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Assessment of Toxicity of Lead and Nickel on the Biochemical and Immunological Parameters of Earthworm, *Eudrilus eugeniae*

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ABSTRACT

Background: Soil heavy metal pollution is an important environmental concern that has serious adverse effects on soil biota. The present study is aimed to assess the toxicity of heavy metals; lead (Pb) and nickel (Ni) on the biochemical and immunological parameters of *Eudrilus eugeniae*.

Methods: Adult earthworms were exposed to various sub-lethal concentrations of Pb and Ni along with their combinations. After exposure for a period of 90 days, the content of bio-molecules such as carbohydrates, crude lipids and crude proteins; the total number of coelomocytes and the number of different types of coelomocytes were determined.

Result: The results showed that the bio-molecular content decreased gradually and the maximum decrease was recorded in T6 (Pb 0.06 ppm) followed by T9 (Ni 0.03 ppm+Pb 0.03 ppm). Similarly, a decline in the total number of coelomocytes and the number of different types of coelomocytes was observed in a dose-dependent manner. Structurally five different types of coelomocytes were distinguished: granulocytes I, granulocytes II, amoebocytes I, amoebocytes II and eleocytes. So, it can be concluded that heavy metals are highly toxic to earthworms and biochemical and immunological parameters can be used in environmental monitoring programs.

Key words: Bio-molecules, Coelomocytes, Earthworms, Heavy metals, Toxicity.

INTRODUCTION

Heavy metal pollution remains a serious concern and one of the major problems faced by the environment because of their bio-accumulative nature, toxicity, the ability to enter the food chain, persistence and negative impact on the living beings and ecosystem (Ali *et al*., 2019; Shefali *et al*., 2018). Soil represents the major sink for metals released into the environment from various sources, such as natural weathering of metal-containing rocks, soil erosion, mining, industrial emissions, smelting, accelerated use of fertilizers and pesticides, wastes and sludge residues (Jaishankar *et al*., 2014A; Calisi *et al*., 2014). Above critical limits, the toxicity of heavy metals in the soil becomes a significant problem from environmental, nutritional and ecological point of view (Jaishankar *et al*., 2014b; Nagajyoti *et al*., 2010). From the soil they can enter soil-dwelling organisms, resulting in toxic effects and bio-magnification to higher trophic levels. Due to the increasing heavy metal contamination of soil, it is necessary to assess the toxicity of heavy metals on soil-dwelling organisms (Kumar *et al*., 2020).

Among other soil-dwelling organisms, earthworms occupy an essential place in the soil ecosystem as they control key soil processes such as pedogenesis, soil structure, nutrient cycling, decomposition of organic matter, water infiltration and bioremediation of toxic chemicals (Giraddi *et al*., 2008; Kumar *et al*., 2020). Because of their close contact with the soil and sensitivity to soil pollution, they are particularly affected and can be considered as potential bio-indicators of soil pollution (Yang *et al*., 2017; Ramesh *et al*., 2022). The main route of exposure of earthworms to these metals is either through their highly permeable skin or by ingestion of soil particles (Jatwani *et al*., 2016). After entering the body of earthworms, heavy metals cause oxidative stress due to the formation of free radicles, which damage bio-molecules like lipids, nucleic acids and proteins (Engwa *et al*., 2019). They also interfere with normal metabolic functioning by interacting with various cellular components and result in cellular injuries (Ukpabi *et al*., 2013). The detailed study of biochemical responses, which are induced by heavy metals in earthworms, is the major biological approach for soil monitoring and assessment (Dedeke *et al*., 2016).

