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Research Article

Heavy Metals Toxicity on Various Growth Parameters and Biomolecules of Earthworm, *Eisenia fetida*

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Abstract

Earthworms are very sensitive bio-indicators of soil pollution. The aim of present investigation was to study the effects of heavy metals toxicity on various growth parameters of earthworms and also on their bio-molecules (carbohydrates, proteins, lipids) which are involved in several processes such as energy storage (carbohydrates), catalyzing biochemical reactions *etc*. Adult individuals of *Eisenia fetida* were collected and kept in the green house. After acclimatization they were transferred either to unpolluted slurry (treated as control) or to the heavy metals' polluted (Cu, Sn, Cu+Sn) slurry. They were kept in these treatments at 22±2°C, RH 35% for 90 days. More cocoons and juveniles appeared in unpolluted slurry as compared to polluted one. The bio-molecules of worms maintained in unpolluted slurry were unaffected whereas heavy metals' polluted worms showed reduction in bio-molecules (%). In contrast, in the Cu polluted slurry samples, high mortality of adults was recorded, body weight and length was reduced, reproduction completely inhibited and bio-molecules also reduced (%).

Keywords: Acclimatization; Carbohydrates; Cocoons; *Eisenia fetida;* Lipids; Proteins

Abbreviations

Cu: Copper; Sn: Tin

Introduction

Heavy metals are a group of metals which are detrimental and toxic even at very low concentration characterized by having high atomic number, atomic weight as well as specific gravity is greater than 5 [43]. These heavy metals have tendency to accumulate and persist in the environment hence they are dangerous. Heavy metals cause changes in gene expression, oxidative stress and lysozomal membrane instability [2,41,48], also reduce growth of earthworm and slow down the sexual development [42]. There are many anthropogenic activities through which heavy metals are released into the soil which are proved to be toxic to soil organisms and the abundance, distribution and diversity of these soil organisms are affected by the toxicity of heavy metals [26].

Earthworms have many interactions with the soil and due to these interactions the earthworms are significantly affected by the pollution originated by the rigorous use of heavy metals and biocides in agriculture, industrial activities, and atmospheric deposition. Hence earthworms have been proved as valuable bio-indicators of soil pollution [25]. Biodegradable compounds can be degraded but unlike biodegradable compounds these heavy metals are non-biodegradable compounds that cannot be decomposed in vermicomposting process [14]. However, these heavy metals can accumulate in soil which may be uptaken by plants and finally affect the health of animals and people through food chain (as we all are a part of food chain). Therefore it is necessary to explore the transformation and changes of heavy metals in environment by earthworms [21].

Earthworm species such as *E. fetida* is almost ubiquitous and easy to maintain in laboratory conditions, also these are the representative for the soil fauna and represent the standard test organism commonly used in terrestrial eco-toxicology [10,17], and has been standardized as the most commonly used species for acute toxicity tests for chemicals [29,33]. This earthworm species, *E. fetida* is an ideal species for predicting the effects of heavy metals on them due to the limited difference between their sensitivity to metals and the ease with which they can easily be reared and handled in the laboratory [9]. Growth rate, reproduction rate, life cycle and various biomolecular contents are some of the most sensitive, ecologically relevant parameters for predicting the effects of pollutants on soil fauna. Information about the effects of heavy metals on survival and biochemistry of earthworm is scanty.

Anthropogenic copper pollution in agricultural soils has become a widespread environmental problem [51] and tin is abundant in rural areas, hence the toxic effect of these two heavy metals were investigated.

Aim of the Study

The aim of the present investigation was to check the effect of heavy metals on the earthworm species, *Eisenia fetida.* We investigated the survivability, fecundity, body weight and length and biomolecules of earthworms after heavy metals (Cu, Sn and Cu+Sn) treatment.

Materials and Methods

Animals

Sexually mature earthworms (clitellated) of *Eisenia fetida* were collected from the stock culture maintained in vermitechnology laboratory of Department of Zoology, Chaudhary Charan Singh Haryana Agricultural University, Hisar. They were transferred to the green house and maintained for 90 days (under constant temperature of 22°C, 12h light/12h dark) in 10 different groups (20 animals in each group) with different concentration of heavy metals and each group in three replicates (R1, R2, R3) (Table 1) tubs with 30 L slurry, provided by Janta Biogas Plant, Department of Microbiology.

