Effect of heavy metals concentration on bio-molecules and immunity parameters of earthworm, *Eisenia fetida*

BY

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CERTIFICATE-I

This is to certify that this thesis entitled, **"Effect of heavy metal on bio-molecules and immunity parameters of earthworm, *Eisenia fetida*"** submitted for the degree of Doctor of Philosophy in department of Zoology to the Chaudhary Charan Singh Haryana Agricultural University, Hisar, is a bonafide research work carried out by **Ms. Chhavi Jatwani** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation has been fully acknowledged.

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CERTIFICATE – II

This is to certify that the thesis entitled, **"Effect of heavy metal on bio-molecules and immunity parameters of earthworm, *Eisenia fetida*"** submitted by **Miss Chhavi Jatwani** to the Chaudhary Charan Singh Haryana Agricultural University, Hisar, in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in the subject of **Zoology,** has been approved by the Student's Advisory Committee after an oral examination on the same in collaboration with an External Examiner.

MAJOR ADVISOR **EXTERNAL EXAMINER**

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DEAN, POSTGRADUATE STUDIES

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Place:

Date:

CONTENT

CHAPTER-I

INTRODUCTION

Earthworms (EWs) are a major component of soil micro fauna communities in most ecosystems and comprise a large proportion of macro fauna biomass. Earthworms seem to accelerate the mineralization as well as the turnover of soil organic matter. They increase nitrogen mineralization, through direct and indirect effects on the microbial community. The increased transfer of organic C and N into soil aggregates indicates the potential of earthworms to facilitate soil organic matter stabilization and accumulation in agricultural systems, and that their influence depends greatly on differences in land management practices. Their biomass constitute up to 60-80% of the animal biomass in some soil ecosystem and their burrowing activity is of utmost important for aeration and stability of soil. Their activity is beneficial because it can enhance soil nutrient cycling through the rapid incorporation of detritus into mineral soils. In addition to this mixing effect, mucus production associated with water excretion in earthworm guts also enhances the activity of other beneficial soil microorganisms. This is followed by the production of organic matter. So, in the short term, a more significant effect is the concentration of large quantities of nutrients (N, P, K, and Ca) that are easily assimilable by plants in fresh cast depositions.

The ever growing demand for population in the world has been closely linked with intensifying the industrialization to facilitate higher need of people. Due to their improper disposal of industrial wastes. There is an increase in heavy metal in agro ecosystems is one of the most critical consequence of industrialization. The long-term accumulation of heavy metals in the soil environment is a concern because they potentially have important consequences for the quality of the human food chain, toxicity to plants and soil microbial processes and once applied they have very long residence times in soil. The principal environmental end-points for heavy metals applied to soil in all organic residual materials including compost (Chaney and Ryan, 1993; Deportes, *et al*., 1995), are:

- Reduced plant growth (phytotoxicity) due to phytoaccumulation in plant tissues above tolerable threshold values (Zn, Cu, Ni; Cr may also be listed here, but there is no evidence of crop damage due to Cr in sewage sludge or compost-amended soil).
- Human food chain via crop uptake (Cd).
- Human food chain via direct child ingestion of compost, e.g. applied to home gardens (Cd, Pb, Hg).
- Human food chain via offal meat from animals ingesting compost treated soil (Cd and Pb);
- Animal health (Cu, Pb).
- Soil microbial processes (Zn).

Earthworms can be exposed by direct dermal contact with heavy metals in the soil solution or by ingestion of pore water, polluted food and/or soil particles. Data reported in the literature indicate that soluble metal concentrations are the best descriptors of bioaccumulation in earthworms. Earthworms could be used to extract toxic heavy metals, including cadmium and lead, from solid waste from domestic refuse collection and waste from vegetable and flower markets, according to researchers writing in the *International Journal of Environment and Waste Management*.

The process of vermicomposting in this way allows such waste materials to be remediated and the compost used subsequently for use in growing human food without the risk of accumulating heavy metals in crops. The team says that up to about three-quarters of the various heavy metals can be removed by the worms from solid waste. The *E. eugeniae* species was the most effective worm at remediating solid waste and producing rich compost. The team's tests on vermicomposting reveal that the heavy metal content of such waste can be reduced to levels significantly below the permissible safe limits.

The worm's digestive system is apparently capable of detaching heavy metal ions from the complex aggregates between these ions and humic substances in the waste as it rots. Various enzyme-driven process then seem to lead to assimilation of the metal ions by the worms so that they are locked up in the organism's tissues rather than being released back into the compost as worm casts. The separation of dead worms from compost is a relatively straightforward process allowing the heavy metal to be removed from the organic waste.

Coelomocytes are considered the immune cells of lower coelomate animals (annelida, mollusca, arthropoda). These cells are types of leukocytes that have long been considered to constitute the major innate (unspecific) immune defense system of these animals (Hostetter and Cooper, 1972; Engelmann, *et al.,* 2005). They respond to environmental and experimental challenge by the activation of stress markers, including heat shock proteins such as hsp70 and metallothioneins (Matranga, *et al.,* 2002; Homa, *et al.,* 2005). These cells types are studied because they provide information about mechanisms governing innate immunity.

Coelomocytes of Oligochaetes are characterized by a pronounced polymorphism, and their quantitative and qualitative compositions vary depending on environmental factors, individual age and physical condition (Aval, 1959). Earlier attempts at coelomocyte classification based on morphological and functional criteria, have not gained common acceptance. The reason for the lack of a uniform classification system is that the coelomic fluid contains cells at various functional states and various stages of maturation. Cooper and Stein (1981) distinguished three types of coelomocytes in *Lumbricus terrestris* - hyaline amoebocytes, granular amoebocytes and eleocytes. However, Jarosz and Glinski (1997) divided eleocytes, called also chloragogenic cells, into I and II eleocytes. Eleocytes originate from chloragogenic tissue surrounding the intestine (Jamieson, 1981; Jamieson, 1992; Affar, *et al.,* 1998) and are present in all the examined Oligochaete species. Chloragocytes, called

also chloragogen cells or eleocytes, constitute a subpopulation of phagocytic coelomocytes. These cells contain characteristic granules, called chloragosomes thought to be involved in the protection of cells and organisms against foreign substances (Adamowicz, *et al*, 2005; Muravev, *et al*, 1994) and have been associated with lytic activities (Kauschke, *et al.*, 2001; Koenig, *et al.*, 2003; Peeters-Joris, 2000).

Chloragogenic cells are responsible for maintaining a constant pH and ionic balance of both coelomic fluid and haemolymph (Prento, 1979). Affar *et al.* (1998) opined that they synthesize extra-cellular respiratory pigments (Needham, 1966, Roots and Johnston, 1966; Fischer, 1977) which are capable of storing endogenous substances, such as glycogen and lipids (Roots, 1957; Roots and Johnston, 1966; Ireland and Richards, 1977) and exogenous substances, such as pigments (Roots and Johnson, 1966; Needham, 1966) or metals (Prento, 1979; Morgan, 1979). Eleocytes play an important part in immune process in lumbricids, producing bactericidal substances participating in reactions of encapsulation and formation of brown bodies. The second type of coelomocytes are amoebocytes among which hyaline and granular cells were distinguished, but till now no unambiguous relation has been found between the hyaline and granular cells, though numerous authors suggest that they may be of common origin (Cooper and Stein, 1981). Amoebocytes participate in the transport and storage of nutritive substances (Valembois and Cazaux, 1970), coelomic fluid coagulation and wound healing (Byzowa, 1974) immune reactions of humoral system (Cooper and Roach, 1986; Jarosz and Glinski, 1997) cellular defense reactions (Cooper, 1996) phagocytosis (Cossoarizza, *et al.*, 1996) as well as encapsulation and nodulation (Valembois, *et al.*, 1992)

Earthworm (*Eisenia fetida*) coelomocytes have been shown to express a protein with features of perforin, which is involved in cytotoxic activities (Kauschke, *et al*, 2001). The five major categories of coelomocytes in the earthworm *Lumbricus terrestris* consist of basophils, acidophils, neutrophils, granulocytes, and chloragogen cells. All cell types, with the exception of chloragogen cells, produce pseudopodia and are capable of phagocytosis (Stein, *et al*, 1977; Linthicum, *et al.*, 1977).