In addition to biochemical parameters, immunological parameters can also provide valuable information about potential risk factors for heavy metal contamination. In the earthworm immune system, coelomocytes function as immune effector cells, which are present in the coelomic fluid and are involved in various physiological functions

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such as, fighting pathogens and coping with environmental stresses (Hockner *et al*., 2020). Coelomocytes are very sensitive to a variety of pollutants and are widely used for the evaluation of the toxicity of a range of toxins and can be considered as biomarkers of toxicity (Yang *et al*., 2017). The entry of heavy metals through the dermal route directly affects coelomocytes and therefore impacts on health of earthworms (Muangphra and Gooneratne, 2011). Thus, evaluation of heavy metals induced changes in the biochemical and immunological profile of earthworms provides a potentially sensitive and accessible means of monitoring the effects of heavy metals on living organisms.

E. eugeniae is an epigeic species of earthworm that plays an important role in assessing the environmental impacts of pollution. They have been widely used as model organisms in various ecotoxicological studies (Doherty *et al*., 2019). Consequently, this investigation aimed to determine the toxicity of Pb and Ni on the biochemical and immunological parameters of *E. eugeniae*.

MATERIALS AND METHODS

Earthworms

The earthworms (*E. eugeniae*) used in the study were healthy and clitellated adults. These were procured from the Vermicomposting unit, situated at Department of Zoology and Aquaculture, CCS HAU, Hisar, Haryana. They were cultured in tubs containing cow dung as a substrate and had no history of input of agrochemicals or heavy metals.

Experimental exposure

The present study was carried out during 2017-18 at Department of Zoology and Aquaculture, CCSHAU Hisar, Haryana, India. Analytical grades of lead nitrate [Pb(II) ($Pb(NO_3)2$)] and nickel nitrate [$Ni(NO_3)2.6H_2O$] obtained from Hi-Media, were used in the present study and the treatments given to earthworms are detailed in Table 1. Clitellated earthworms were exposed to different doses of Pb and Ni for 90 days in tubs of 40-litre capacity. Three replicates per dose were maintained with controls. Proper temperature, moisture and aeration were maintained throughout the experiment.

Biochemical analysis

Earthworms were removed from each dose along with controls, before and 90 days after exposure to heavy metal stress. They were washed thoroughly in distilled water, blotted carefully and their gut contents were cleared by filter paper feeding method to avoid contamination. They were then washed with distilled water and left at 25°C for a period of 72 h. They were then homogenized with a pestle and mortar to obtain a brown colored paste. The paste was dried at 60±2°C for 24 h and kept at 4°C for further biochemical analysis. Total body tissue carbohydrates were analyzed by the Standard phenol sulphuric method (Masuko *et al*., 2005), total body lipids were estimated by the Soxhlet extraction method (Soxhlet, 1879) and total crude protein content was analyzed by following the Micro-Kjehdhal's method.

Harvesting of coelomocytes

The gut cleaned earthworms were placed in a sterile petri dish and used for the extraction of coelomocytes. Coelomic fluid containing coelomocytes was extracted by applying an electric shock of 6 volt for a period of 1 minute (Cooper *et al*., 1995). Due to this, the coelomic fluid was expelled through the dorsal pores. The extruded fluid was then diluted 1:100 with Ca^{2+} free LBSS (Lumbricus Balanced Salt Solution) containing 4.8 mM KCl, 71.5 mM NaCl, 0.4 mM KH_2PO_4, 1.1 mM $MgSO_4.7H_2O$, 4.2 mM $NaHCO_3$ and 0.3 mM Na_2HPO_4 (Diogene *et al*., 1997), which maintains the cell structure and physiological integrity.

Quantitative and qualitative analysis of coelomocytes

The total number of coelomocytes was counted visually by using a Neubauer haemocytometer in a light microscope. For qualitative analysis of coelomocytes, a drop of the coelomic fluid was placed on a slide to make a thin smear and was dried at room temperature for 24 hours. It was then stained with Leishman's stain and the number of each type of coelomocyte was calculated based on the morphology of coelomocytes such as granulocytes, amoebocytes and eleocytes by analyzing the slides under a light microscope.

Statistical analysis

All data are the mean±SE of three replicates. One way ANOVA was used as a statistical tool to analyze the critical difference at 5% (0.05) probability levels using Software 'OPSTAT', developed at Computer Centre, College of Basic Sciences and Humanities, CCS Haryana Agricultural University, Hisar.