Slurry

Slurry samples were collected from the Department of Microbiology after 8 weeks of digestion (Table 2). Prior to analysis, the slurry samples were air-dried. Slurry pH was measured by using Elico pH meter. Total solids (TS) was estimated by first drying the samples at 80˚C and for volatile solids 1.0 gram of fine dried powder sample was taken in a pre-weighed China crucible and incinerated in a muffle furnace at 550˚C for 1 h. The loss in weight of TS on combustion was taken as volatile solids (VS). Total volatile fatty acids (TVFA) in the sample were determined by steam distillation method. Organic Carbon (OC) content of the samples was calculated as:

$$\%OC = \frac{\%\text{ Volatile Solids}}{1.724}$$

Sr. No.	Treatments	Description
1.	T0	0.00 (ppm) Control
2.	T1	Copper (0.06 ppm)
3.	T2	Copper(0.08 ppm)
4.	T3	Copper (0.10 ppm)
5.	T4	Tin (0.06 ppm)
6.	T5	Tin(0.08 ppm)
7.	T6	Tin (0.10 ppm)
8.	T7	Copper+Tin (0.03+0.03 ppm)
9.	T8	Copper+Tin (0.04+0.04 ppm)
10.	T9	Copper+Tin (0.05+0.05 ppm)

Table 1: Description of treatments given to the earthworms along with control.

pH	8.70
TS (%)	10.94
VS (%)	81.0
TVFA (mg/Kg)	690
OC(%)	46.98

Table 2: Physico-chemical characteristics (mean ± SD) of slurry samples after 8th week of digestion.

Earthworm's various growth parameters

Earthworm body weight and length in particular slurry samples were checked at 30th, 60th and 90th day of experiment. Mortality and survival were measured at different intervals (30th, 60th and 90th day) by counting the surviving worms in each container. Cocoons were collected and counted by hand sorting during different intervals (30th, 60th and 90th day).

To study the effect of heavy metals toxicity on the biochemical parameters such as total tissue carbohydrate, crude lipid and crude protein content in *Eisenia fetida*

- Estimation of total tissue carbohydrate content by standard phenol sulphuric acid method [27]
- Estimation of crude Lipid content by Soxhlet Extraction Method [36]
- Estimation of crude Protein content by Micro-Kjehdahl's Method [23].

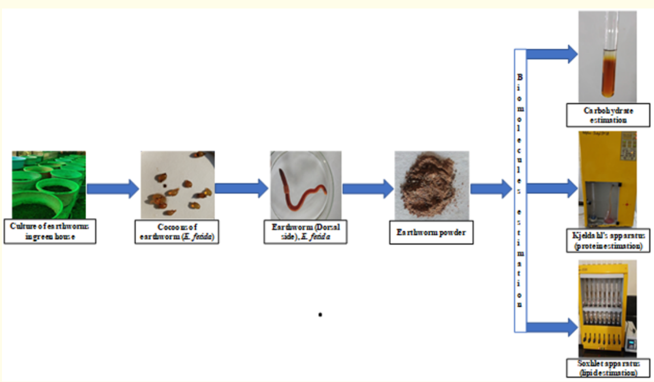

Figure a

Statistical analysis

The standard statistical tools were used for analysis of data recorded in different experiments. The experimental design for screen house study was completely randomized block (three factor analysis) with three replicates (tubs). Critical difference (CD) was calculated between the treatments by CRD (*in vitro*), accordingly, using 'OPSTAT' software, available on CCS Haryana Agricultural University, Hisar website www.hau.ac.in.

Results

Animal survivability

The exposure of earthworms to different concentrations of heavy metals significantly reduced the number of adult earthworms, further the number of survived worms decreased with increase in concentration of metals as well as increase in exposure time. The effect of metals i.e. Copper and Tin was observed on the survivability of earthworm and graphically shown in figure 1. The study revealed that maximum reduction in number of worms was observed after 90 days of exposure. The minimum number of adult earthworm survived in substrate treated with combined dose of Cu+Sn @0.05+0.05 ppm, which is 55% of 1st day. In T3 and T8 groups only 33.33% and 30% worms survived after 90 days of exposure as compare to 1st day of experiment.