Lumbricus terrestris coelomocytes can act as effector cells that can specifically kill non self target cells (Suzuki and Cooper, 1995; Engelmann, *et al.*, 2004). Graft rejection in *Lumbricus terrestris*, is mediated by granulocytic coelomocyte (Linthicum, *et al.*, 1977). Cytotoxic small coelomocytes of the earthworm have been shown to express CD11a, CD45RA, CD45R0, CD49b, CD54, CD90, CD24. They also express Beta-2-Microglobulin and TNF-alpha. Phagocytic large coelomocytes do not express these markers. (Cossarizza, *et al.*, 1996). Engelmann *et al.* (2002) have detected cytokines (TNF-alpha, TGF-alpha alpha peptide hormone, thyroid stimulating hormone) in sub populations of earthworm coelomocytes with monoclonal antibodies developed originally against human and/or mouse antigens. Two coelomocyte subtypes express CD90, CD24 and TNF-alpha antibodies, whereas another subpopulation is negative for these markers.

Antioxidant activity is directly attributed to phenolic compounds that play a vital role in neutralizing the free radicals because phenolics have a hydroxyl group (Balamurugan, *et al*., 2007). Limited studies have been done on the extraction of phenolic compounds from the earthworm species *Lumbricus rubellus* (red worm) and *Eudrilus eugeniae* (African night crawler). Most of the earlier studies focused primarily on the activity of different biologically active compounds such as glycolipoprotein. Some of these studies assessed the activity of glycolipoprotein molecules (G-90) (Grdisa, *et al*., 2001; Balamurugan, *et al*., 2008).

Keeping in view the above facts and need for search of effect of heavy metals concentration on bio-molecules and immunity parameters of earthworm, *Eisenia fetida,* it was decided to investigate its damage potential and management under the following objectives:

- To estimate the effect of heavy metals on the protein, carbohydrate and lipid constituents of *Eisenia fetida*
- To study the effect of heavy metals on the qualitative and quantitative parameter of coelomocytes of *Eisenia fetida*
- To study the effect of heavy metals on antioxidant activity of earthworm, *Eisenia fetida*

CHAPTER-II

REVIEW OF LITERATRE

The main thrust of ecotoxicological work is to provide information to estimate ecological risk. Biomarkers are used as a sensitive; "early warning" tool for impact assessment of various contaminants on terrestrial ecosystem. The term biomarkers has been defined as any functional measure of exposure that is characterized at a suborganism level of biological organizations (Adams, *et al*., 2003). The National Research Council of US (NRC, 1987, 1989) defines biomarkers as "Indicators of events in biological systems or samples" and was further described as "tools that can be used to clarify the relationship, if any, between exposure to a xenobiotic substance and disease". Also, the NRC and WHO (2001) classified biomarkers into three categories based on their relation to the exposure-disease continuum: biomarkers of exposure, effect and susceptibility. Good biomarkers are sensitive indices of both pollutant bioavailability and early biological responses. The success of biomarkers in supporting ecological risk assessment depends importantly on the identification of valid biomarkers and the establishment of process-level linkages between biomarkers and higher-level responses.

The popularity of earthworms as excellent bioindicators makes them a robust model for assessing soil pollution. In ecotoxicology of earthworm, there exist a range of biomarkers of toxic compounds, including biomarkers from the molecular to the organismal level like metallothioneins, stress proteins, cholinesterases, detoxification enzymes, parameters of oxidative stress and others. Measurement of biomarkers in invertebrates or earthworms certainly provides valuable information on both the ecological impact of long-term chemical contamination (diagnostic approach) and the conditions of biological restoration of damaged ecosystems (predictive approach). The present communication will highlight the use of earthworm biomarkers to have impact assessment of toxicological compounds in use.

Soil is a complex mixture of mineral matter, organic matter and its fauna. Management of soil quality i.e. fertility and functioning of tropical ecosystem is dominantly depends upon its soil fauna which are the main consumers and decomposers of the soil ecosystem (Handrix, 2000) Recently, growing interests on soil fauna have made them attractive model for monitoring adverse ecological impact of pollutants. The plentifulness or activity of soil macrofauna has been used as an indicator for evaluating the biological health of soils (Bauble and Schmidt, 1997). Earthworms are excellent bio-indicator of relative health of ecosystem among other terrestrial invertebrates, as they possess a number of qualities that predispose them for use in environmental monitoring and remediation of soil (Reinecke and Reinecke, 2004; Ricketts, *et al*., 2004). A wide range of biomarkers have been developed in

earthworms like behavioral, reproductive, enzymological, lysosomal, genetic, immunological, neurological and histopathological, including the effect of both organic and inorganic substances (Ali, *et al*., 2012).

Soil pollution has enormously increased during the last decades due to the intensive use of biocides and fertilizers in agriculture, industrial activities, urban waste and atmospheric deposition. Its occurrence is related to the degree of industrialization and intensity of chemical usage. Soil pollution causes decrease in soil fertility, alteration of soil structure, disturbance of the balance between flora and fauna residing in the soil, contamination of the crops, and contamination of groundwater, constituting a threat for living organisms. The most diffusive chemicals occurring in soil are heavy metals, pesticides, petroleum hydrocarbons, polychlorobiphenyl (PCBs), dibenzop- dioxins/diben-zofurans (PCDD/Fs).

The total metal contents and the DTPA-extractable fractions of soils are positively and significantly related to the concentrations of metals in the earthworms. Similar trends for the ratios of the total metal contents in soils to earthworm contents have been observed by Morgan and Morgan (1993, 1999), Abdul Rida and Bouche (1997), and Kennette, *et al*. (2002). The concentrations of Cu and Pb in the earthworm tissues were of the same order than those reported from the literature for other contaminated soils by Kennette *et al*. (2002). The fractions of metals extracted by DTPA shows similar correlations with the concentrations of the metals in earthworm tissues than those of the total metal content in soils.

Bioaccumulation of metals in earthworms is their ability to eliminate the excess of metals. Spurgeon and Hopkin, (1999) showed that for essential metals, such as Cu and Zn, a fast initial uptake was followed by equilibrium after a few days of exposure, highlighting a physiological control and a possible excretion of these elements. For the xenobiotic metals, such as Cd and Pb, the excretion was slow or absent. The same bioconcentrations of Cu in earthworms leading to the decrease of the BSAF for polluted soils. For Zn, even if the concentrations were high, the BSAF were also lower in the most contaminated soils. Excretion of a part of Zn could contribute to the regulation of the metal concentrations in earthworms

2.1 Effect of heavy metal toxicity on protein carbohydrates, lipid and protein content of earthworm

Earthworms are among the most sensitive soil invertebrates when it comes to metal toxicity (Bengtsson and Tranvik, 1989) and given the ecological importance of these organisms earthworms have been of particular concern in metal-contaminated areas (Edwards and Bohlen, 1996). Sublethal stress from toxicants such as heavy metals can induce compensatory changes in the energy metabolism of organisms due to increased energy expenses associated with detoxification processes. Since most of an organism’s energy consumption is used for growth, reproduction and basal metabolism, coping with toxic stress

can lead to reductions in growth and reproduction and thus the scope for population growth (Sibly and Calow, 1989; Calow, 1989; Maltby, 2005). It has been shown that the difference between available energy reserves (glycogen, fat and protein) and energy consumption is indicative of an organism's overall condition, and that this correlates with higher levels of biological organization such as population growth rates (De Coen and Janssen, 2003). A decrease in the available energy reserve can therefore be used as a biomarker of metal stress (Scott-Fordsmand and Weeks, 2000).

Carbohydrates represent the principal and immediate energy precursors for earthworm exposed to stress condition while protein is spared during chronic period of pollutant stress (Umminger, 1970). The observed depletion in carbohydrates may be due to hypoxia, since hypoxia increase carbohydrate consumption (Dezwaan and Zandee, 1972). Severe environmental hypoxia caused by pollutants is responsible for rapid depletion of stores of carbohydrate (Heath and Pritchard, 1965).

The animal utilized carbohydrate as a source of energy in tissue. It is therefore obvious that depletion of carbohydrate may be due to its direct utilization for energy generation demand caused by pesticide induced hypoxia. Dezwaan and Zandee (1972) reported that carbohydrate metabolism gets altered during toxic stress and decrease may be due to the prevalence of hypoxic and anoxic conditions which normally increase carbohydrate utilization.

Another approach to identifying the toxic pressure of metals to soil organisms is by determining uptake and elimination fluxes, also known as kinetic modeling. As demonstrated for isopods by Van Straalen, *et al* (2005). the use of fluxes increases the predictability of toxic effects as compared to total body burdens. This is due to the ability of isopods of metal sequestration. Like isopods, earthworms are capable of sequestering metals at high internal levels, and different strategies may be used for this purpose. Specific strategies of metal handling can also be expected by essential metals.