RESULTS AND DISCUSSION

Effect of heavy metals on the biochemical parameters

Biochemical responses such as total tissue carbohydrates, crude lipid and crude protein content in *E. eugeniae* were determined under differing sub-lethal concentrations of Pb and Ni. After 90 days of exposure, increased heavy metals concentrations had a negative impact on total tissue carbohydrates, crude lipid and crude protein content. Heavy metal exposure significantly ($P<0.05$) decreased the content

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

Treatment	Description
Control	No heavy metals present
T1	Nickel 0.02 ppm
T2	Nickel 0.04 ppm
T3	Nickel 0.06 ppm
T4	Lead 0.02 ppm
T5	Lead 0.04ppm
T6	Lead 0.06 ppm
T7	Nickel 0.01ppm + Lead 0.01ppm
T8	Nickel 0.02 ppm + Lead 0.02 ppm
T9	Nickel 0.03 ppm + Lead 0.03 ppm

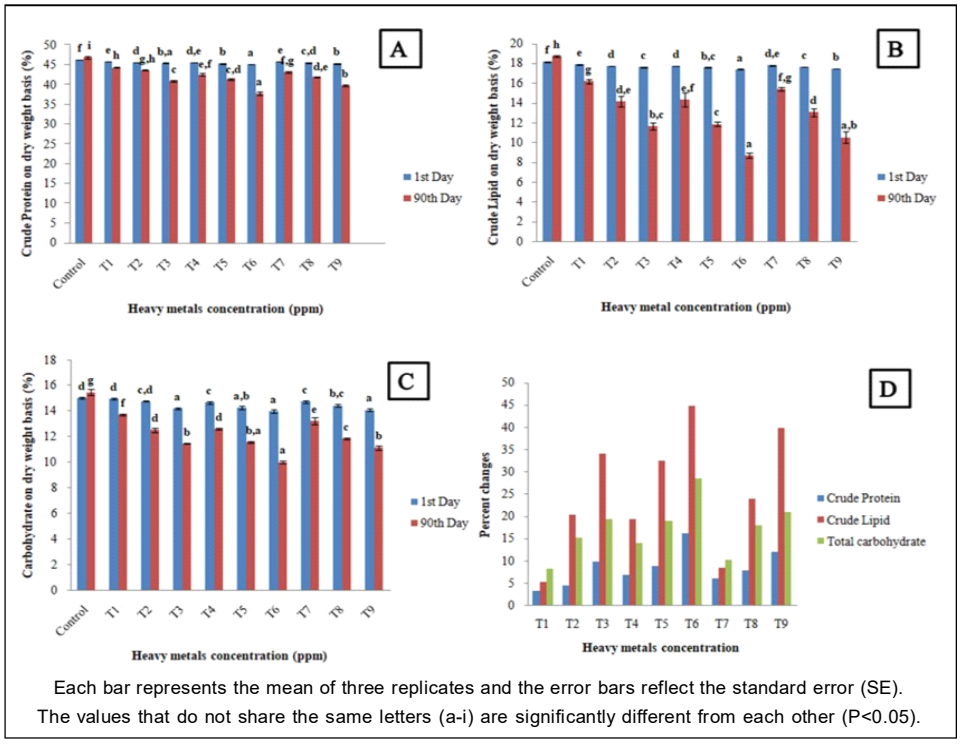
of bio-molecules in a dose-dependent manner as compared to control. T6 treatment showed the maximum reduction in total tissue carbohydrates (28.55%), crude lipid (44.74%) and crude protein (16.23%). The content of these parameters was also reported to decrease in the presence of Ni in dose-dependent manner. However, exposure to Pb was determined to be more harmful, as it resulted in greater loss in content of studied bio-molecules (Fig 1).