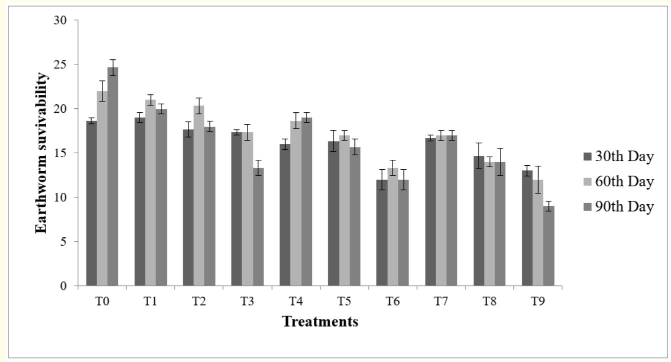

Figure 1: Effect of heavy metals toxicity on survivability of earthworm.

Cocoon production

The number of cocoons collected from the control group was higher than those collected from the Cu and Sn treated groups. A significant reduction in cocoon production during the course of the exposure was observed when compared with the control ($p < 0.05$). The worms exposed to T9 (Cu+Sn) had the lowest cocoon production after 30, 60 and 90 day exposure, which only accounted for 55.05% of control. While the average numbers of cocoons produced in the different concentration of Cu group T1, T2 and T3 are 0.72%, 14.59% and 23.35% at 90th days of exposure as compare to control (T0), respectively. Nevertheless, no significant difference was observed in total number of cocoons exposed to Sn (0.06 ppm) polluted soil (Figure 2). In the Cu+Sn (0.04+0.04 ppm), Cu+Sn (0.05+0.05 ppm), the number of cocoons increased by 3.79% and 38.8% at 60th day and by 18.93% and 32.65% at 90th day, respectively.

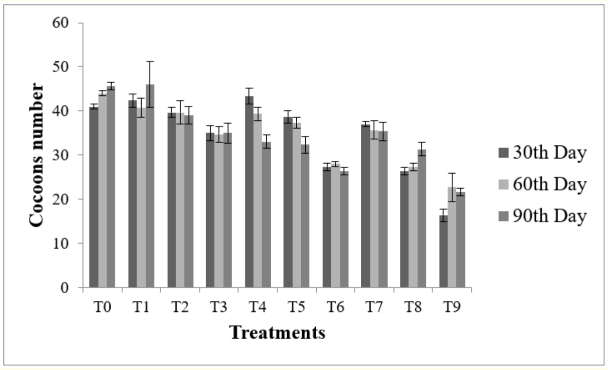

Figure 2: Effect of heavy metals toxicity on cocoon number of earthworm.

Body weight (gms)

The earthworms in all treatments survived after 90 days of exposure. The relative weight loss rates of earthworms in Cu treatment changed significantly compared to the control (Figure 3). In the control, the relative weight gain rates were 4.58% and 25.25% after 60 and 90 days. And, the relative weight loss rates were significantly increased to 24.32% 27.47% and 44.59% compared to the 30th day after 90 day of exposure at T2, T5, T9 respectively (Figure 3). The ANOVA analysis revealed that Cu+Sn at highest concentration had significant effects on weight loss rates ($p < 0.05$). However, there were no significant difference of relative weight loss rates between treatments and control at 60th day of exposure with metals (Figure 1A). The joint toxicity test showed sufficient decrease of the relative weight loss rates at 60th day i.e. 40.42% at T9.

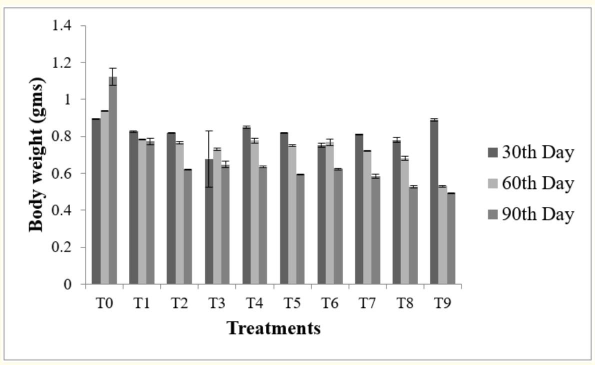

Figure 3: Effect of heavy metals toxicity on body weight (gms) of earthworm.