Essential metals may be subject to regulation either by limiting metal uptake at the level of the total body content, or by involving organism-specific accumulation strategies with active excretion from the metal excess pool, storage in an inert form, and/or excretion of stored (detoxified) metal (Rainbow, *et al*,. 2002). Nevertheless, tissue- and organ-specific metal accumulation are ultimately determined by cellular mechanisms. At the cellular level, biota have evolved control mechanisms to minimize accumulation of reactive metal species and to facilitate optimal utilization of essential metals. The excess of metal ions are potentially toxic and must be removed from the vicinity of important biological molecules. This is achieved by the various chemical forms in which metals can be present, including binding in the active center of functional proteins and enzymes, binding in the active center of enzymes, binding to metallothionein (MT), transport proteins, or other sequestration proteins,

and precipitation in extracellular granules, mineral deposits, residual bodies, and exoskeletons(Vijver, *et al*., 2004).

The decrease in protein content may be attributed either to increased turnover rate of protein or interference of the toxicant at certain site during protein synthesis (Rao, *et al.*, 2003). It is established fact that proteins are used as alternate energy source especially under stress conditions. It is expected that *Eisenia fetida* and *Perionyx excavatus* also must have utilized protein for the production of energy to mitigate the stress caused by heavy metals. These results are supported by several investigators who reported decline in protein content in different organisms under the influence of heavy metals (Sturzenbaum, *et al*., 2001).

Earthworm acute and chronic toxicity tests are used to assess the potential hazards of various environmental pollutants to soil ecosystems, as well as in risk assessment procedures. Earthworms, especially the compost worm *Eisenia fetida*, are model organisms for assessing the eff ects of various chemicals on terrestrial invertebrates (Spurgeon, *et al*., 2003; Nahmani, *et al*., 2007). Earthworm survival and reproductions tests are relatively easy to conduct, require little maintenance and are low-cost. Acute and chronic earthworm toxicity tests were successfully applied to assess the toxicity of contaminated soils in urban areas (Pizl-Josens, 1995; Jager *et al*., 2005), in mining and smelting areas (Tervihuo, *et al.*, 1994; Honsi, *et al*., 2003; Alvarenga, *et al*., 2008), in industrial (Saterbak, *et al*., 1999) and other areas (Berthelot, *et al*., 2008; van Gestel, *et al*., 2009). Earthworms are also frequently used in various mesocosms and test batteries (Gunderson, *et al*., 1997; Rombke, *et al*., 2006). Screening of contaminated soils is conducted using and combining field and laboratory tests.

Growth is an integral parameter easy to measure, and it integrates a suite of biochemical and physiological effects. It represents changes in individual energy budgets as the exposed organisms have to expand their energy to metabolism, detoxification or sequestration, and excretion of the contaminants. This additional energy requirement results in a decrease of growth. The growth and reproduction of earthworms exposed to metals were negatively correlated, i. e. if the earthworms did not grow, they produced cocoons, and vice versa, if they did not produce cocoons, they continued growing (Burgos, *et al*., 2005).

The earthworm reproduction test is generally considered to be more sensitive than the acute toxicity test because it can evaluate the effects of sublethal concentrations of chemicals far below a given LC50 concentration for earthworms. Furthermore, the earthworm reproduction test is widely regarded to be of greater signifi cance for predicting the impacts on soil ecosystems. Reproduction is very important in ecotoxicological assessment as it influences the population dynamics. Reproduction rate was shown to be a sensitive endpoint in toxicity tests of various metals and organic compounds (such as hydrocarbons, PCB, pesticides) (Van Gestel, *et al*., 1989; Hubalek, *et al*., 2007).

Lipids are one of the most important sources of energy and structural components in animal. Decrease in lipid content suggests that mobilization of energy rich lipids for production of energy during toxic stress caused by metals. The decrease in lipid content correlates with the increased activity of lipase, the energy responsible for the breakdown of lipids into free fatty acid and glycerol. Decrease in tissue lipid under toxicant stress could be due to formation of lipoproteins, which are used to provide energy.

Effect of heavy metal on coelomocytes of earthworm

Diogene *et al.* (1997) extruded Coelomocytes from three earthworm species: *Lumbricus terrestris, Eisenia fetida* and *Octolasion tyrtaeum.* Featuring a simple low-vacuum holding device, the proposed methodology allows the recovery of cells with minimum risk of contamination by faecal material. The viability of O. *tyrtaeum* coelomocytes was highly reproducible (average 93%), with an average yield of 0.92 x 10^6 viable cells per earthworm. Cell viability for *L. terrestris* and *E. fetida* averaged -68% but the cell yields were higher (respectively 1.67 x 10^6 and 1.28 x 10^6). Large inter-individual differences in cell yields were observed with *L. Terrestris.* Flow cytometric analyses indicated species to species differences in cell populations. Coelomocytes from *E. fetida* were the smallest with -57% of the total viable cells recovered being monitored between 2 and 10 μm. Large granulated cells I~20 μm were detected in fairly large proportions in *L. terrestris* and *O. tyrtaeum* [-52 and -96%, respectively I while they were less abundant in *E. fetida* (-9%). Using the vital dye neutral red to assess functional integrity, average cellular uptakes were significantly higher for 1. *terrestris* and O. *tyrtaeum* than for *E. fetida*.

Kasschau, *et al.* (2006) stated that coelomocytes from *L. terrestris* respond to an increase in environmental osmotic pressure from isotonic conditions (170 mOsm) to hypertonic conditions (715 mOsm) by changing from a round/petalloid morphology to a filopodial morphology. Cytoskeletal fluorescent staining studies indicate that for filopodia to form, the actin cortical ring, present in most coelomocytes in isotonic conditions, must be disrupted. Breakdown of the actin ring by exposure to a hypertonic environment or actin disrupting drugs allows the formation of actin or tubulin-based filopodia.

Formdsmand, *et al.* (1998) studied toxic effects of nickel on survival, growth, and reproduction of *Eisenia veneta* were investigated following 4 weeks of exposure to a nickel-chloride spiked loamy sand soil. The lysosomal membrane stability, measured as neutral-red retention time, was reduced at soil nickel concentrations similar to those that reduced reproduction, and demonstrated a dose-response relationship. The neutral-red retention time showed large individual variation for the earthworms within each exposure concentration. It was concluded that the lysosomal membrane stability, measured as neutral red retention time, has a potential role in risk assessment. One important group of terrestrial invertebrates are earthworms. They are abundant and widespread in soils and play a key role in the functioning of

such ecosystems. Although information on the toxicity of a range of metals toward earthworms is available (e.g., Bengtsson and Tranvik, 1989; Morgan, *et al*., 1993; Spurgeon, *et al*., 1994; Spurgeon and Hopkin, 1995; Spurgeon and Hopkin, 1996; van Gestel, *et al*., 1989).

The first alterations caused by xenobiotics in organisms may be found at the subcellular level, e.g., in the lysosomes (Moore, 1990). The neutral red retention time (NRRT) is a commonly used nonspecific biomarker technique that measures changes in lysosomal membrane stability, especially the increased membrane permeability in response to stress. The NRRT provides a rapid and sensitive indication of response to altered environmental conditions. The NRRT assay, originally developed for marine organisms and fish (Lowe, *et al*., 1992; Lowe and Pipe, 1994) has been modified for earthworms by Weeks and Svendsen (1996).

Coelomocytes retrieved from the heavy metal soil survivors exhibited significant impairment of pinocytosis and plastic adherence. Perhaps impairment of immune functions contributed to the poor survival under conditions of heavily polluted soil samples (Homa, *et al*.,2005).

