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ACCUMULATION OF LEAD AND ZINC IN EARTHWORM *LAMPITO MAURITII* (KINBERG): EFFECT ON SURVIVAL, GROWTH AND ACETYLCHOLINESTERASE ACTIVITY

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Abstract

Well clitellate earthworm, *Lampito mauritii* (Kinberg) were exposed to different concentrations (75, 150, 300 mg kg^{-1}) of Pb and Zn separately for 28 days. The effects of the selected metals were studied on survival and growth of the earthworm, *Lampito mauritii*. In addition to that the levels of Pb and Zn in the specimens were assessed and the response of acetylcholinesterase to metal pollutants was also studied. The results revealed that presence of the Pb and Zn at the selected concentrations did not affect the survival and growth of the earthworm. Pb and Zn were found to gradually accumulate in the earthworm but without recording any significant change in tissue metal concentration after day 14 (Pb 150 and Zn 300 mg kg^{-1}) and day 21 (Pb 75, 300 and Zn 150 mg kg^{-1}). No considerable acetylcholinesterase inhibition was recorded in Zn treated earthworm. Whereas the present study is the evidence of neurotoxic potential of Pb in terms of acetylcholinesterase inhibition though the enzyme activity attains the control level at the end of the experiment. Thus it may be surmised that these low levels of metal treatment do not apparently harm the earthworm in respect of their survival, growth and AChE activity. These factors need to be investigated in future studies with the increasing metal treatment in the soil.

Keywords: *Lampito mauritii*; Growth; Survival; Acetylcholinesterase; Metal accumulation

Introduction

Industrial processes are responsible for discharging toxic heavy metals to the terrestrial environment that might get biomagnified at higher trophic levels, contaminating the entire food chain (Turgut et al. 2004). Heavy metals endanger all types of terrestrial life of which earthworm is an important member, playing a major role in the development and maintenance of soil structure and is also a source of food for other organisms (Edwards and Bohlen 1996). Earthworms have the ability to tolerate many kinds of chemical contaminants including heavy metals (Corp and Morgan 1991; Spurgeon and Hopkin 1999a). This tolerance may be a positive attribute for assessing bioaccumulation or toxicity studies of severely contaminated sites. In both standard laboratory and field tests earthworms are regarded as bioindicators of soil contaminated with heavy metals, pesticides and other organic pollutants (Fitzpatrick et al. 1996; Saint-Denis et al. 1999; Spurgeon and Hopkin 1999a; Booth et al. 2000; Scott-Fordsmand and Weeks 2000). This invertebrate organism is generally recognized as macroconcentrators of various metals (Neuhauser et al. 1995; Cortet et al. 1999; Łaszczyca et al. 2004). Metals having the greatest potential for causing disease are the ones, which accumulate in the body. Owing to their ability to bind to macromolecules and react with sulfhydryl groups, numerous studies have shown that metals are involved, either in vitro or in vivo, in the inhibition of enzymes. Due to the biocidal and non-biodegradable properties, the excess use of Pb causes the environment to become contaminated at different levels of ecosystem. After entering the body, Pb acts as a broad-spectrum toxin. It disturbs almost every metabolic function in body chemistry (David et al. 1976) and is deposited in different tissues. It has been shown to inhibit enzymes and alter biological membranes related to the formation of lipid peroxides (Lawton and Donaldson 1991; Labrot et al. 1996).

In eukaryotic organisms the function of Zn in both nutrition and physiology is widely appreciated, especially its importance in many enzymes (Hughes and Poole 1989). On the other hand, anthropogenic activities increased the circulation of this element in the biosphere may represent a source of toxic stress. Because of this dual character, the concentration and availability of Zn in soil may disturb soil ecosystems by affecting the productivity and biodiversity. Zn is one of

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the most commonly encountered heavy metals in contaminated agricultural soils and is more phytotoxic than other commonly occurring metals (Bidwell and Dowdy 1987). It is presumed that in many cases zinc is the critical toxic metal for earthworms (Spurgeon and Hopkin 1999b, 2000).

