems. This metal is an essential cofactor to many classes of enzymes, such as oxidoreductases, transferases, ligases, hydrolases (LAW, 1998). However, soil enrichment with manganese resulting from different activities endangers terrestrial ecosystems (BORDEAN et al., 2014). Several studies have shown the harmful effects of exposure to manganese by skin touch and / or by ingesting soil for a wide range of soil invertebrates. Manganese exists in the soil in a number of oxidation states, i.e., 0, + 2, + 3, + 4, 6, 7 (POST, 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 common forms in biological systems (MILLALEO et al., 2010).

Earthworms can colonize contaminated soils if climatic conditions, organic matter, soil texture and pH are appropriate, so that, when organic matter is included in the contaminated soils, earthworms are likely to colonize, resulting in changes in the soil chemical, biological and physical properties.

It was studied the impact of earthworms in terms of mobility and accessibility of metals and it was shown that earthworms increase the mobility of metals in soil (MA, 1982). Recent experiments have identified that this may be due to the impact of earthworms on organic matter degradation and subsequent release of organically bound elements that dissolve organic acids, lower soil pH and lead to subsequent mobilization of potentially toxic elements (POST, 1999).

Bioaccumulation of metals by earthworms was associated with metal concentrations in soil (SANTORUFO et al., 2012). Earthworms are widely used in ecotoxicological tests because they contribute to the decomposition of litter and recycling of organic matter. Since they feed directly with decaying matter and fungi in soil, they provide faster clues about changes in soils than other animals (COLE et al., 2001; DIDDEN & RÖMBKE, 2001). In addition, they are suitable for ecotoxicity testing because they are easy to obtain in crops, have relatively short life cycles and are cost effective (FOUNTAIN & HOPKIN, 2005; LOWE & BUTT, 2007).

The overall objective of this study was to evaluate the effects of zinc and manganese in Dithane M45, on various species of earthworms sampled from maize cultivated soils.

The specific objectives pursued: 1. survival rate (%) after applying toxic and correlations between survival and toxic concentrations; 2. initial biomass (g) and biomass resulting from (g) intoxication; 3. bioaccumulation (ppm) of Zn and Mn by earthworms.

MATERIAL AND METHODS

Collection and preservation of lumbricidae. Lumbricidae were sampled from maize cultivated soil, Dobrești, Furești village, Argeş County, Cândeşti Piedmont, below the mixed deciduous forests. The geographical coordinates are: (44^0 $56^{'}$ 06"N; 25^0 06' 28"E). Pedogenetic conditions described a glacis relief on the right side of Cârcinov River, slightly inclined surface (10°), eastern exhibition and 327 m altitude. Groundwater, 5-10m in depth, had an overall good drainage. Following the soil profile there were established five pedogenetic horizons (Apcol, AC, C_1, C_3, C_4). The soil formula was *aluviosol coluvic- eutric on coluvo-proluvial deposits, loamy / sandy loam. Lumbricidae* have been sampled manually, (RAW, 1960), randomly on soil levels 30cm in depth, 25/25 cm control sample. To identify species, earthworms were taken to the laboratory. They were measured up to species level using the stereomicroscope, the Identification Manual *Lumbricidae in Romania* (POP, 1949) and *A guide to the valid names of Lumbricidae* (*Oligochaeta)* (EASTON, 1983). Individual biomass of lumbricidae was determined by the analytical balance (g).

Preparation of the testing underlayer. The testing underlayer was prepared according to OECD 207/1984; OECD (2004). Soil in the maize cultivated area was used as basic underlayer to test earthworms. The underlayer / soil was air-dried, cleaned of gravel and large debris after sampling. Water content was determined by oven-drying at 105°C for 8 hours. After determination of humidity the testing underlayer was filled with 1 litre of distilled water. The soil pH = 6.0 was also measured.

Acclimatization. Experimental samples for acclimatization were prepared before applying the 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 fifth 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 analysed, and they were placed again in the pots with underlayer / soil. The fine toxic powder 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 the 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 analysed: survival rate, individual biomass and bioaccumulation of zinc and manganese.

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 1.000 mg

/ l. Dithane M45. According to Regulation (EU) no. 1907/2006, as amended by Regulation (EU) No. 453/2010 and Regulation (EU) no. 1272/2008, Dithane M45 is contact fungicide containing 80% mancozeb. It is part of the fourth 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.

Statistical analysis. Statistics of the results was made using SPSS 16 for Windows. For the analysed 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 (RSquare) 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.