Three-day dermal exposure of *Dendrobaena veneta* to metal ions differentially disrupts the immunocompetence/pathogen balance. Zn does not accumulate in the earthworm body, Cu accumulation is temperature-independent while Cd accumulation is stronger at 22 °C than at 10 °C. During in vitro incubation with metal ions at 22 °C, growth of coelom-derived bacteria is enhanced by Zn, but significantly or almost completely inhibited by Cu or Cd. In contrast, under in vivo conditions at 22 °C, bacterial load is decreased only after Cd exposure, but increased after Zn and Cu exposures. At 10 °C bacteria growth is almost completely inhibited in all groups except Cu-treated animals. Coelomocyte number is unaffected in animals exposed to Zn, but significantly decreased after exposure to Cd (at 22 °C) and Cu (at 22 °C and 10 °C) with concomitant changes of amoebocyte-to-eleocyte ratio in favour of amoebocytes. Metal exposure up-regulates expression of metallothioneins in coelomocytes, mainly amoebocytes. (Olchawa, *et al*.,2006)

Scaps, *et al*. (1997) showed that although cadmium and lead are bio concentrated in *Eisenia fetida* tissue, bioaccumulation is not shown for concentrations below 100 ppm for lead, individuals eliminating as much metal as they ingest or the interactions between lead and organic matter in our substratum reduce the bioavailability of lead at low concentration. The cholinesterase activity was not inhibited when individuals were exposed for 8 weeks to either 8 or 80 ppm of cadmium or 100 or 2,000 ppm of lead. Results were different from those reported in another species *Eisenia fetida andrei* (5 *E. andrei*) showing an inhibitory effect of lead on ChE activity; thus, differences in cholinesterase inhibition reflects the existence of two separate species. No effect of cadmium and lead on the activity of esterases, malate dehydrogenase, phosphoglucomutase and glutamate oxalate transferase was found in

our experimental conditions, but they observed the disappearance of the fast moving band after electrophoretic separation for phosphoglucose isomerase. Earthworm *Lampito mauritii*, found throughout in India has been reported to have antimicrobial, anti-inflammatory, antioxidative and antiulceral properties (Balamurugan, *et al.*, 2007; Prakash, *et al*., 2007).

Homa, *et al.* (2005) provides direct evidence that earthworm immune cells, coelomocytes, are exposed to bio-reactive quantities of metals within 3 days after dermal exposure, and that they respond by upregulating metallothionein (MT) and heat shock protein (HSP70, HSP72) expression. Indirect support for the hypothesis that coelomocytes are capable of trafficking metals was also obtained. Coelomocytes were expelled from adult individuals of *Eisenia fetida* after 3-day exposure either to metal ions (Zn, Cu, Pb, Cd) or to distilled water (controls) via filter papers. The number of coelomocytes was significantly decreased after Cu, Pb, or Cd treatment. Cytospin preparations of coelomocytes were subjected to immunoperoxidase staining with monoclonal antibodies against human heat shock proteins (HSP70 or HSP72), or rabbit polyclonal antibodies raised against metallothionein 2 (w-MT2) of *Lumbricus rubellus*. Applied antibodies detected the respective proteins of *E. fetida* and revealed that the expression of HSP70, HSP72 and w-MT2 proteins was either induced or significantly enhanced in coelomocytes from metal-exposed animals.

Fitzpatrick, *et al*. (1996) studied the LC_{10} and LC_{50} values of Cd for earthworms were 15-fold and 7-fold lower, respectively, than those of Pb, indicating that Cd was more acutely toxic to *P. peguana* than Pb. The exposure time of 48-hours to Cd used in the study was adequate to induce coelomocyte DNA damage.

Effect of heavy metal on antioxidant activity

Many agrochemicals such as OP insecticides are able to induce oxidative stress (Lukaszewicz-Hussain, 2010), a situation in which the production of reactive oxygen species (ROS) overcomes the cellular antioxidant mechanisms (molecular and enzymatic), leading to the oxidative damage of biomolecules (e.g., lipids, proteins or DNA). Glutathione level is one of the most used biomarker of pro-oxidant exposure in fish (van der Oost, *et al*., 2003) and birds (Koivula and Eeva, 2010). Glutathione transferases (GSTs) form a ubiquitous superfamily of multi-functional dimeric enzymes (w50 kDa) with roles in detoxification. Several studies have been concerned with changes in glutathione concentration and glutathione-dependent enzymes in terrestrial invertebrates i.e. earthworm on metals and pesticide exposure (Salam Aly and Schroder, 2008; Maity, *et al*., 2008; Lukkari, *et al*., 2004; Saint-Denis, *et al*., 2001; Booth, *et al*., 2001). Biomarkers of oxidative stress have been mainly explored in earthworms exposed to, or inhabiting in, metalpolluted environments. For example, earthworm GST activity is a noteworthy detoxication system (Stenersen, 1981), which is induced in earthworms exposed to organochlorine pesticides. However, no effects on this enzyme activity were observed in earthworms exposed to the OP fenitrothion (Booth and

Halloran, 2001) or the CM carbaryl (Ribera, *et al*., 2001). Herbicides also induce the GST activity of earthworms.

Biochemical responses in organisms against environmental stress are regarded as early warning indices of pollution in the environment. Many enzymatic activities and low molecular mass molecules, such as the glutathione of the phase II biotransformation system, have been considered as biomarkers of environmental pollution (Saint-Denis, *et al*., 1999). These enzymes and low molecular mass molecules of living organisms possess antioxidant capabilities and can protect cells against adverse effects of reactive oxygen species (ROS) and xenobiotics (Saint-Denis, *et al*., 1998). On the other hand, very few studies on oxidative stress and antioxidant defences in earthworms have been performed.

Stenersen, *et al*. (1979) and Stenersen and Oien (1981) reported the existence of the glutathione (GSH) eglutathione-Stransferase (GST) system in earthworms. Saint-Denis, *et al*. (1998) studied the activities of enzymes (catalase, glutathione peroxidase, glutathione- S-transferase and glutathione reductase) involved in antioxidant defence systems in *Eisenia fetida andrei*, which are mainly localized in the cytosolic fractions. In response to organic and inorganic pollutant perturbations of glutathione concentration, the activity of glutathione-S-transferases, glutathione reductase and glutathione peroxidase has been reported in earthworm (Saint-Denis, *et al*., 1999, 2001).

Among metals, methyl mercury might be more easily absorbed by and accumulated in earthworms, suggesting that the earthworm is an ideal candidate for monitoring methyl mercury. Metal bioaccumulation by earthworms could be used as an ecological indicator of metal availability. The presence of contaminants in earthworms poses a serious risk of secondary poisoning of vertebrate predators due to biomagnifications (Reinecke and Reinecke, 2004).

Burgos, *et al*. (2005) studied the potential of a suite of novel molecular biomarkers as early warning indicators of environmental state and damage. Transcriptional responses of four genes, metallothionein 1 and 2, amine oxidase, and the lysosomal associated glycoprotein, were measured in the earthworm Lumbricus rubellus exposed to increasing concentrations of cadmium and copper in OECD soil. These responses were compared to metal body concentrations and lifecycle parameters: survival, cocoon production, and growth. Adverse physiological effects were observed at concentrations 1/3rd to 1/10th those of the artificial soil LC_{50}. Multivariate statistics, principal component analysis (PCA), was used to investigate the correlations between the different variables. Three key components were derived explaining 77.6% of the variance, with component 1 contributing 32.4%, component 2 contributing 26.7%, and component 3 contributing 18.5%. These components were interpreted in terms of population health, pollutant exposure, and detoxification pathways, respectively.

Markad, *et al*. (2012) carried out bio-monitoring and risk assessment of fly ash in earthworms as a model system. In which *Dichogaster curgensis* were allowed to grow in presence or absence of fly ash (0–40%, w/w) for 1, 7, and 14 d. The biochemical markers viz. catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), glutathione S-transferase (GST), and malondialdehyde (MDA) level were measured. The comet and neutral red retention assays were performed on earthworm coelomocytes to assess genetic damages and lysosomal membrane stability. The results revealed increased activities of SOD, GPx, GST, and MDA level in a dose–response manner while GR activity was decreased with increasing concentrations of fly ash. No obvious trend was observed in the CAT activity and fly ash concentration. Lysosomal membrane destabilization was noted in the earthworms exposed to 5% and more fly ash concentration in a dose and time dependent manner. The comet assay demonstrated that the fly ash induced DNA damage and DNA–protein crosslinks in earthworm coelomocytes.

Ausland, *et al*. (2011) studied response of *Eisenia fetida* earthworms raised from juveniles for 20−23 weeks in soil spiked with either 20 or 200 mg/kg of a commercially available uncoated titanium dioxide (TiO_2) nanomaterial (nominal diameter of 5 nm). Multivariate statistical analysis of the 1 H NMR spectra for aqueous extracts of *E. fetida* tissue suggested that earthworms exhibited significant changes in their metabolic profile following TiO_2 exposure for both particle sizes. The observed earthworm metabolic changes appeared to be consistent with oxidative stress, a proposed mechanism of toxicity for nanosized TiO_2.