Nervous system is perhaps the primary target for any toxic material and thus most effective for chemically upsetting normal body function (Hoar 1991). Consequently, acetylcholinesterase, one of the important enzymes responsible for the hydrolysis of acetylcholine in the vertebrate and invertebrate nervous system is used as a reliable parameter for assessing the poisoning due to pesticides and heavy metals (Stenersen et al. 1992; Patnaik and Dash 1992 a, b; Pradhan and Mishra 1998).

On the basis of the above discussion and also in order to find out the metal tolerance capability of earthworms, the present study was designed to determine the growth, survival capacity, body metal concentration along with the AChE activity of earthworm *Lampito mauritii* (Kinberg) exposed separately to different doses of Pb and Zn for 28 days.

Materials and Methods

Chemicals

All chemicals used were of analytical grade purchased from Sigma Chemical Company (USA), Sisco Research Laboratories (India) and E. Merck (India).

Animals

It is generally felt that in vermitechnology, the endemic species are preferred because the exotic species might carry fungal and other pathogens, which may create additional problems to the crops (Dash 1999). Hence, the earthworm species, *Lampito mauritii* (endemic species) was selected for the present study. This is believed to be indigenous to Indo- Pacific region (Julka and Senapati 1987). Native species are also well adapted to local conditions (Goswami and Kalita 2000). It was observed that *Lampito mauritii* is efficient in amending metal treated soil (Maity et al. 2008a) and is also well equipped to metabolize electrophilic xenobiotics (Maity et al. 2008b). *Lampito mauritii* is the predominant species of earthworm in Santiniketan, Birbhum District (23°30' to 24°45'N and 7° to 88°10'E), West Bengal, India, the place where this research was carried out. Considering the above mentioned characteristics we have conducted the present study with *Lampito mauritii* (Kinberg) as the main bioagent.

The earthworms (*Lampito mauritii*) were collected from the meadow, which had no history of input of either heavy metals or agrochemicals. They were carefully brought to the laboratory along with the moist soil and acclimatized for 1 month under laboratory conditions in polyethylene buckets (culture pot) containing soil and farmyard manure mixture (9:1), at a temperature of 28 ± 2° C. The pH of the soil and farmyard manure mixture was 6.8 ± 0.05. Sufficient deionised water was added to the dry soil to achieve a moisture content of 30% by weight. This was maintained throughout the experiment by adding deionised water. Adult earthworms were removed from the culture pot 24 hours prior to use, rinsed in water and kept on moist filter paper in the dark at 28 ± 2° C as a routine procedure to allow voiding of gut contents.

Selection of concentrations of metals

Heavy metals are toxic to most life forms at some level of exposure (Pizl and Josens 1995). Animals in the natural environment are usually exposed to low level of xenobiotics. In our study, the highest concentration was chosen on the basis of above discussion and also to mimic the average concentration of Pb and Zn which is mostly found in urban soils of India (Jeevan Rao and Santaram 2003; Krishna and Govil 2004, 2005, 2007). No avoidance behaviour and nervous symptoms were noted in the *Lampito mauritii* exposed to the selected concentrations of Pb and Zn.

Preparation of Earthworm beds

All the experiments were carried out under laboratory conditions. Twenty replicates of six sets of experimental beds containing 75, 150 and 300 mg of Pb and Zn kg^{-1} separately were prepared in 500 ml polythene culture pots by mixing fine sieved air dried soil (2 mm) and farmyard manure (FYM) mixed at a ratio of 9:1 in culture pots covered with nylon net to prevent escape of earthworms. Soil for beds was taken from the site of collection of earthworms, which has no history of input of heavy metals and agrochemicals. Two types of experimental beds were prepared as follows: i) Metal treated bed (soil + FYM +metal +earthworm); ii) Control bed (soil + FYM + earthworm). To obtain metal treated beds containing 75, 150, 300 mg of Pb and Zn kg^{-1} of soil separately, the soil was treated by adding appropriate amount of lead nitrate and zinc sulphate solution made in distilled water. The same procedure using distilled water was applied to prepare a set of twenty control beds without Pb and Zn. The soil was mixed thoroughly to ensure a homogenous mixture. The moisture content was adjusted to 30% of the final weight in all experimental beds. The average height of each bed was 10 cm and initial pH of the soil was 6.8 ± 0.05. Temperature was maintained at 28 ± 2°C throughout the study period. The experimental pots were left for one week undisturbed prior to experimentation for softening of wastes or thermostabilization. For removing gut

contents, earthworms were kept overnight on moist filter papers without food. After one week, 18 adult (well clitellate) gut evacuated earthworms were inoculated in each experimental metal treated beds and control beds.