RESULTS AND DISCUSSIONS

Species identified and used in the toxic test were: *Octodrilus complanatus* (Dugès 1828), *Dendrobaena octaedra* (Savigny 1826), *Octolasion lacteum (*Őrley 1885), *Lumbricus rubellus* (Hoffmeister 1843), *Dendrodrilus rubidus rubidus (*Savigny 1826), *L. castaneus* (Savigny 1826), *L. terrestris* (Savigny 1826). These belong to the three ecological groups (epigeic, endogeic, anecic), each of them with an important role in the soil level they populate.

Epigeic species live in litter, consume considerable amounts of crude organic matter and have a wide range of enzymatic capacities mainly from ingested microflora (CURRY & SCHMIDT, 2007). *Endogeic* species live in the soil. They feed mainly on soil organic matter and dead roots. Living roots are rarely eaten by endogeic earthworms (LAVELLE, 1983). *Anecic* species feed on plant debris, but live in underground galleries. The behaviour of these species may vary depending on environmental conditions (EDWARDS & BOHLEN, 1996).

Survival. After the acclimatization period, the survival rate of samples was 100% in all experimental variants. For the survival rate of individuals and body biomass change there are commonly used tests to determine the impact of hazardous substances on lumbricidae. Survival analysis is needed to calculate LC_{50} i.e. to know if concentration of pollutants causes mortality to 50% of individuals in the population exposed. This is characteristic of a species substance with a determined exposure time.

Figure 1 shows the values of survival rate in experimental variants after applying the toxic (Dithane M45). Survival values were 100% in control variant (V5) and V4 (100mgkg^{-1}), so there were no significant differences. Therefore, the lowest toxic concentration (100 mgkg^{-1}) did not produce a significant change in the survival rate (p = 1). The survival rate in concentrations of 250 mgKg^{-1} (V1), 200 mgKg^{-1} (V2) and 150 mgKg^{-1} (V3) showed a significant decrease, with average values ranging from 68% (V1) -78% (V3) compared to control variant (V5-100%) (Table 1). Increasing concentration of Dithane M45 in V1 (250 mgKg^{-1}) caused a significant decrease in the survival percentage compared to V4, and V5. Significant regressions between metal concentrations and survival rate were obtained by NAHMANI et al. (2007).

The correlation between survival rate of the 5 experimental variants and toxic concentrations (Fig. 2) indicated a significant decrease in the survival rate of individuals with increased toxic concentration (R^2=(0.646; p<0.0001). However, in their findings, ADEOLA & HASSAN (2013) stated that growth and development of earthworms exposed to contamination with various metals were more sensitive than survival.

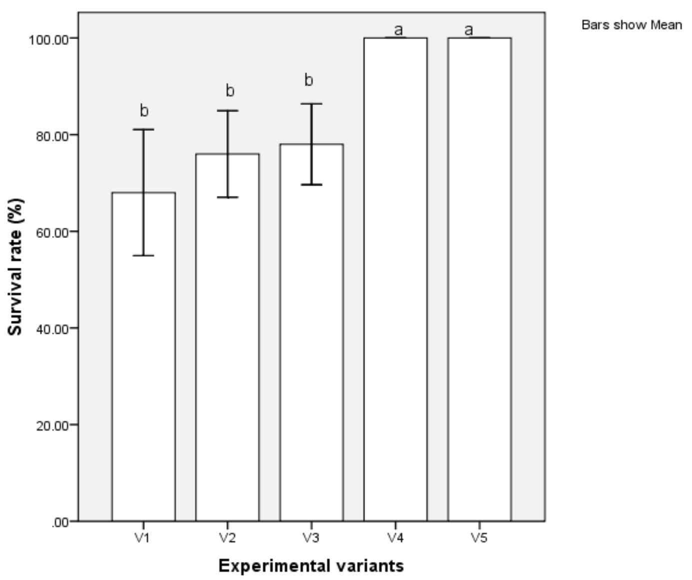

Figure 1. Survival rate (%) of individuals exposed to intoxication with Dithane M45 in the 5 experimental variants V1 (250 mg·Kg^{-1}); V2 (200 mg·Kg^{-1}); V3 (150 mg·Kg^{-1}); V4 (100 mg·Kg^{-1}); V5 (control variant).
Bars with the same letters are not significantly different at 5% level, according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