Brulle, *et al*. (2006) developed a PCR approach to characterize partial mRNA sequences of selected effectors in the laboratory model, *Eisenia fetida*. After cloning, levels of expression of each gene were analyzed following exposures (80 and 800 mg/kg) to cadmium spiked soils using real-time PCR. An implemented approach was allowed to test quickly potential biomarkers in *Eisenia fetida*. Selected effectors were calmodulin, heat shock proteins, superoxide dismutase, catalase, metallothionein, â-adrenergic receptor kinase, pyruvate carboxylase, trancriptionally controlled tumor protein, protein kinase C, and ubiquitin. Most of the selected effectors did not show variations of expression level after exposure. Others expressed weak changes of expression as heat shock proteins. At last for catalase and metallothionein, early suitable variations of expression were observed.

CHAPTER-III

MATERIAL AND METHODS

The present investigation entitled "Effect of heavy metals concentration on bio-molecules and immunity parameters of earthworm, *Eisenia fetida*" was undertaken broadly in three phases. The first phase covered the effect of heavy metals on protein, carbohydrates and lipid content of earthworm. The second phase was concerned with the effect of heavy metals on the qualitative and quantitative parameter of coelomocytes of Eisenia *fetida*. The last phase of the study included the effect of heavy metals on the antioxidant activity of earthworm, *Eisenia fetida*. Studies were carried out in Vermicompost laboratory and in screen house of Department of Zoology, CCS Haryana Agricultural University, Hisar. Material used and methodology adopted for different experiments under laboratory are outlined below under relevant sub-headings.

3.1 Methodology of vermiculture

Earthworms were reared on biogas slurry from Biogas plant of Department of Microbiology, CCS HAU Hisar each tub had 12 kg of dry weight. Percent moisture in the mixture was calculated by keeping 1 kg of mixture in oven at 600 □C overnight. Moisture content was maintained constant between 40 to 45% and the temperature was not allowed to exceed 50-55 °C. 45 clitellated earthworms were weighed and released in each tub after 15 days. All the tubs were covered with gunny bags and water was sprinkled every day to maintain moisture.

3.2 Heavy metal introduction into soil containing earthworm *Eisenia fetida.*

To record the effect of heavy metals, such as mercury and cobalt on earthworm *Eisenia fetida* the treatments were given according to the table given below. In this investigation five replicates were maintained for each treatment. The experiment was planted on 9th January, 2014 in the research area, of Vermicompost Laborartory, Department of Zoology, CCS Haryana Agricultural University, Hisar (Haryana). Treatments were given by direct spraying of heavy metals in corresponding tubs. Each investigation was done in 15 days of interval.

Sr. No.	Treatment	Concentration (ppm)
1.	Control	No treatment of heavy metals
2.	Mercury	0.02, 0.04 and 0.06
3.	Cobalt	0.02, 0.04 and 0.06
4.	Mercury + Cobalt	0.01+ 0.01 ,0.02 + 0.02 and 0.03+0.03

3.3 Preparation of earthworm powder

After 15 days, 15 sexually mature clitellated earthworms were taken from each tub (approximate 900 mg/worm) were washed with running tap water to remove any dirt from body surface and then fed with wet blotting paper for 18-20 hours to clear their gut. The gut cleared earthworms were again washed with distilled water. The living earthworms were left there at a temperature of 25˚c for a period of 72 hours. Thereafter the earthworms were ground in homogenizer, mucus and coelomic fluid that oozed out digested the dead worms forming the brown coloured paste-earthworm paste which was dried and stored at 4˚C for further use.

3.1.1 Estimation of total proteins content in earthworm powder

About 0.5 ml of earthworm powder dissolved in distilled water was taken. The volume was made up to 1 ml with 0.1 N sodium hydroxide. 5 ml of reagent (alkaline copper solution) was added. The contents of tube were mixed well and allowed to stand for 10 minutes at room temperature. Then 0.5 ml of reagent sodium tartarate solution was added. It was mixed well and incubated at room temperature in dark for 30 minute. Blue colour indicated the presence of protein. The reading was taken at 660nm. Bovine serum albumin was taken as standard (Lowry, *et al*., 1951).

3.1.3 Estimation of total tissue carbohydrate in earthworm powder

25 mg of earthworm powder was taken into glass test tube and 0.1 ml of 12 M sulfuric acid was added after hydrolysis (16 hours at room temperature) 2.4 ml of water were added and the content of the test tube was heated on boiling water bath for 8 hours. To determine the carbohydrate content, o.5 ml of hydrolysate (corresponding to 5mg of earthworm powder) was mixed with 0.5 ml of water and 1 ml of 5% phenol solution and immediately 5 ml of concentrated sulfuric acid were added rapidly so that the solution were well mixed. The content of the test tube was vortexed for 10 seconds and allowed to stand for room temperature for 1 hour. In a blank the phenol solution was replaced by water. The absorbance of samples were measured at 485 nm against diluted (5+2, v/v) sulfuric acid at the same wavelength. The difference between these two reading was then used for calculation of carbohydrate content. Standard curve was plotted using graded concentration of D-glucose.

3.1.4 Estimation of total lipid content in earthworm powder

3 gm of earthworm powder were taken into thimble 80 ml of petroleum ether was poured in the beaker along with thimble was loaded in the soxhlet apparatus. keeping in view that the boiling temperature should be 20˚C more than that of solvents maximum boiling point. After 60 minutes of processing time increase the temperature i.e double of solvent's maximum boiling point is known as recovery temperature. Now thimble was removed from all the beakers and beakers were kept into a hot air oven at 100˚C. After 20-30 minutes, all the beakers were placed in dessicator in 10-15 minutes for cooling up to the room

temperature. After cooling final weight of the beaker (**FBW**) was taken and the percent (%) fat was calculated by following formula.

$$\%\mathbf{FAT} = \frac{\mathbf{W2 - W1}}{\mathbf{SW}} \times \mathbf{100}$$

W1- Weight of empty beaker

W2- Weight of flask and extracted fat (g)

SW- Sample weight

3.4 Estimation of Coelomocytes

3.4.1 Coelomocytes Retrieval

Adult earthworms were obtained from each tub containing heavy metal treatment on 15, 30, 45 and 60 days of treatment. Sexually matured earthworms from each tub was taken coelomic fluid was obtained by means of electric shock (Cooper and Suzuki., 1995), stimulating the earthworm with galvanic current of 6 volt for a duration of 1 minute. The volume of thus obtained coelomic fluid averaged 80µl. The fluid was diluted 1:1000, LBSS (Lumbricus Balance Salt Solution) (Diogene @ 1997), which is used in laboratory to maintain cell and tissue structure and physiologic integrity. LBSS contain KCl-5.30mM, KH_2PO_4-0.396mM, $NAHCO_3$-4.09mM, $NA_2HPO_4.7H_2O$-0.298Mm, NA_2SO_4-0.401mM, Dextrose- 5.50mM, Hepes-10mM, GGE-50.4mM, Nacl-58.5Mm, Penicillin-G sodium salt(U/ml)-100mM, Streptomycinsulfate (µg/ml)-100mM, Amphotericin B(ng/ml)-25mM.

3.4.2 Quantitative analysis of Coelomocytes

The coelomocyte were counted with the chamber method in Neubauer haemocytometer. This gives the coelomocyte density, i.e., cell number per ml. then this was multiplied by factor three (volume of extrusion of fluid), giving the cell number per animal.

Total number of coelomocytes = cell number per ml× 3

The concentration of cells in the sample suspensions was determined in triplicate by Neubauer haemocytometer using the following formula:

Number of coelomocytes ml^{-1} of suspension = X ×400 ×10 × 1000 × D

Where,

X	=	Average number of cells per small square
400	=	Number of small squares
10	=	Depth factor
1000	=	Conversion factor for mm^3 to cm^3
D	=	Dilution factor

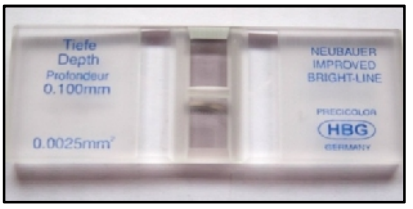

Plate 2: Tubs in screen house

Plate 3: Cocoons of Earthworm

Plate 4: Clitellated Earthworm

Plate 5: Earthworm powder

Plate 1- Haemocytometer

3.2.3 Qualitative analysis of coelomocytes

A thin smear of coelomic fluid of earthworm was taken on the slide and were dried at room temperature for 24 hours and stained with leishman's stain. The slides were analysed by light microscope and percentage of each type of coelomocyte were calculated on the bases of morphology of coelomocytes such as Amoebocytes , Eleocytes and Granulocytes.