Determination of Heavy metal concentration (Pb/Zn) in wet body mass of Lampito mauritii

The earthworms were collected carefully at 0, 2, 7, 14, 21 and 28 days of metal treatment and kept overnight on a moist filter paper without food for evacuating their gut contents, weighed, killed by freezing and digested in HCl-HNO_3- $HClO_4$ tri-acid mixture and metal concentrations (Pb and Zn) were estimated in a GBC 908AA flame Atomic Absorption Spectrometer (AAS).

Estimation of in vivo acetylcholinesterase (AChE) activity in Lampito mauritii

The AChE activity of *Lampito mauritii* (Kinberg) was estimated spectrophotometrically (Ellman et al. 1961) using acetylthiocholine iodide as the substrate. Briefly, four worms (well clitellate) from each concentration (one from each replicate specified for a particular day) were selected randomly at an interval of 0, 2, 7, 14, 21, 28 days of exposure. Each AChE assay sample consisted of pooled extracts from four specimens. All assays were done in triplicate. To prepare a 10% homogenate, worms were gut cleaned, weighed and homogenized at 4 ^{o}C for 1 min in ice-cold phosphate buffer (0.1M, pH 8). The homogenate was filtered in the cold through cheesecloth and the AChE activity of the filtrate was determined. The following reagents were then added; 0.1M of phosphate buffer (pH 8), 0.2M of 5, 5'-dithiobis, 2-nitrobenzoic acid (DTNB) and 1.83 mM Acetylthiocholine iodide (AChThI). The rate of enzyme reaction was recorded at 405 nm as ΔOD per minute in a Beckman DU 640 Spectrophotometer, and the specific activity of the enzyme expressed in terms of nmoles thiocholine produced min^{-1} mg^{-1} protein, with the help of a freshly prepared thiocholine standard curve (Jash et al. 1982). The protein content of the homogenate was determined according to Lowry et al. (1951) using bovine serum albumin as standard.

Statistical analysis

The experimental values of AChE activity and metal concentrations in the earthworms were tabulated as mean ± standard error (S.E.) of three replicates, each comprising four worms. Test of significance was performed by one-way analysis of variance (Snedecor and Cochran, 1967) followed by Duncan multiple range test (Duncan, 1955) considering $P<0.05$ for both metal concentration of wet body mass and AChE activity of earthworm.

Results

Survival and growth:

During the present investigation, no mortality and avoidance behavior were noted in the earthworms exposed to metal treated beds. There was no significant alteration in body weight of earthworm in metal treated beds with respect to the control ones for the specific period of exposure.

Lead / Zinc concentration in wet body mass of *Lampito mauritii*

Prior to exposure (0 day), in contrast to the presence of Zn in tissues (28 mg kg^{-1}wet wt) Pb was not found in the wet body mass of the earthworm. Metal concentration in the earthworm maintained in the control beds till day 28 was found to be same as that observed on 0 day. Pb was under the threshold of determination of the atomic absorption spectrometer in the wet body mass of earthworm.

It is evident from Table-1 that Pb and Zn concentrations in earthworm increase with increasing metal contamination. It is also apparent that tissue metal concentrations in *Lampito mauritii* increase with a concomitant increase in exposure duration to the respective metal contaminated soil. Pb concentration in earthworm increased drastically on day 7 and 21 at 75 and 300 mg kg^{-1} Pb treatment, while on day 7 and 14 a significant increase in Pb concentration was observed in 150 mg kg^{-1} Pb exposed *Lampito mauritii*, which continued till the end of exposure period. A sudden significant increase in Zn concentration in earthworm was also observed on day 14 and 21 of exposure to 150 mg kg^{-1} of Zn and on day 7 and 14 in 300 mg kg^{-1} of Zn contaminated beds, thereafter metal concentration remains more or less unaltered. However, no significant change was observed in the level of Zn in *Lampito mauritii* exposed to 75 mg kg^{-1} Zn contaminated soil.