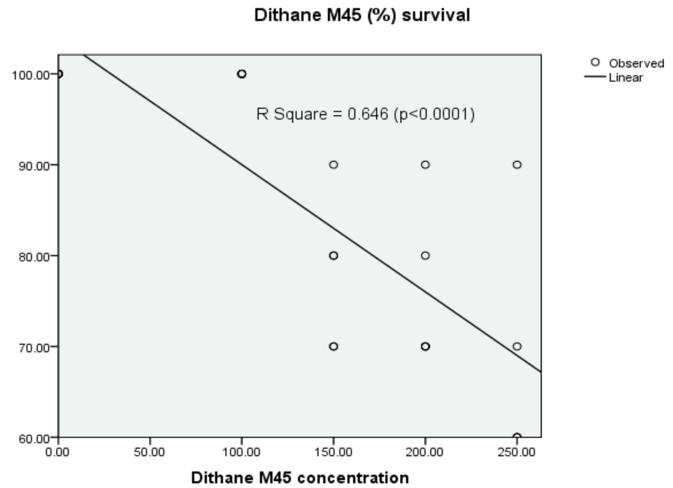

Figure 2. Correlation between survival rate of individuals (%), represented by the trend line in the 5 experimental variants exposed to toxic action (Dithane M45): V1 (250 mg·Kg^{-1}); V2 (200 mg·Kg^{-1}); V3 (150 mg·Kg^{-1}); V4 (100 mg·Kg^{-1}); V5 (control variant).

Biomass. Biomass change is the difference between the initial and final weight of earthworms after exposure to contaminants. Biomass change is more sensitive than ecological survival (MABOETA et al., 2004; PANDARD et al., 2006; SANTORUFO et al., 2012).

Average values of initial biomass (Fig. 3) (before applying the toxic) in the experimental variants did not differ significantly for variants V2, V3, V4 and V5 ($p > 0.05$). V1 biomass was significantly higher (4.23 g) than all the other variants ($p < 0.05$) (Table 1). Average values of initial biomass in the 5 experimental variants were between 2.84g-4.23g (Table 1). Biomass determined after intoxication (Fig. 4) with Dithane M45, in the concentrations corresponding to variants V2, V3 and V4 was significantly lower ($p < 0.05$) compared to control variant (V5).

V1 biomass values (2.00g) were close to control variant (2.18g). Weight loss in earthworms intoxicated with Dithane M45 did not exceed 20%. This decrease in biomass may have been due to the fact earthworms were not fed during the test. This hypothesis corresponds to NAHMANI et al. (2007), who in their studies observed that individual biomass decreased when no food was provided. HELLING et al. (2000) reported that earthworms receiving food once a week during the toxicity test showed no weight loss in experimental variants.

SPURGEON et al. (2003) suggest that weight loss should be less than 15%. Also, GONZALO et al. (2013) in their studies, noted that weight loss of earthworms intoxicated did not exceed 20%.

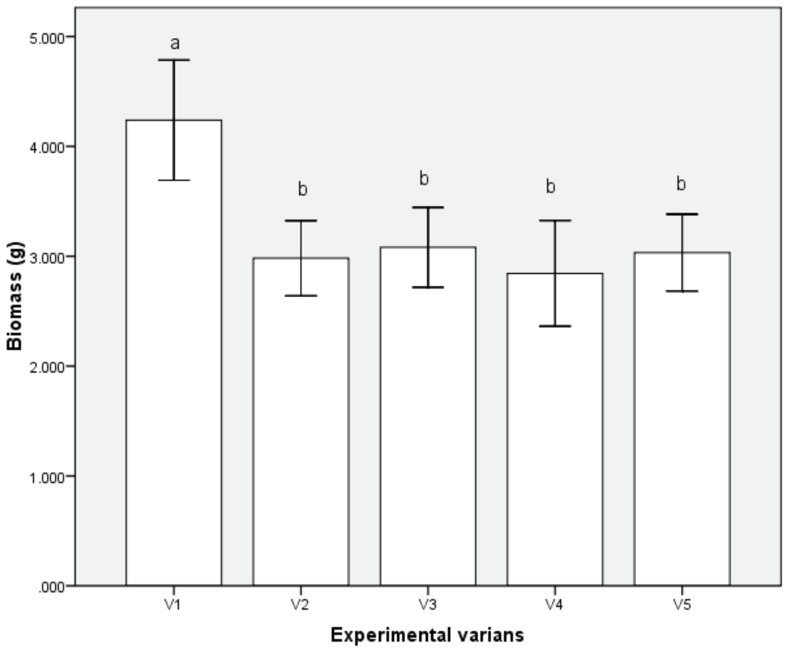

Figure 3. Biomass of individuals (g) in the 5 experimental variants before applying the toxic (Dithane M45).
Bars with the same letters are not significantly different at the 5% level, according to Duncan's multiple range test.

