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Synergistic Effect of Lead and Nickel on Metallothinein Response in Epigeic Earthworm *Eisenia fetida* (Savigny 1826)

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ABSTRACT

Earthworms are provided with metallothionein, a molecular biomarker that assists to decode the heavy metal contamination of soil. In this laboratory based ecotoxicological study, the L_{50}, bioconcentration factor and metallothionein response had been analyzed. In acute toxicity test (14 days), the LC_{50} value of lead (Pb) and nickel (Ni) were established in *Eisenia fetida* in both artificial and natural garden soil.Low observed effective concentration (LOEC) of mixture of both metals (Pb and Ni) were also determined through repetitive experimental acute toxicity test. The sub-lethal doses of lead and nickel and their combined LOEC doses were applied on natural garden soil for chronic toxicity test (28 days). The experimental set up had been arranged as control (C), T_1 (1506.25 mg Pb), T_2 (3012.5 mg Pb), T_3 (193.75 mg Ni), T_4 (387.5 mg Ni), T_5 (753.125 mg Pb and 96.875 mg Ni) and T_6 (1506.25 mg Pb and 193.75 mg Ni) per kg of dry soil. After end of chronic periods, the bioconcentration factor and methallothionein responses were determined by spectrophotometric method. The lead and nickel concentrations in the tissue of earthworms and culture medium were evaluated and showed a significant positive correlation ($p<0.01$). The mean difference of recorded methallothionein values were also significant ($p<0.05$).

Keywords Lead, Nickel, *Eisenia fetida*, Bioconcentration, Metallothionein.

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INTRODUCTION

Heavy metal pollution of soil was responsible for beginning of bioaccumulations followed by biomagnifications in terrestrial ecosystem. Edwards and Bohlen (1992) determined that the earthworm, *Eisenia fetida,* an epigeic species acts as the standard species for eco-toxicological study by OECD guideline 207 in biological laboratory (OECD 1984, 2004). Earthworms acts as good sentinel organisms of soil pollution because they contact with soil pore water directly (Kammenga et al. 2000, Joy and Chakravorty 1991). Contaminants wre entered to body of earthworms through permeable skin of the body including via feeding materials and finally absorbed in their tissue (Jager et al. 2003) as a result, metallothionein (MT) protein became expressed for reducing the metallic effects in their tissue (Morgan et al. 2004). This protein is cysteine rich small metalbinding protein that act as significant molecular biomarker of earthworms for heavy metal pollution and involved in suppression of heavy metal toxicity including oxidative stress (Amiard et al. 2006) . Previous several studies exhibited that earthworms accumulate metals, such as cadmium, chromium, copper, cobalt, nickel (Ni), lead (Pb), zinc from soil under both field and laboratory conditions (Li et al. 2010). Maity et al (2011) reported that several studies have been demonstrates metallothionein induction assay in different epecies of earthworms serve as reliable biomarker cum soil biomonitoring and assessment for metal pollution in terrestrial enviroment. In the last decades, diffusible heavy metals contamination were increased in soil ecosystem enormously from variable sources, such as agricultural field, Industrial wastes, mining, sludge residues and especially from coal-fired thermal power station (Wuana and Okieimen 2011, Khillar et al 2012). MTs are low-molecular weighted proteins found ina wide range of phylogenetic groups including earthworms (Ali and Kashem 2018), Viarengo et al. 1999). Earthworms were provided with efficient detoxification systems of which metallothionein acts as a good biomarker during metal exposure (Suriya et al 2012). The present work has been done for evaluating the LC_{50} of lead and nickel in acute toxicity test followd by chronic toxicity test that determing bioconcentration factor (BCF) and metallothionein response in *Eisenia fetida* after exposure with sub-lethaldoss of lead, nickel and their combination.