Table 1. Pb and Zn concentrations (mg kg^{-1} wet wt) in *Lampito mauritii* exposed for 28 days in contaminated soil.

Treatment	Days of exposure					
	0	2	7	14	21	28
Pb-75	BDL	0.59 ± 0.1^{c}	1.5 ± 0.3^{b}	2.21 ± 0.1^{b}	3.22 ± 0.2^{a}	3.68 ± 0.7^{a}
Pb-150	BDL	0.92 ± 0.2^{c}	2.66 ± 0.4^{b}	3.26 ± 0.4^{a}	4.13 ± 0.1^{a}	4.42 ± 1.1^{a}
Pb-300	BDL	3.39 ± 0.3^{c}	6.89 ± 0.6^{b}	9.25 ± 0.8^{b}	12.58 ± 1.1^{a}	14.74 ± 1.1^{a}
Zn-75	28.03 ± 0.4^{a}	28.83 ± 0.7^{a}	29.48 ± 0.5^{a}	31.39 ± 0.3^{a}	31.25 ± 0.2^{a}	31.89 ± 2.0^{a}
Zn-150	28.03 ± 0.4^{c}	28.91 ± 0.2^{c}	30.33 ± 0.5^{c}	38.94 ± 0.8^{b}	42.70 ± 0.3^{a}	45.41 ± 3.2^{a}
Zn-300	28.03 ± 0.4^{c}	39.04 ± 1.6^{c}	53.32 ± 2.7^{b}	67.5 ± 5.4^{a}	69.48 ± 5.2^{a}	70.06 ± 4.3^{a}

[All values indicate mean ± S.E. of three replicates; Significance was tested statistically by one way ANOVA ($P\leq0.05$) and Duncan multiple range tests within treatment (Pb/Zn) for different period; Values with same superscript letters in a row are not significantly different from each other. BDL = Below detection limit.]

In vivo Acetylcholinesterase activity of *Lampito mauritii*

The AChE activity in *Lampito mauritii* following application of 75, 150 and 300 mg kg^{-1} of Pb and Zn are given in Table-2 and 3 respectively. The data show that the effects of Pb and Zn on the AChE activity of *Lampito mauritii* were different. In case of Pb treated worm no significant change in AChE activity was observed after the first and second day of exposure. Significant *in vivo* inhibition of AChE activity by Pb as found to be dose dependent only after day 7 of exposure recording 8.94%, 16.46% and 50.90% in the worms treated with 75, 150 and 300 mg kg^{-1} of Pb respectively compared to control. After a period of 14 days of exposure, the inhibition of AChE activity recovered in 75 and 150 mg $kg^{-1}$of Pb treated *Lampito mauritii* while a slight inhibition (4.52%) was recorded at 300 mg kg^{-1} of Pb treatment. This inhibition was found to increase after 21 day of exposure that was 14.28% in respect of control. However, after 28 days of exposure the activity of AChE regained at the control level in respect of all the three doses of Pb.

Table2: *In vivo* effect of Pb on AChE activity of *Lampito mauritii* exposed for 28 days to Pb contaminated soil.