Earthworms (*Eisenia fetida* and *Eudrilus eugeniae*) exposed to sub-lethal concentrations of heavy metals have previously been reported to have decreased content of biomolecules such as carbohydrates, lipids and proteins (Jatwani *et al*., 2016; Urmila *et al*., 2019). Our results also corroborate the study of Zhang *et al*. (2012) who reported that Al caused a decrease in protein content in *Eisenia fetida*. Similarly Bilalis *et al*. (2013) in their experiment found that the protein composition decreased in Al treated earthworms (*Octodrilus complanatus*) compared to control. Novais *et al*. (2013) demonstrated that in *Enchytraeus albidus,* cadmium (Cd) exposure resulted in lipid depletion followed by complete carbohydrate consumption. Vaidya (2016) reported that in the mercuric chloride (25 and 30 mg/kg soil) treated earthworm (*Perionyx excavates*), total protein level decreased significantly by 13% and 26.05% in the ovary and 10.99% and 24.21% in the testis. When exposed to pollutants, earthworms reduce the toxicity of the chemical by changing their internal biochemical reactions before affecting the growth. Therefore, the biochemical changes are very important to assess the potential negative effects of pollutants on earthworms (Vaidya, 2016). Heavy metals generate free radicles and induce oxidative stress and nitrative stress, which further lead to oxidation of sulfhydryl groups, consumption of thiol proteins, protein depletion, lipid peroxidation, DNA damage and reactions with DNA and nuclear proteins that cause deterioration of bio-molecules such as lipids, nucleic acids and proteins (Mathew *et al*., 2011; Engwa *et al*., 2019; Jan *et al*., 2015; Morcillo *et al*., 2016). According to Damien *et al*. (2004) reactive oxygen species such as superoxide radicals, hydroxyl radicals and hydrogen peroxide mainly affect proteins, carbohydrates, lipids and nucleic acid. Lead causes toxicity in living cells by using ionic mechanism and by exerting oxidative stress (Jaishankar *et al*., 2014b; Jan *et al*., 2015).

Effect of heavy metals on the total number of coelomocytes

The total number of coelomocytes per unit volume (ml) of the coelomic fluid was calculated and the results showed that the exposure of earthworms to various concentrations of Pb and Ni for 90 days resulted in a significant ($P<0.05$) reduction in the total number of coelomocytes as compared to control (Fig 2). Dose and time-dependent decrease in all treatments were reported. The maximum reduction in the number of coelomocytes *i.e*. 67.39% on day 90 was observed in worms exposed to T6 followed by a 51.53% reduction in T9.

Different studies have confirmed the sensitivity of coelomocytes towards heavy metals exposure (Hayashi *et al*., 2012; van der Ploeg *et al*., 2014; Irizar *et al*., 2014a, b;



Each bar represents the mean of three replicates and the error bars reflect the standard error (SE). The values that do not share the same letters (a-i) are significantly different from each other ($P<0.05$).

Fig 1: A. Effect of heavy metals on the crude protein content; B. Effect of heavy metals on crude lipid content; C. Effect of heavy metals on total tissue carbohydrate content; D. Per cent changes in bio-molecules concentration of *E. eugeniae*.