MATERIALS AND METHODS

Acute toxicity test

Adult age synchronized *Eisenia fetida* worms (270-290 mg/worm with visible clitellum) were collected from organic vermicomposting pit. Then worms were blotted on filter paper and weighed individually. The worms were acclimated for 24 h after removal from the mother culture prior to experimental use, washed with redistilled water and then hold on wet filter paper in the dark environmental chamber at desirable temperature (28˚C ± 0.5˚C) and humidity (60-65%). This process was allowed to defecation of gut contents (Rathi et al 2011). Acclimated *Eisenia fetida* was used for the acute toxicity test in both artificial soil and natural ground soil medium as per OECD guideline 207. Lead acetate trihydrate $(CH_3COO)_2Pb_3H_2O$ and *nickel acetate tetrahydrate* $(CH_3COO)_2Ni_4\ H_2O$) are used for contamination of the experimental soil. Three replicates waskept up for each set of experiments together with control set simultaneously.

The experimental set up was performed in an Environmental Test Chamber at a constant temperature of 28 ±0.5˚C and 69-65% relative humidity.The physio-chemical parametrs of both artificial soil and natural soil such as organic carbon content, moisture content and pH were determined in constant room temperatureand moisture content. Infrared Torsion balance moisture meter utilized for determination of moisture content of the soil (Chakravorty 1990). The pH and organic carbon content of both soil were determined by the method of Piper (1966) in stable temperature and moisture. Those specimens showed no observable evidence of life after every 7 days of interval, even when poked with a blunt needle, were considered as dead and were removed from the box due to avoid unwanted contamination. The soil was checked at specific regular interval (weekly) for detection of moisture loss by weighing the test containers and replenished with redistilled water if required.

Chronic toxicity test

The sub-lethal doses of LC_{50} of two selected heavy metals (Pb and Ni) were used individually and jointly for chronic toxicolgoical study of bioaccumulation and metallothionein response in the above described specimen. The chronic toxicity test was performed in similar way as described above in acute toxicity test in ground soil for a period of 28 days of study. Very finely grinded (0.25 mm) and dried cow dung manure (5 g dry weight) was added to the test soil medium weekly to provide food for the growing worms (Chakravorty et al. 2017) . Two individual sub-lethal doses of lead (pb), nickel (Ni) and combination of lead with nickel (Pb-Ni) were applied in ground soil, along with control (C) for determining the bioconcentration factor and metallothionein response. 25% (T_1-1506.25 mg) and 50% (T_2 -3012.5

mg) of LC_{50} values of the lead (Pb) and 25% (T_3 — 193.75 mg) and 50% (T_4-387.5 mg) of the LC_{50} values of the nicket (Ni) were applied on gardensoil (kg) for metallic exposure. Thetest concentration of heavy metals combination were determined after repetitive conduction of preparatory trial experiment. The lowst observed effective concentration (LOEC) for each metals was chosen for final expermentation (Todorova et al. 2015). The mixture of both havy metals. lead and nickel was applied on soil (kg) as 12.5% (T_5 -753.125 mg Pb and (6.875 mg Ni) and 25% (T_6 —1506.25 mg Pb and 193.75 mg Ni) of the LC_{50} values of thrspectivelead and nickelmetals. The specimens were introduced in the experimental boxes, after that the boxes, after that the boxes are placed in an Environmental Test Chamber and maitaining costant temperature of 28°C (±0.5°C) and 60—65% relative humidity.

Determination of bioconcentration factor (BCF)

The BCF of earthworms was quantified by the ratio of the metal concentrations of earthworms was quantified by the ratio of the metal concentrations of earthworm tissue and the soil substrate (Mountouris et al. 2002). The heavynmetal analysis were done after Li et al. (2010).

Determination of metallothionein (MT)

Metallothionein concentration was determined by simple spectrophotometric method as described by Viarengo et al. (1997). The results were expressed in terms of µg GSH/mg of protein or expressed as µg MT/mg of proteins. The standard GSH curve has been prepared byusing know microgram GSH in the same experimental (metallothionein procedure) process against the value of spectrophotometric absorbance (412 nm). Here GSH used instead of worms tissue. Protein concentration was quantified by the Lowry's method. A standard curve was drawn using BSA and the amount of protein was calculated from the liner regression equatuion bassed on the standard curve (Lowry et al. 1951).

Table 1. Physico-chemical parameters of the artifical and natural garden soil acts as test medium used in our experiment.