Duration of exposure	n mole thiocholine produced / mg protein			
Day	Control	Pb-75	Pb-150	Pb-300
1	307.48 ± 2.65^{a}	300.98 ± 4.06^{a}	301.60 ± 2.23^{a}	298.56 ± 1.74^{a}
2	294.72 ± 2.20^{a}	292.66 ± 1.90^{a}	290.14 ± 1.78^{a}	286.14 ± 3.60^{a}
7	286.34 ± 7.44^{a}	260.73 ± 4.50^{b}	239.18 ± 3.18^{c}	140.59 ± 6.07^{d}
14	281.17 ± 2.63^{a}	273.14 ± 2.58^{a}	276.81 ± 2.74^{a}	268.46 ± 2.27^{b}
21	303.45 ± 4.68^{a}	302.70 ± 3.50^{a}	294.36 ± 7.29^{a}	259.75 ± 7.62^{b}
28	293.51 ± 6.38^{a}	288.53 ± 7.56^{a}	284.56 ± 9.07^{a}	292.16 ± 5.88^{a}

[All values indicate mean ± S.E. of three replicates; Significance was tested statistically by one way ANOVA and Duncan multiple range tests ($P<0.05$) within same exposure duration between different doses of Pb. Values with same superscript letters in a row are not significantly different from each other.]

In case of Zn treatment an inhibition of 13.75% AChE was recorded only at Zn (300 mg kg^{-1}) after 14 days of exposure (Table-3). No significant change in AChE activity was noted in any of the other experimental doses or experimental durations of Zn exposure.

Table 3. *In vivo* effect of Zn on AChE activity of earthworm, *Lampito mauritii* exposed for 28 days in Zn contaminated soil

Duration of exposure	n mole thiocholine produced/ mg protein			
Day	Control	Zn-75	Zn-150	Zn-300
1	307.48 ± 2.65^{a}	301.82 ± 2.11^{a}	298.62 ± 3.08^{a}	302.04 ± 3.68^{a}
2	294.72 ± 2.20^{a}	292.31 ± 2.24^{a}	291.49 ± 3.68^{a}	299.55 ± 4.19^{a}
7	286.34 ± 7.44^{a}	295.39 ± 3.42^{a}	282.22 ± 1.73^{a}	281.79 ± 1.08^{a}
14	281.17 ± 2.63^{a}	274.64 ± 2.56^{a}	268.70 ± 5.53^{a}	242.37 ± 8.76^{b}
21	303.45 ± 4.68^{a}	298.30 ± 5.01^{a}	296.97 ± 4.95^{a}	290.73 ± 4.42^{a}
28	293.51 ± 6.38^{a}	289.38 ± 5.96^{a}	292.84 ± 4.12^{a}	300.23 ± 7.38^{a}

[All values indicate mean ± S.E. of three replicates; Significance was tested statistically by one way ANOVA and Duncan multiple range tests ($P<0.05$) within same exposure duration between different doses of Zn. Values with same superscript letters in a row are not significantly different from each other.]

Discussion

Survival and growth:

Candidate heavy metals, Pb and Zn, at selected concentrations (75, 150, and 300 mg kg^{-1} soil), did not exert any visible harmful effect on earthworm *Lampito mauritii*, in terms of their survival and growth. This was similar to the findings of Reinecke et al. (1997) in Pb nitrate exposed *Eisenia fetida*. Our result indicates strong resistance of *Lampito mauritii* to the different concentrations of Pb and Zn tested.

Metal concentration (Pb / Zn) in wet body mass of *Lampito mauritii*

Earthworms are known to accumulate metals from the soil efficiently as observed by various authors (Ireland 1975a, 1975b; Wright and Stringer 1980; Morgan and Morgan 1988; Labrot et al. 1998). The toxicity of heavy metal for earthworms increases with increasing the soil metal concentration (Marinussen et al. 1997). This suggests that the heavy metal availability is determined by heavy metal concentrations in soil. In the present study Pb and Zn were detected in *Lampito mauritii* exposed to Pb and Zn contaminated soils. With increasing metal contamination Pb and Zn concentration in earthworm also increased. This is in agreement with other studies which have clearly demonstrated that concentrations of metals in whole worms increase concomitantly with increased soil metal concentration (Morgan and Morgan 1988; Neuhauser et al. 1995). Honda et al. (1984) reported that accumulation of Pb, Hg, Cd and As in the earthworm *Pheretima hilgendorfi* depends on the exposure duration whereas the accumulation of Fe, Mn, Zn, Cu, Ni and Co is dependent upon the metabolic turnover. Thereafter metal concentrations remain constant throughout the entire life span. The present study clearly demonstrates that statistically significant accumulation of Pb and Zn in the earthworm does take place as reported earlier (Honda et al. 1984). It is known that earthworm chloragosomes function as the cation exchange system capable of taking up and retaining heavy metals (Ireland 1978; Morgan and Morgan 1998), which are subsequently excreted by fractionation of the chloragocytes (Fischer 1976). The low level of metal accumulation in *Lampito mauritii* when exposed for a longer duration may be attributed to either the presence of the cation exchange system of chloragosomes or due to the induction of the metal binding proteins. Marcano et al. (1996) studied the patterns of accumulation and elimination of copper and zinc in the tropical polychaete worm *Eurythoe complanata* in relation to levels of metal-binding proteins during sublethal exposure. During the uptake and depuration phases, these authors found that heavier metalloprotein (6 kDa) displayed a higher affinity for copper. Metallothionein- like proteins (molecular mass 10-20 kDa) showed higher affinity for zinc than copper, suggesting that different biochemical mechanisms underlie the control of zinc and copper metabolism.