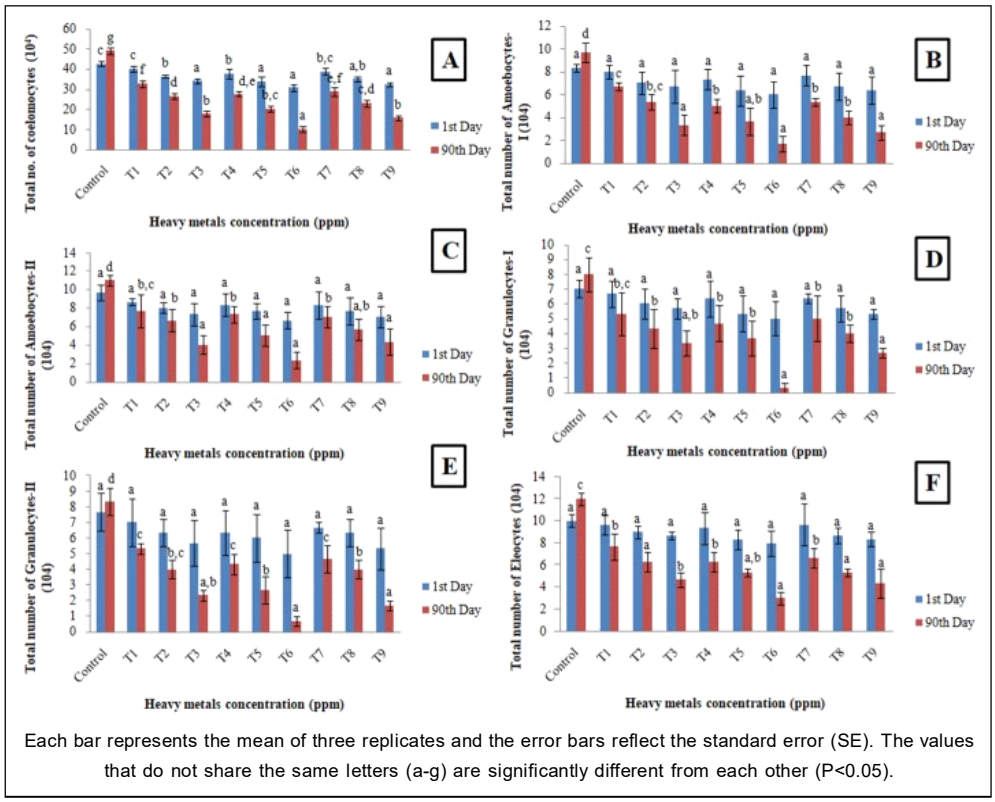


Fig 2: A. Effect of heavy metals on the total number of coelomocytes; B. Effect of heavy metals on the total number of Amoebocytes-I; C. Effect of heavy metals on the total number of Amoebocytes-II; D. Effect of heavy metals on the total number of Granulocytes-I; E. Effect of heavy metals on the total number of Granulocytes-II; F. Effect of heavy metals on the total number of Eleocytes in *E. eugeniae*.

Irizar *et al*., 2015a, b). A similar pattern of results was obtained in the study of Homa *et al*. (2010) who reported that heavy metals significantly reduced the viability and activity of coelomocytes in earthworm *A. chlorotica*. Moreover, Homa *et al*. (2005) observed that coelomocytes were particularly sensitive to certain heavy metals as they showed differential up-regulation of metallothionein when exposed to a 1.32 μ g cm^{-2} concentration of each metal ion. Ray *et al*. (2019) reported in their study that the total numbers of coelomocytes of earthworm *M. posthuma*, collected from various metal contaminated sites were declined in each season. Earlier Podolak *et al*. (2011) in their study found that coelomocytes of Zn exposed worms displayed a significant reduction compared to control.

Effect of heavy metals on the different subpopulation of coelomocytes

On the basis of morphology, three major types of coelomocytes were distinguished; Granulocytes, amoebocytes and eleocytes. Of these, granulocytes and amoebocytes were further divided into type I and type II (Fig 3 and 4). Along with these five cell types, some other cells were also observed that either represent immature stages or represent disintegrated parts of the main coelomocyte types.

Granulocytes

Based on the distribution of the granules, two types of granulocytes were distinguished: granulocytes I and granulocytes II (Fig 4A and B). Granulocytes I had abundant numbers of characteristic dense granules distributed evenly. They ranged from spherical to oval in shape with an eccentrically located nucleus. Granulocytes II on the other hand had distinctive prominent vesicular structures or blebs on their cell surface. They were medium in size and ranged in shape from spherical to club-like with an eccentric nucleus. Granulocytes showed no tendency to form aggregates.