Soil perameters	Artifical soil	Natural garden soil
pH	6.40	6.80
Organic carbon content	0.76%	0.88%
Moisture	62.2%	61.4%

RESULTS AND DISCUSSION

In our experiment, some important physico-chemical paramical parameters of both artifical and artifical soil had been determined which are given in Table 1. The p^H and organic carbon content of natural ground soil was slightly higher than artificial soil. But moisture content of ground soil was lower than articial soil. From acute toxicity test, the mortality were assessed by EPA probit analysis program 1.5 (US EPA 2006) in both natural garden soil and artifical soil and the LC_{50} value were given in Table 2. In this experiment nickel shows more toxic than lead in both type of soil

Table 2. LC_{50} values of two heavy metals (mg/kg) used in the Acute toxicity test.

		LC_{50} (14 days) (mg/kg)					
		Artificial soil			Natural garden soil		
Heavy metals	Commercial compound	LC_{50}	95% confidence limit Lower	Upper	LC_{50}	95% confidence limit Lower	Upper
Lead (Pb)	Lead acetate trihydrate $(CH_3COO)_2\ Pb._3H_2O)$	6250	1.065	1.484	6025	0.755	1.994
Nickel (Ni)	Nickel acetate tetrahydrate $(CH_3COO)_2\ (Ni.4H_2O)$	790	0.137	0.183	775	0.135	0.179

experiment. On the other hand, both metals revealed more toxic in garden soil than artificial soil. Neuhauser et al., (1985), Suurgeon et al., (1994) indicate that LC_{50} for Pb to range from 4480 to 5941mg/kg in artificial soil for 14 days of study in *Eisenia frtida.* In this time, Neuhauser et al. (1985) also determine LC_{50} of nickel for adult *Eisenia fetida* after 14 days, the LC_{50} was 757 mg/kg in artificial soil. Although in our acute toxicity study of lead and nickel showed LC_{50} values were quite high tat means 6250 mg/kg for lead and 790 mg/kg for nickel in artificial soil and 6025 mg/kg for lead and 775 mg/kg for nickel in natural garden soil. This result might occurred due to variable ecophysiological condition or acquisition of adaptive tolerance in the test specimens.

Sanchez-Hernandez (2006) pointed out the earthworms are important biological tools in soil biomonitoring and bioassessment in respect of adverse ecological effects such as metallic pollution. The bioaccumulation of heavy metals varies in several species of earthworms. In *Apporectodea tuberculata*. Lukkari et al. (2004) observed a significant correlation were found between MT activitives and the metal concentrations when specimen treated with Zn and Cu. He interprets MT is a potential representative of an efficient molecular biomarker for monitoring the metal contamination in soil. Heavy metal level of earthworm tissue is directly related to their proportion amount of metal contaminated in a given soil (Gupta et al 2005). We got similar results in this work that means bioconcentrations of heavy metals in test specimen were increased if concentration of metallic expoures were increased to soil. Based on AAS result, the metal concentration are tabulated in given Table 3. The heavy metal concentrations of soil and earthworm tissue showed a significant positive correlation ($p<0.05$ level). The BCF was showed in the same table that is the ratio between the specific metal concentration in specimen tissue and in test soil medium. The highest BCF was found in T_2 for lead and lowest in control for nickel.

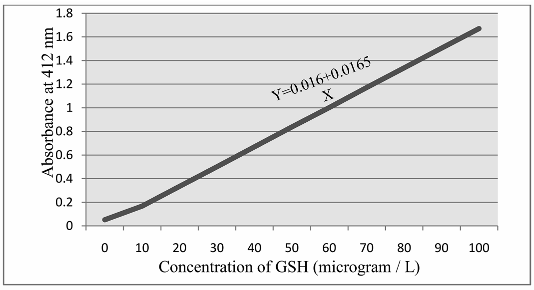

Fig. 1. Standard GSH curve. Concentrationof GSH (microgram / L)

Dedeke et al. (2016) showed that significant positive correlations were present between MT and heavy metal concentrations in tissue of the several species of earthworms. The standard GSH curve was produced by Microsoft software and shown in Fig. 1. The regression equation was achieved by computation of liner regression statistics between absorbance (412 nm) and known concentration of GSH (g/L). From the regression equations, the MT concentration was evaluated in respect of its specific absorbance (412 nm). The MT concentrations were significantly different among all metallic treatments

Table 3. The calculated BCF values and concentrations of metal (mg/kg) in tissue respective to soil treatment. The values of metal concentrations are presented as arithmetic mean in mg/kg with standard deviation.