3.3 Meaurement of Antioxidant activity

The antioxidant activity was adopted from Alam et al., (2010). Different concentrations of the samples of earthworm paste were prepared in 75% methanol ,80% methanol and 80% ethanol in five concentration (40, 80, 120, 160, 180 and 200 ppm). DPPH solution was prepared in methanol by adding 0.0025 g in order to get the final concentration of DPPH 25 ppm. 0.5 ml of each concentration in methanol was added to 3.5 ml of DPPH solution. The mixture was shaken and incubated for 30 min in the dark at room temperature. The absorbance was read at 517 nm by using the spectrophotometer. The control sample was prepared by following the same method without the sample. Ascorbic acid was used as positive control standard. Inhibition of DPPH free radical in percent was evaluated according to the formula:

$$\text{Inhibition (\%)} = \frac{[(\text{Acontrol} - \text{Asample})] \times 100,}{\text{Acontrol}}$$

where, Acontrol represents the absorbance of the control reaction (containing all reagents except the test compounds),

Asample represents the absorbance of test samples.

L-ascorbic acid was used as positive control.

CHAPTER-IV

RESULTS

The present study was carried out in Vermitechnology laboratory in Department of Zoology, CCS HAU, Hisar to study the effect of two heavy metals i.e. Mercury and Cobalt on the earthworms biomolecular content and on immunity parameter. Tubs containing vermiculture was maintained and different concentration of heavy metals as well as in combination was sprayed. Five replicates were maintained for each treatment.

4.1 Effect of heavy metals on the protein, carbohydrate and lipid constituents of *Eisenia fetida*

The dry matter of an earthworm's body consists of 60-70% protein, 7-10% fat, 8-20% carbohydrate, 2-3% minerals, and a variety of vitamins. They can be used as a feed additive for fish, pigs, and poultry. The worms accumulate metal within their tissue by binding with some metalloproteins called as metallothioneines. Generally, earthworms need to consume great amount of soil to achieve their daily nutrition, during which the digestive process liberates heavy metals in their free forms in the gut lumen. These freely available metals are then absorbed by the gut epithelial lining. This way, earthworms tend to accumulate considerable amount of the heavy metals within the cells in alimentary canal. In the present study changes in protein, carbohydrates and lipid content was studied in different heavy metal treated earthworms at the interval of 15 days till 2 months. Table 1 indicates changes in protein content of earthworm when treated with heavy metals.

4.1.1 Effect of heavy metal toxicity on protein content of *Eisenia fetida*

Table 1 indicates the effect of heavy metal concentration on the protein content of earthworm, *Eisenia fetida*. During the experiment period of 60 days total protein content decreased gradually. Maximum decrease was seen in case of earthworms reared in soil containing 0.06 ppm Hg which was 28.296 µg/mg at day 1 that had been reduced to 19.860 µg/mg at day 60. Similar pattern of decrease was seen in earthworms treated with combination of Hg and Co at 0.03 ppm concentration which was 28.224 µg/mg at day 1 had been reduced to 26.252, 24.504, 22.182 and 20.442 µg/mg at day 15, 30, 45 and 60 respectively which is second most toxic concentration. However, at day 60 protein concentration of earthworm treated with Co-0.06, Hg-0.04 and Hg- 0.02ppm concentration were found significantly at par with Hg-0.03+Co-0.03, Hg-0.02+Co-0.02 and Hg-0.01+Co-0.01 ppm concentration respectively.

Table 1: Effects of different concentration of heavy metals on protein content of earthworm, *Eisenia fetida.*

Sr. No.	Treatment (ppm)	Protein concentration (µg/mg of body tissue)				
		Day 1	Day 15	Day 30	Day 45	Day 60
1	Control	28.324±0.055[a]	28.404±0.051	28.312±0.114	28.422±0.013	28.514±0.173
2	Hg-0.02	28.178±0.069[a]	27.162±0.038	26.132±0.029[c]	24.974±0.039	24.100±0.017[c]
3	Hg-0.04	28.236±0.068[a]	26.802±0.043[b]	25.522±0.014[b]	23.758±0.037[a]	22.362±0.096[b]
4	**Hg-0.06**	**28.296±0.093[a]**	**25.942±0.052**	**24.012±0.018**	**21.800±0.039**	**19.860±0.081**
5	Co-0.02	28.268±0.045[a]	27.344±0.045	26.522±0.022	25.652±0.038	24.840±0.131
6	Co-0.04	28.832±0.059[b]	27.492±0.033	26.202±0.029[c]	24.546±0.046	23.488±0.125
7	Co-0.06	28.144±0.064[a]	26.240±0.036[a]	24.656±0.029[a]	23.760±0.042[a]	20.806±0.172[a]
8	Hg-0.01 + Co-0.01	28.754±0.081[b]	27.704±0.048	27.566±0.002	25.452±0.032	24.466±0.123[c]
9	Hg-0.02 + Co-0.02	28.512±0.082	26.828±0.047[b]	25.476±0.009[b]	24.148±0.026	22.208±0.360[b]
10	Hg-0.03 + Co-0.03	28.224±0.056[a]	26.252±0.033[a]	24.504±0.008[a]	22.182±0.021	20.442±0.162[a]
	SE(m)± CD (P=0.05)	**0.069 0.197**	**0.043 0.124**	**0.075 0.216**	**0.035 0.099**	**0.167 0.480**

*** Values in the column having same subscript do not differ significantly**

4.1.2 Effect of heavy metal toxicity on carbohydrate content of *Eisenia fetida*

Effect of heavy metal concentration on the carbohydrate content of earthworm *Eisenia fetida* was summerised in Table 2. During the experiment period of 60 days carbohydrate content decreased gradually. From the above table it was concluded that most toxic heavy metal from the above treatments was Hg-0.03 ppm then Hg-0.03 + Co-0.03 and Co-0.03 so on. Maximum decrease was seen in earthworms treated with Hg-0.03 ppm concentration which was 15.312µg/mg at day 1 had been reduced to 14.333, 13.629, 12.884and 12.009 µg/mg at day 15, 30, 45 and 60 respectively. In case of earthworms reared in soil containing Hg-0.03+Co-0.03 ppm protein concentration in earthworm was 15.544µg/mg at day 1 that had been reduced to 12.257µg/mg at day 60 which is second most toxic concentration. However, at day 60 protein concentration of earthworm treated with Hg-0.04 ppm concentration were found significantly at par with Hg-0.03+Co-0.03 ppm concentration respectively.

Table 2: Effects of different concentration of heavy metals on carbohydrate content of earthworm, *Eisenia fetida*

Sr. No.	Treatment (ppm)	Carbohydrate concentration (µg/mg of body tissue)				
		Day 1	Day 15	Day 30	Day 45	Day 60
1	Control	15.842±0.036	15.700±0.073	15.632±0.080	15.712±0.085	15.786±0.040
2	Hg-0.02	15.246±0.108	14.671±0.032^{c}	13.978±0.001^{c}	13.370±0.019^{b}	12.623±0.04
3	Hg-0.04	15.346±0.0102	14.510±0.075^{b}	13.807±0.015^{a}	13.082±0.015^{a}	12.263±0.04^{a}
4	**Hg-0.06**	**15.312±0.101**	**14.333±0.038^{a}**	**13.629±0.015**	**12.884±0.015**	**12.009±0.02**
5	Co-0.02	15.436±0.110	14.870±0.023^{d}	14.186±0.016	13.519±0.016^{c}	12.860±0.07
6	Co-0.04	15.492±0.158	14.656±0.035^{c}	14.084±0.09	13.346±0.09^{b}	12.644±0.005
7	Co-0.06	15.418±0.071	14.246±0.027^{a}	13.887±0.07^{a}	13.104±0.07^{a}	12.356±0.03
8	Hg-0.01 + Co-0.01	15.482±0.147	14.867±0.026^{d}	14.197±0.026	13.516±0.0026^{c}	12.741±0.005
9	Hg-0.02 + Co-0.02	15.456±0.106	14.701±0.030^{c}	13.966±0.019^{c}	13.236±0.0019	12.484±0.004
10	Hg-0.03 + Co-0.03	15.544±0.171	14.502±0.043^{b}	13.854±0.012^{a}	13.053±0.012^{a}	12.257±0.05^{a}
	SE(m)±	**0.117**	**0.044**	**0.028**	**0.031**	**0.013**
	CD (P=0.05)	**NS**	**0.126**	**0.079**	**0.089**	**0.038**