Amoebocytes

Based on the distribution and size of pseudopodia, two types of amoebocytes were identified: amoebocytes I and amoebocytes II (Fig 4B, C, D and E). Amoebocytes I formed one or two small and regularly distributed pseudopodia called lobopodia. They had large, oval and centrally located nuclei. Their cytoplasm contained a large number of lysosomes, vacuoles and round vesicles. Amoebocytes II had a large bean-shaped nucleus and usually formed numerous radiating and long pseudopodia, usually concentrated at one pole of the cell.

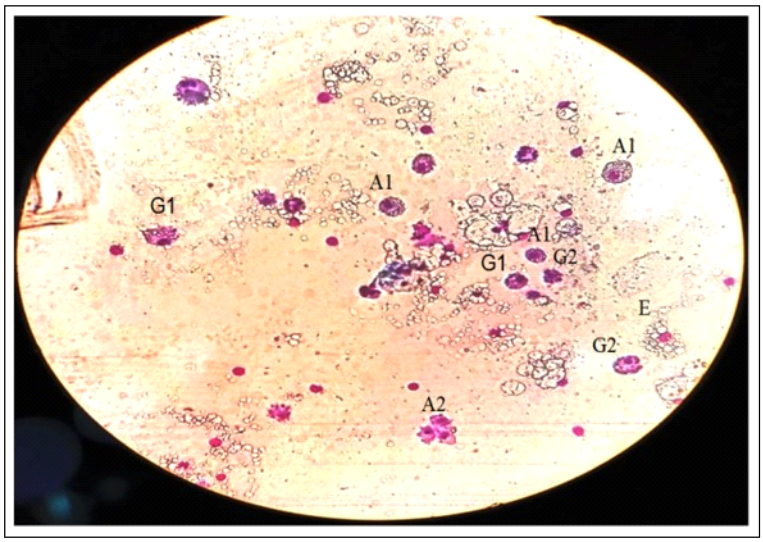


Fig 3: Different types of coelomocytes of *E. eugeniae* (A1- Amoebocyte I, A2- Amoebocyte II, G1- Granulocyte I, G2- Granulocyte II and E- Eleocyte). The cells were stained with leishman's stain. Objective utilized 40 ×.

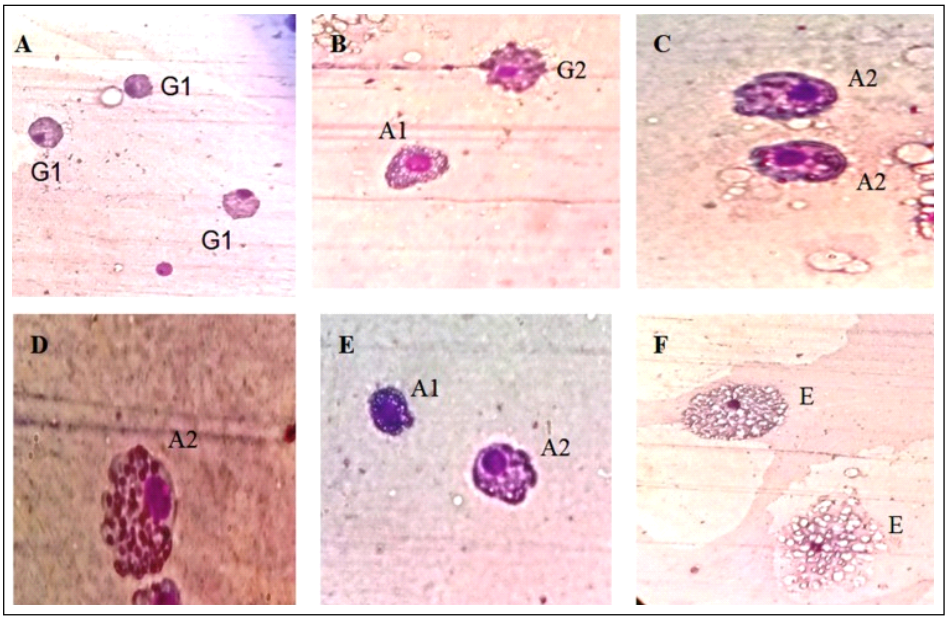


Fig 4: Coelomocytes of *E. eugeniae*: A. Granulocytes I B. Granulocytes II and Amoebocytes I C. Amoebocytes II D. Amoebocytes II E. Amoebocytes I and Amoebocytes II F. Eleocytes. The cells were stained with leishman's stain. Objective utilized: 100 × oil immersion.