Individual metal or conbination of both metals	Control		Lead (Pb)		Nickel (Ni)		Lead with mickel (Pb-Ni)			
	Pb	Ni	T_1	T_2	T_3	T_4	T_5		T_6	
							Pb	Ni	Pb	Ni
Con. of metal in soil	5.89 ±0.4	2.37 ±0.23	1506.25 ±6.25	3012.5 ±11.12	193.75 ±1.80	387.5 ±2.10	753.12 ±2.16	96.85 ±1.08	1508.35 ±6.45	194.25 ±1.95
Con. of metal in tissue	0.89 ±0.07	0.21 ±0.012	1373.7 ±6.01	2855.85 ±10.09	115.28 ±1.75	241.42 ±2.01	587.44 ±3.75	44.95 ±1.06	1235.12 ±4.07	97.84 ±1.20
Bioconcentration factor (BCF)	0.15	0.09	0.912	0.948	0.595	0.623	0.78	0.464	0.82	0.503

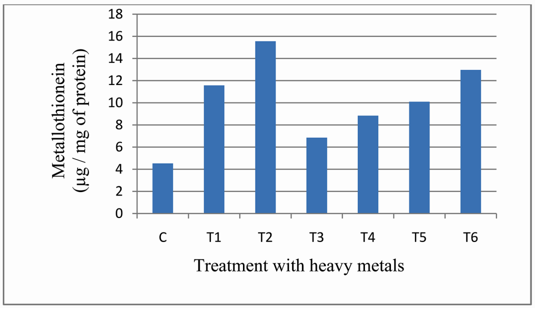

Fig. 2. Metallothionein (MT) concentrations earthworm tissue. Treatment with heavy metals.

($p<0.05$). The MT concentrations of all different treatment was graphically represented in Fig. 2. The highest level of MT concentration occurred in T_1 and lowest was recorded in control. Lead revealed that it was responsible for higher expression of MT response than that of nickel. But by comparison of the graphical representation (Fig.2), when we apply both metallic mixture in T_5 and T_6, where T_6 showed comparatively higher MT response than control, T_1, T_3 and T_4. Although T_5 showed that it produce higher MT response than control, T_3 and T_4 experimental sets. Earlier workers showed showed that metallic mixture of copper and cadmium to soil could enhance MT concentration in *Lumbricus terrestries*. The present investigation showed that metallothionein concentrations were higher in lead exposure than nickel exposure to soil. But in combination of both metals (Pb-Ni) in T_6 at LOEC dose showed synergistic effect due to the concentration of metallothionein is higher as compared with individual sub-lethal metallic doses (T_1, T_3 and T_4).

Statistical analysis

All data were provided as artihmetic mean and the mean + /- standard deviation (SD). The LC_{50} of both metals were determined individually through probit analysis by EPA probit analysis program, version 1.5 (US EPA 2006) in 95% confidence limit. Statisical analyses for other measurements were performed by Statistical Package for Social Sciences (SPSS) version 20.0. The heavy metal concentrations of both soil and earthworm tissure wereanalyzed by correlation and showed positive type ofcorrelation ($p<0.05$). MT concentrations were significantly different after the analysis of variance (ANOVA) followedd by Post Hoc test ($p<0.05$).

CONCLUSION

In our above investigation, it was clearlyindicated that LC_{50} value of lead was higher than nickel that means nickel, that means nickel was more toxic than lead. We had also established a positive correlation between heavy metal concentration of soil and earthworm's tissue. The metallothionein concentration of earthworm's tissue acts as molecular biomarker that that were increased after treatment the soil with heavy metals. The combination of heavy metals had synergstic effect to the earthworms than individual metal.

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