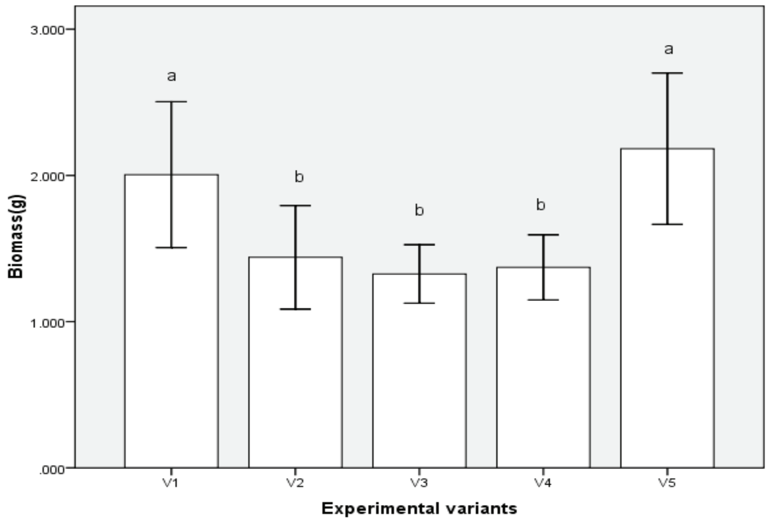

Figure 4. Biomass of individuals (g) in the 5 experimental variants after applying the toxic (Dithane M45).
Bars with the same letters are not significantly different at the 5% level, according to Duncan's multiple range test.

Bioaccumulation. There was a significant positive correlation (R^2= 0.793; p<0.0001) between concentration of Dithane M45 used in experimental variants and Zn amount bioaccumulated by earthworms (Fig. 5). A significant positive correlation was also recorded for Mn bioaccumulation (R^2=0.971; p<0.0001) (Fig. 6). In general, earthworms consume a large amount of soil to get their food during the digestive process that releases heavy metals in their free forms in the intestinal lumen (SUTHAR et al., 2008). Metals are then absorbed by intestinal mucosa. Thus, earthworms accumulate significant amounts of heavy metals in cells of the digestive canal (SUTHAR & SINGH, 2008).

Exposure to high levels of manganese, particularly in powder form leads to side effects (WALDBOH, 1978). Interference with iron metabolism especially haemoglobin formation was one of the first toxic effects of manganese (OTHMER, 1978). The largest amount of bioaccumulated Zn was observed in variant V1 = 1.333.47ppm, and the lowest in variant V4 = 408.626ppm (Table 1). The same was observed for Mn bioaccumulation (V1 = 81.904ppm V4 39.598ppm), which showed that the highest concentration of Dithane M45 (250mgkg^{-1}) caused higher bioaccumulation in the tissues of earthworms.

Our results are in agreement with ENUNEKU & AYOBAHAN (2014) according to whom the highest metal bioaccumulation in earthworms produced the highest concentration of the toxic. Similarly, the results of this study are in agreement with HEIKENS et al. (2001) who studied the bioaccumulation of heavy metals in terrestrial invertebrates. Bioaccumulation was also observed in control variants, Zn (V5 122.114ppm) and Mn (V5 14.854ppm) leading to the idea that there were already traces of these elements in the underlayer used in the toxicity test. Several studies have demonstrated that the adsorption of cationic metals like Cu, Ni, and Zn is influenced by soil pH, CEC, $CaCO_3$, iron (Fe), manganese oxides (Mn), clay and OM content MELLIS et al. (2004), ADHAMI et al. (2008). This is in agreement with HODGE et al. (2000), REINECKE et al. (2002) who stated that earthworms can sometimes tolerate certain chemicals.

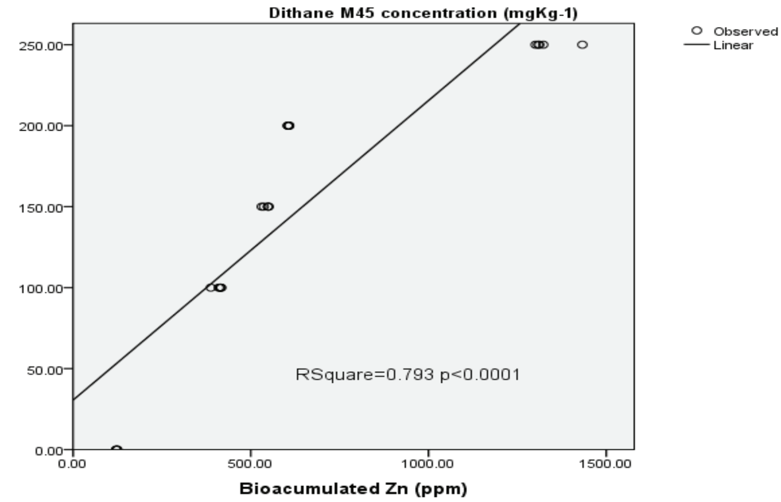

Figure 5. Trend line of Zn amount (ppm) bioaccumulated by earthworms depending on Dithane M45 concentration (V1 =250 mg·Kg^{-1}; V2 = 200 mg·Kg^{-1}; V3 = 150 mg·Kg^{-1}; V= 100mg·Kg^{-1}; V5 = control variant).