In vivo acetylcholinesterase activity in *Lampito mauritii*

Inhibition in AChE activity of *Lampito mauritii* was recorded after treatment with 75, 150, 300 mg kg^{-1} of Pb and 300 mg kg^{-1} of Zn. Inhibition of AChE activity after 14 days of Zn (300 mg kg^{-1}) exposure might be associated with adaptive mechanisms. The inhibition was, however, more pronounced in Pb treated worms indicating that the toxic potential of Pb is greater than that of Zn. This may be due to the different biochemical mechanisms involved in biotransformation of Pb and Zn also due to the indispensable nature of of Zn in metabolism of the organism.

Various reports are available indicating metal mediated AChE inhibition. Lead and uranium inhibited AChE activity in *Eisenia fetida andrei* (in vivo and in vitro) upto 60% (Labrot et al. 1996). In oligochaetes (*Limnodrilus hoffmeisteri*) lead exposure caused AChE inhibition (Martinez-Tabche et al. 2001) while zinc exposure does not affect AChE activity either in scallop, *Adamussium colbecki* (Corsi et al. 2004) or in zebra fish, *Danio rerio* (Senger et al. 2006). Our findings also clearly indicated the innocuousness of Zn so far as AChE inhibition is concerned in *Lampito mauritii*. AChE inhibition in *Lampito mauritii* exposed to Pb contaminated soil has been found to be dose dependent, which agrees with earlier findings on earthworms (Labrot et al. 1996). The statistical analysis of the present data reflects that the AChE inhibition of *Lampito mauritii* had started to recover after 14 days of exposure in Pb for all three concentrations in the present study. Such recovery of AChE activity substantiates earlier findings obtained in earthworm by Saint-Denis et al. (2001) exposed to lead contaminated soil and also in other animals exposed to pesticides (Dembélé et al. 1999). The recovery of AChE activity in *Lampito mauritii* may be attributed to adaptation of the worms to the habitat through antioxidant responses as evidenced by our earlier work (Maity et al. 2008) or may be their capacity to detoxify and sequester the metal in biologically inactive forms by the induction of metal binding protein (Stürzenbaum et al. 2001; Homa et al. 2005). Moreover, in a companion study, we observed a significant induction in the level of metallothionein on *Lampito mauritii* exposed to Pb and Zn at the same condition (communicated elsewhere).

Conclusions

The present study clearly indicates a dose dependent increase in the body burden of metals (Pb and Zn) without recording any morphological alteration,

which signifies the survival efficiency of earthworm, *Lampito mauritii* in presence of heavy metals (Pb and Zn). No considerable AChE inhibition was recorded in Zn treated earthworm because Zn is one of the essential metals in body metabolism. Whereas the present study is the evidence of neurotoxic potential of Pb in terms of AChE inhibition though the enzyme activity attains the control level at the end of the experiment. Thus it may be surmised that these low levels of metal treatment do not apparently harm the earthworm in respect of their survival, growth and AChE activity. These factors need to be investigated in future studies with the increasing metal treatment in the soil.

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