Body length (cm)

Significant changes took place in the length of earthworms after different treatments of heavy metals individually and in combination, minimum length (0.557±0.152cm) was recorded after 90th day of treatment in the tub treated with T9 (Copper and Tin @0.05+0.05 ppm), whereas in T3 (Cu 0.10 ppm) 0.630±0.027cm was observed (Figure 4). Also in control i.e. unpolluted slurry 6.41% increase in body length of worms observed after 90 days. Sn individually found not to be toxic for the worms as compare to copper. Maximum reduction in body length of earthworms was observed in combined metal exposure (T7, T8, T9) which prove that these metals produced synergistic effect on the worms.

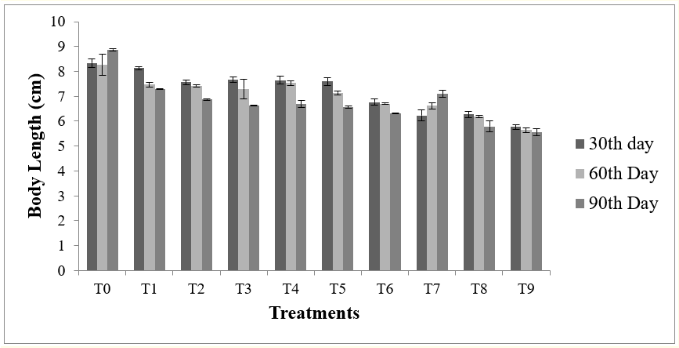

Figure 4: Effect of heavy metals toxicity on body length (cm) of earthworm.

Bio-molecules content (%)

Carbohydrates (%)

Figure 5 shows the effects of Cu, Sn and Cu+Sn on the carbohydrate content in earthworms. The carbohydrate content was explored after 45 and 90 days of heavy metal exposure. After 45 and 90 days of exposure carbohydrate content in all treatments except T0 at 45th day decreased significantly ($p < 0.05$) when compared to 1st day. The maximum percent reduction in carbohydrate content observed was 32.08% and 35.22% @T4 and T10 on 90th day as compare to 1st day of heavy metals exposure. Sn individually was found not to be toxic for the worms but in combination with Cu it causes synergistic effect and proved to be toxic.

Protein (%)

The protein content was markedly lower at T4 (Cu 0.10 ppm) and T9 (0.05+0.05 ppm) by 21.06% and 26.33% respectively af-

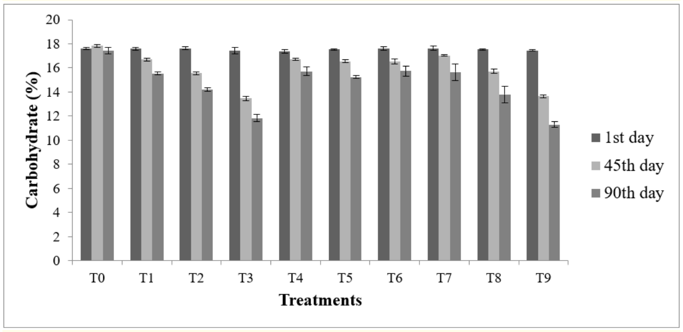

Figure 5: Effect of heavy metals toxicity on carbohydrate content (%) of earthworm.

ter 90 days of exposure ($p < 0.05$) (Figure 6). Protein content was determined by kjeldahl method. The result showed protein content decreased depending on metal concentration. No significant change in protein content of earthworms was observed when exposed to Sn (T4, T5, T6). After 45 day exposure protein content found to be decreased by 11.43%, 11.47% and 16.67% in T3, T8 and T9, respectively. Whereas after 90 days in T4, T9, T10 protein content decreased by 21.06%, 17.21%, 26.33% as compare to control.