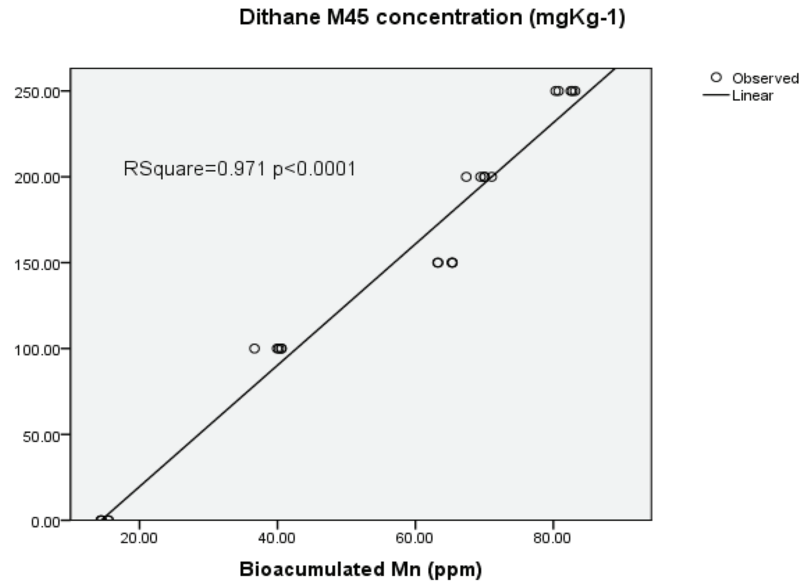

Figure 6. Trend line of Mn amount (ppm) bioaccumulated by earthworms depending on Dithane M45 concentration (V1 =250 mg·Kg^{-1}; V2 = 200 mg·Kg^{-1}; V3 = 150 mg·Kg^{-1}; V= 100mg·Kg^{-1}; V5 = control variant).

Table 1. Result of chronic toxicity bioassays (OECD 207).

Variant (V1...5)	**Survival**	**Mn bioaccumulated in earthworms**	**Zn bioaccumulated in earthworms**	**Initial biomass**	**Biomass after intoxication**
	(%)	(ppm)	(ppm)	(g)	(g)
V1	68±1.3^b	81.904±1.2^a	1333.47±55.1^a	4.23±0.5^a	2.00±0.4^a
V2	76±8.9^b	69.588±1.3^b	605.97±1.9^b	2.98±0.3^b	1.44±0.3^b
V3	78±8.3^b	64.504±1.1^c	542.749±8.7^c	3.08±0.3^b	1.32±0.1^b
V4	100±0.0^a	39.598±1.6^d	408.626±12.1^d	2.84±0.4^b	1.37±0.2^b
V5	100±0.0^a	14.854±0.5^e	122.114±1.7^e	3.03±0.3^b	2.18±0.5^a

Notes: The values are mean of five replicates ± standard deviations. Values with different superscripts within the some column show significant differences (p<0.05).

CONCLUSIONS

Statistics regarding survival rate revealed that the highest Zn and Mn concentrations corresponding to V1, V2, V3, caused a significant decrease in survival rate compared to V5. Initial biomass in the 5 experimental variants ranged between 2.84g - 4.23g and after exposure to toxic substance, in concentrations corresponding to variants V2, V3 and V4 it was significantly lower (p <0.05) compared to control variant (V5). There was a significantly positive correlation (Zn=R^2= 0.793; p<0.0001), (Mn=R^2=0.971; p<0.0001) between Dithane M45 concentration in the experimental variants and Zn and Mn amounts bioaccumulated by earthworms (R2 = 0.793 Zn; p <0.0001) (Mn = R2 = 0.971; p <0.0001). The study suggests that increasing the concentration of Zn and Mn in the soil can be toxic to lumbricidae.

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***. Regulamentul (CE) nr. 1907/2006 al Parlamentului European şi al Consiliului din 18 decembrie 2006, privind înregistrarea, evaluarea, autorizarea şi restricţionarea substanţelor chimice (REACH), de înfiinţare a Agenţiei 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.

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