Eleocytes or chloragocytes

They had small and spherical nuclei and a variety of large specialized polymorphic granules or chloragosomes in their cytoplasm (Fig 4F). They were the largest cells found in the coelomic fluid with very little motility. They often disintegrate upon contact with the substrate and release chloragosomes due to the presence of a delicate cell membrane (Adamowicz, 2005).

As compared to control group, all kinds of earthworm coelomocytes decreased significantly ($P<0.05$) with an increase in heavy metal concentration and with time over the 90-day experiment (Fig 2). On the other hand, no significant changes were observed on the first day. T6 treatment resulted in a significant ($P<0.05$) decrease in granulocytes-I (93.4%), granulocytes-II (86.6%), amoebocytes-I (72.17%), amoebocytes-II (65.07%) and eleocytes (62.50%). According to findings, *E. eugeniae* coelomocytes, particularly granulocytes-I and granulocytes-II, were very sensitive to even the lowest sub-lethal concentration of heavy metals and reacted in a dose-dependent way.

Despite a lot of morphological and structural studies, the classification of coelomocytes of earthworms is still not well standardized. It is difficult to determine whether particular cells represent different types of coelomocytes or

different developmental stages of same coelomocytes (Adamowicz, 2005). Calisi *et al*. (2009) in their study observed five types of coelomocytes in *Eisenia foetida*: granulocytes, eleocytes, neutrophils, leukocytes I and leukocytes II. Kurek *et al*. (2007) distinguished three main types of cells in the coelomic fluid of *Allolobophora chlorotica*. These were granular amoebocytes, hyaline amoebocytes and eleocytes. Adamowicz (2005) identified three major types of coelomocytes in *Dendrobaena veneta*: granulocytes, amoebocytes and eleocytes.

The results of our study confirmed the five distinct types of coelomocytes, as previously reported (Adamowicz and Wojtaszek, 2001; Adamowicz, 2005; Manazhy *et al*., 2010; Hatti, 2013). The reduced number of amoebocytes may be due to exhaustion of proliferation or development of multicellular bodies, which are too large and cannot be extruded by electric shock (Takacs *et al*., 2016). Chatterjee *et al*. (2017) reported the reduced number of auto-fluroscent eleocytes and amoebocytes in the coelomic fluid of earthworms treated with heavy metals compared to control. Similarly Irizar *et al*. (2015b) observed the massive mortality of eleocytes and amoebocytes in *Eisenia fetida* upon exposure to various heavy metals. They also found that eleocytes were more sensitive to metal exposure as compared to amoebocytes.

CONCLUSION

Earthworms exposed to Pb and Ni showed a significant reduction in the content of biochemical parameters such as crude protein, crude lipid and total tissue carbohydrate. Similarly immunological parameters such as total number of coelomocytes and number of different sub-population of coelomocytes (*i.e*. granulocytes I and II, amoebocytes I and II and eleocytes) in heavy metals (Pb and Ni) exposed *E. eugeniae* displayed a mark reduction in numbers, indicating heavy metals toxicity to the earthworm's immune system. Although both Pb and Ni heavy metals were highly toxic to earthworms, Pb was found to be more toxic as it caused greater bio-molecular loss and decreased number of coelomocytes. The present investigation clearly demonstrates that the biochemical and immunological parameters of *E. eugeniae* are highly sensitive to heavy metal contamination and can be used as biomarkers to assess heavy metals toxicity to earthworms.

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Conflict of interest

All authors declare that they have no conflict of interest.

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