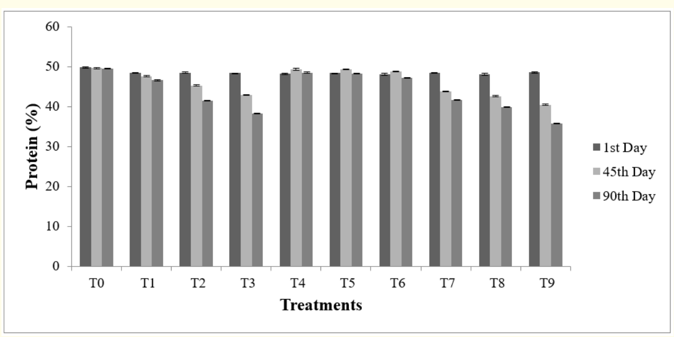

Figure 6: Effect of heavy metals toxicity on protein content (%) of earthworm.

Lipid (%)

Lipid content of earthworms was not significantly affected after 1st day of heavy metal exposure, while significant decrement were measured in T3, T4 and T9 by 31.25%, 40.79% and 31.07% after 90 days of exposure, respectively (Figure 7) as compare to 1st day. After 45th day in T8 group lipid content increased by 0.54% whereas in T3 and T9 group lipid content decreased by 18.57% and 11.53%, respectively. Lipid content did not decrease significantly in Sn treated groups.

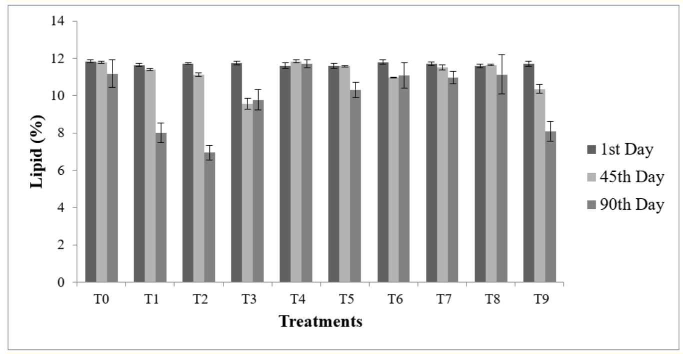

Figure 7: Effect of heavy metals toxicity on lipid content (%) of earthworm.

Discussion

Earthworms have the ability to decompose loads of organic matter into rich compost. Secondary these are helpful indicator of environment contamination and the survivability, reproduction and biomass of earthworm can be evaluated for the degree of contamination in environment/substrate. Nowadays earthworms are facing critical phase in the natural environment resulting in their depletion whose main reason is high degree of metal contamination and also increase in the pollution [6]. Earthworm biomarkers represent useful tools in soil monitoring and assessment as an early warning of adverse ecological effects [32,34]. They have becoming increasingly important in the last years in the evaluation of the effects of contaminants on soil micro/macro-biota [34]. In our study, the effects of heavy metals (Cu and Sn) on various growth parameters and biomolecules were evaluated in the earthworm *E. fetida* as potential biomarkers for soil heavy metal contamination.

[24] found that the number of earthworm species decreased significantly with increasing copper and zinc concentrations, which is similar to our results. A similar finding was reported by [38] in the surroundings of a smelter. A study on zinc toxicity

showed a significant mortality of *E. fetida* at Zn concentration 1200 µg/g dry weight [38]. [47] also observed mortality of earthworms in soil having Cd > 1000 mg/kg but Pb and Cr were found to be least toxic. [13] found addition of Cu as a soluble @≥ 2500 mg/kg in 100% mortality within 1 week whereas no mortality was observed in the control throughout experimental period. No significant deaths occurred upon increasing concentration of cadmium [53]. [55] observed decreased mortality rate also halted Cu uptake in the presence of Glyphosate (GPS). They proved that Cu toxicity reduced in the presence of GPS.

[1] showed that the production of cocoon and the viability of earthworm in the soil at concentrations >500 mg/g of copper and < 3.5% organic matter is decreased. But [4] found no clear relationship between cadmium concentrations in artificial soil and cocoon production. Reduction in cocoon production due to lead and cadmium exposure was reported in several studies [35,37,45,46]. High concentration of cadmium caused malformation of cocoons and also made the cocoons infertile [16]. Low lead levels were not affected in *Eudrillus euganiae* [39]. In a review it is mentioned that heavy metals significantly affected reproduction of earthworms by increasing embryonic time or by reducing the number and hatchability of egg capsules [31]. Hence it can be concluded that cocoon production is a sensitive parameter of toxicity for various metals and pollutants and this can be proved by laboratory exposures of various metals and pollutants to worms in artificial soil.