***Values along the column having same subscript do not differ significantly**

4.1.3 Effect of heavy metal toxicity on lipid content of *Eisenia fetida*

During the experiment period of 60 days lipid content decreased gradually with increase in heavy metal concentration (Table 3). Maximum decrease was seen in earthworms treated with Hg-0.03 ppm concentration which was 7.272 µg/mg at day 1 had been reduced to 6.524, 5.772, 4.272 and 4.271 µg/mg at day 15, 30, 45 and 60 respectively. However, at day 60 protein concentration of earthworm treated with Hg-0.02 ppm concentration were found significantly at par with Hg-0.03+Co-0.03 ppm concentration respectively. For earthworms treated with Co-0.03 ppm the lipid content was 7.520 µg/mg at day 1 which was reduced to 5.774 µg/mg at day 60. In case of earthworms reared in soil containing Hg-0.03+Co-0.03 ppm protein concentration in earthworm was 7.286 µg/mg at day 1 that had been reduced to 4.922 µg/mg at day 60 which is second most toxic concentration. From the above table it was concluded that most toxic heavy metal from the above treatments was Hg-0.03ppm then Hg-0.03 + Co-0.03 and Co-0.03 so on. Table 4 indicates the percent decrease in bio molecular concentration of earthworm with different heavy metal treatment. Maximum decrease was found in case of lipid concentration of earthworm i.e. it decreased up to 41.25% when earthworms were reared in soil containing Hg-0.06 and in this concentration carbohydrate and protein content decreased up to 21.57% and 29.813%. Figure 1 represents percent decrease in biolmolecular concentration of *Eisenia fetida*.. Maximum decrease in protein, carbohydrate and protein content was found in case of toxicity of Hg-0.06ppm in soil

Table 3: Effects of different concentration of heavy metals on lipid content of Earthworm, *Eisenia fetida*

Sr. No.	Treatment (ppm)	Lipid concentration (µg/mg of body tissue)				
		Day 1	Day 15	Day 30	Day 45	Day 60
1	Control	7.286±0.067	7.448±0.148	7.306±0.065	7.326±0.180	7.326±0.18
2	Hg-0.02	7.272±0.087	6.656±0.001[a]	6.05±0.087[a]	4.828±0.014[a]	4.823±0.01[a]
3	Hg-0.04	7.304±0.085	6.635±0.002[a]	5.959±0.085[a]	4.612±0.012	4.610±0.04
4	**Hg-0.06**	**7.272±0.081**	**6.524±0.002[a]**	**5.772±0.081[a]**	**4.272±0.010**	**4.271±0.05**
5	Co-0.02	7.428±0.198	7.129±0.001	6.828±0.198	6.167±0.019	6.181±0.09
6	Co-0.04	7.450±0.071	7.030±0.002[c]	6.621±0.179[c]	5.771±0.007[b]	5.763±0.07[b]
7	Co-0.06	7.520±0.179	6.958±0.001[b]	6.472±0.009[c]	5.525±0.012	5.521±0.34
8	Hg-0.01 + Co-0.01	7.441±0.114	6.853±0.013[b]	6.321±0.010[b]	5.237±0.019	5.245±0.01
9	Hg-0.02 +Co-0.02	7.410±0.171	6.688±0.001	6.084±0.021[b]	4.922±0.014[a]	4.922±0.03[a]
10	Hg-0.03 + Co-0.03	7.286±0.067	6.958±0.001[b]	6.472±0.009[c]	5.525±0.012	5.521±0.34
	SE(m)±	**0.124**	**0.047**	**0.101**	**0.044**	**0.058**
	CD (P=0.05)	**NS**	**0.135**	**0.290**	**0.127**	**0.167**

***Values along the column having same subscript do not differ significantly**

Table 4: Percent decrease in biomolecules of Earthworm, *Eisenia fetida.*

Sr. No.	Treatments (ppm)	Percent decrease in		
		Protein	Carbohydrates	Lipid
1	Control	-	-	-
2	Hg-0.02	14.47	17.20	33.61
3	Hg-0.04	20.80	20.09	36.86
4	**Hg-0.06**	**29.81**	**21.57**	**41.25**
5	Co-0.02	12.12	16.68	16.92
6	Co-0.04	18.53	18.38	22.72
7	Co-0.06	26.07	19.86	23.26
8	Hg-0.01 + Co-0.01	14.91	17.70	25.74
9	Hg-0.02 + Co-0.02	22.10	19.22	29.32
10	Hg-0.03 + Co-0.03	27.57	21.14	32.44

***Values along the column having same subscript do not differ significantly**

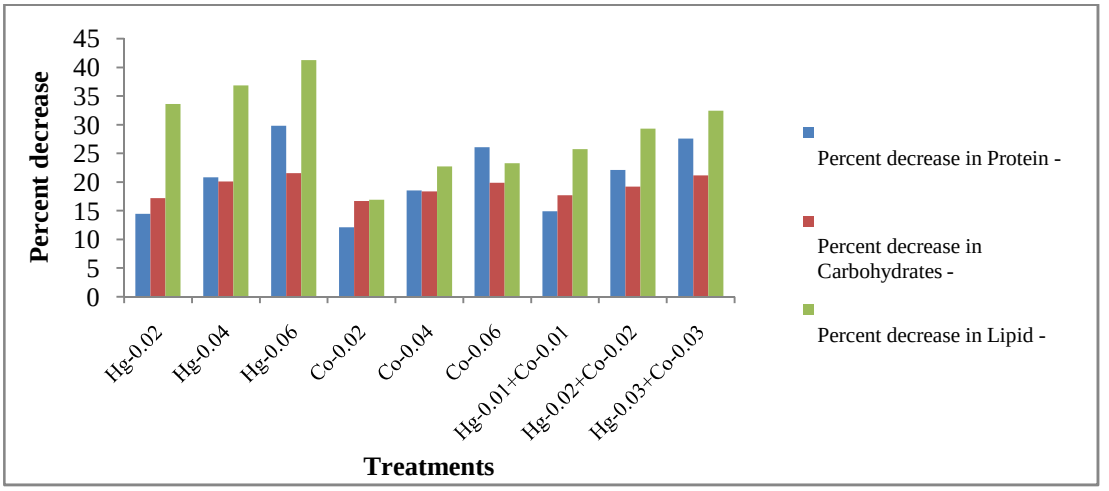

Figure 1: Percent decrease in biomolecules concentration of Earthworm, *Eisenia fetida*

4.2 To study the effect of heavy metals on the qualitative and quantitative parameter of coelomocytes of *Eisenia fetida*.

Effect of heavy metal concentration on total number of coelomocytes is presented in Table 5. Consistant with the results the total number of coelomocytes were same initially which decreases with a very fast rate with increasing concentration of heavy metals (Hg-0.06ppm).

Table 5: Effects of different concentration of heavy metals on total number of Coelomocytes

Sr. No.	Treatment (ppm)	Total number of coelomocytes (10^4)		
		Day 1	Day 30	Day 60
1	Control	22.698±0.538 (4.867)	22.310±0.23 (4.828)	24.379±0.532 (5.037)
2	Hg-0.02	23.042±0.320 (4.903)	14.396±0.040 (3.924^b)	7.670±0.042 (2.945)
3	Hg-0.04	23.268±0.720 (4.924)	13.097±0.053 (3.755^a)	5.678±0.042 (2.584^a)
4	**Hg-0.06**	**22.314±0.758 (4.826)**	**11.372±0.045 (3.517)**	**3.916±0.048 (2.217)**
5	Co-0.02	22.286±0.642 (4.824)	15.318±0.064 (4.040^c)	9.491±0.070 (3.239)
6	Co-0.04	22.640±0.489 (4.861)	14.463±0.048 (3.932^b)	7.941±0.052 (2.990)
7	Co-0.06	23.080±0.276 (4.907)	13.115±0.063 (3.757^a)	5.850±0.046 (2.617^a)
8	Hg-0.01 + Co-0.01	22.898±0.871 (4.885)	15.101±0.071 (4.013^c)	8.622±0.047 (3.102)
9	Hg-0.02 + Co-0.02	22.258±0.611 (4.821)	13.630±0.056 (3.825)	7.073±0.046 (2.841)
10	Hg-0.03 + Co-0.03	23.218±0.450 (4.920)	12.084±0.060 (3.617)	4.707±0.055 (2.389)
	SE(m)±	**0.596**	**0.092**	**0.175**
	CD (p=0.05)	**NS**	**0.030**	**0.054**

***Values along the column having same subscript do not differ significantly**
****Figures in parenthesis are square root transformation**

The number of coelomocytes of earthworm at day 60 treated with Co-0.06ppmwas found significantly par with the number of coelomocytes treated by Hg-0.04ppm. in case of earthworm reared in soil containing Hg-0.06ppm was found to be $23.314x10^4$, $11.372x10^4$ and $3.916x10^4$ at day 1, 30 and 60 respectively. In Co-0.06ppm toxicity the total number of coelomocytes were $23.080x10^4$ at day1, $13.115x10^4$ at day30 and $5.850x10^4$ at day60. When combination of both the heavy metals were sprayed on the soil at 0.03 ppm each the total number of coelomocytes were $23.218x10^4$, $12.084x10^4$ and $4.707x10^4$ at day 1, 30 and 60. The CD (P=0.05) was found to be 0.263 and 0.502 at day30 and 60.