[15,37,49] indicated that earthworms exposed to higher Cu concentrations in soil tended to lose more weight than those in control, in accord with our studies. The weights of the earthworms in all treatments including the control were inhibited, probably due to the lack of suitable food in the OECD standard soil medium used [53]. A dose dependent reduction in growth, survivability, cocoon production and no. of hatchlings/cocoon was observed. Cd was found more toxic than Zn and Cd along with Zn act synergistically, thus proves more detrimental for worms [52]. [50], in an experiment using the earthworm *Aporrectodea caliginosa*, showed that Cu at 110 mg/g (based on dry soil) did not affect body weight relative to the control, whereas Zn at 1100 mg/g resulted in a reduction of about 50% of body weight. Body weight reduction due to metal exposure was observed in other studies [2,48] but no significant impact on body weight, or even an increase in weight, were also repeated [45].

A study of [21] concluded abnormal functioning of major physiological systems such digestion and absorption; they also reported a significant reduction in earthworm biomass after exposure to different concentration of copper chloride. [20] clearly demonstrated that the metal salts mixed into soil had a negative concentration related effect on the growth of *Aporrectodea caliginosa*. In ecotoxicological trials in the laboratory without plants glyphosate herbicide has also been shown to decrease the growth of *A. caliginosa* and reproductive output of compost worms (*E. andrei* and *E. fetida*). [22] explored reduction in body length after exposure to pesticide, this may be due to non-availability of energy, which resulted in the low gain in length in earthworms exposed to chemicals.

Biochemical changes are also better indices of change to animal tissue by pollution then conventional physiological changes. Various biochemical studies have been carried out at the level of whole animals, organ, tissue, sub-cellular organs [5]. After ingestion these metals in acid medium of the stomach converted to their respectively stable oxidation states (e.g. Zn^{2+}, Pb^{2+}, Hg^{2+}, Cd^{2+}, Ag^{2+}, As^{2+}, As^{3+}) and then they combine with the biomolecules of body i.e. proteins, enzymes to form stable and strong chemical bonds [30]. Earthworm biomolecules and physiological fluids including coelomic fluid and blood, offer an interesting field for utilization of novel sensitive non-destructive biomarkers.

As we know that carbohydrate present the principle and immediate energy precursor for earthworm exposed to stress conditions while protein is spared during chronic period of stress [44]. The depletion in carbohydrate was observed by [8] may be due to hypoxia as we know that hypoxia cause carbohydrate consumption. Similarly in one research, in response to earthworm exposure, secreted proteins bind metal cations, thereby reducing their toxicity [6,11]. [7,12], explored toxicity of metals, including Pb, may also bind to soluble or membrane biomolecules, such as enzymes, or react with sulfhydryl group of biomolecules, such as peptides or proteins. Influx of metals can also result to the degradation of macromolecules such as protein, lipid and DNA [19] thus leading to deleterious conditions such as lipid/protein peroxidation and genotoxicity [26]. The carbohydrates level has been decreased by 17.65% @0.06 ppm Hg, lipid and protein content decreased by 41.25% and 42.47 % respectively @0.06 ppm Hg [18]. [3] indicated that Aluminium caused reduction in earthworm protein content. The protein concentration was significantly negatively correlated with the soil aluminium content. [54] also observed that protein content in earthworms was much higher in control treatment as compare with the aluminium treatments. [28] concluded that highest mercury concentrations was able to cause depletion of GSH level and the organism damage determined through increased protein content.

Conclusion

This study demonstrated that there are significant impacts of high Cu and Sn on various growth parameters including survivability, body weight and length, cocoon numbers of *E. fetida*. Carbohydrates, Proteins and Lipids content (%) decreased, indicating the impact of heavy metals on earthworms.

Conflict of Intearest

The author(s) confirm that this article content has no conflict of interest.

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