Table 6: Effects of different concentration of heavy metals on total number of Coelomocytes per body weight

Sr. No.	Treatment (ppm)	Total number of Coelomocytes/ Body Weight (10^4)		
		Day 1	Day 30	Day 60
1	Control	24.862±0.042	24.320±0.249	24.942±0.036
2	Hg-0.02	24.874±0.036	19.032±0.060[b]	13.082±0.048[a]
3	Hg-0.04	24.898±0.041	17.483±0.061[a]	10.072±0.060
4	**Hg-0.06**	**24.784±0.044**	**16.116±0.050**	**7.674±0.092**
5	Co-0.02	24.828±0.054	20.083±0.064	15.176±0.077
6	Co-0.04	24.816±0.040	19.166±0.053[b]	13.296±0.082[a]
7	Co-0.06	24.850±0.037	17.742±0.074[a]	10.594±0.078
8	Hg-0.01 + Co-0.01	24.828±0.072	19.740±0.069	14.240±0.068
9	Hg-0.02 + Co-0.02	24.736±0.065	18.357±0.064	12.128±0.079
10	Hg-0.03 + Co-0.03	24.786±0.053	16.667±0.069	9.332±0.220
	SE(m)± **CD (P=0.05)**	**0.050** **NS**	**0.099** **0.284**	**0.097** **0.277**

***Values along the column having same subscript do not differ significantly**

The total number of coelomocytes per body weight (table 6) was almost same initially which decreases with a very fast rate with increasing concentration of heavy metals (Hg-0.06ppm). CD (P=0.05) was found to be 0.284 and 0.277 at day30 and 60. In case of earthworm reared in soil containing Hg-0.06ppm the number of coelomocytes per body weight was found to be 24.784 $x10^4$, 16.116 $x10^4$ and 7.674 $x10^4$ at day 1, 30 and 60 respectively. In Co-0.06ppm toxicity the total number of coelomocytes per body weight were $24.850x10^4$ at day 1, 17.742 $x10^4$ at day 30 and 10.594 $x10^4$ at day 60. When combination of both the heavy metals were sprayed on the soil at 0.03 ppm each the total number of coelomocytes per body weight were 24.786 $x10^4$, $16.667x10^4$ and 9.332 $x10^4$ at day 1, 30 and 60. The number of coelomocytes per body weight of earthworm at day 60 treated with Co-0.04ppm was found significantly par with the number of coelomocytes per body weight

treated by Hg-0.02ppm. Statistically comparable data shows that Hg-0.02 and Co-0.04 found to be par from each other.

Qualitative analysis of coelomocytes:

Qualitative analysis of coelomocytes of earthworm indicates total number of eleocytes, granulocytes I, granulocyte II, amebocyte I and amebocyte II. Types of coelomocytes decreases with increase in heavy metal toxicity and with passage of time. From Table 7-11, these types of coelomocytes decreases maximallywith toxicity of mercury 0.06 ppm. In body of earthworm, eleocyte constitute 30%, granulocyte I 13%, granulocye II 14%, amebocyte I 22% and amebocyte II 22%. Depending upon the morphology of cell type they are categorized. From the treatments given in the soil in terms of heavy metal toxicity mercury at 0.06 ppm found to be most toxic then the combination of mercury + cobalt to be 2nd most toxic concentration. In these treatments it was concluded that cobalt is less toxic even at 0.06 ppm to earthworm as compared to mercury.

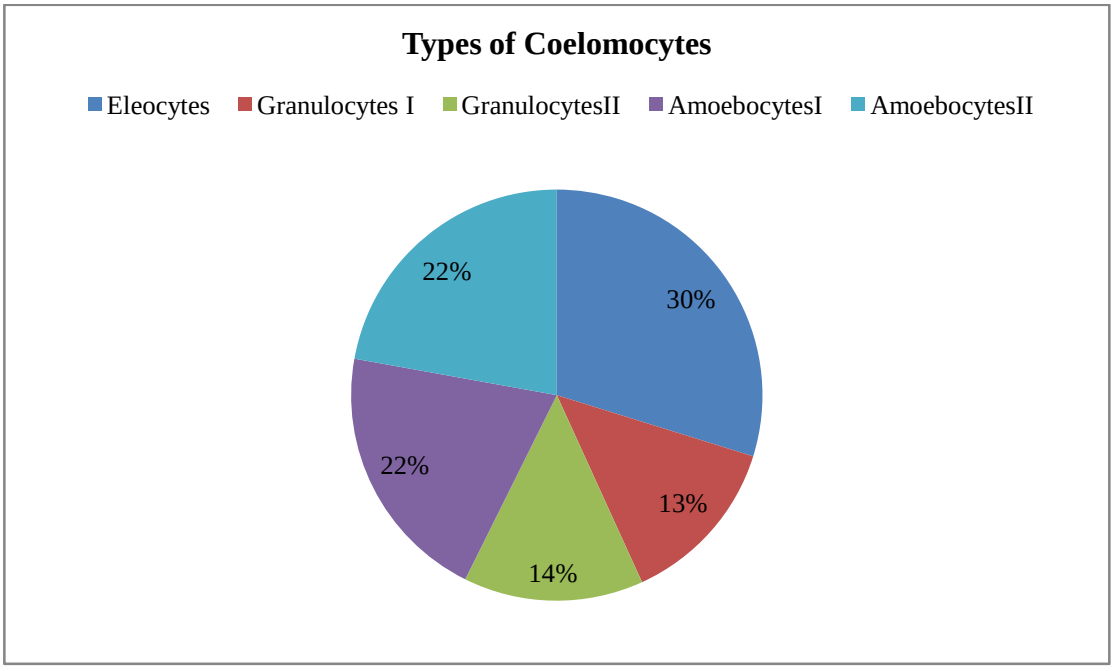

Figure 2: Diagram illustrating percent of each type of coelomocytes present in earthworm, *Eisenia fetida*

4.2.2.1 Changes in number of eleocytes

Eleocytes constitute about 30% population of coelomocytes in all the three species studied. These cells are round or oval and smaller than amoebocytes and granulocytes. The nuclei are located eccentrically and polymorphic granules are present in the cytoplasm Table 7 indicates the effect of heavy metal concentrations on total number of eleocytes. In case of mercury toxicity at 0.06 ppm in soil leads to changes in total number of eleocytes which were 6.636 x10^4 at day 1, which reduced to 1.162 x10^4 at last day of two month of experiment. In another case of cobalt's highest concentration i.e 0.06 ppm, number of eleocytes were not that

much affected as in case of mercury which were found to be 6.836 $x10^4$, 3.854 $x10^4$and 1.740 $x10^4$ at day 1, 30 and 60 respectively. In case of combine toxicity of both the heavy metals at 0.03 ppm each was found to be more toxic to earthworm in terms of eleocytes as compare to cobalt alone in this soil containing at day 1 (6.878 $x10^4$) as compared to day 60 (1.390 $x10^4$).

Table 7: Effects of different concentration of heavy metals on total number of Eleocytes

Sr. No.	Treatment (ppm)	Total number of eleocytes (10^4)		
		Day 1	**Day 30**	**Day 60**
1	Control	6.720±0.148 (2.778)	6.606±0.055 (2.758)	7.232±0.171 (2.869)
2	Hg-0.02	6.688±0.169 (2.772)	4.284±0.019 (2.299^b)	2.270±0.016 (1.808^b)
3	Hg-0.04	6.916±0.213 (2.812)	3.884±0.029 (2.210^a)	1.684±0.016 (1.638^a)
4	**Hg-0.06**	**6.636±0.242 (2.762)**	**3.372±0.018 (2.091)**	**1.162±0.017 (1.470)**
5	Co-0.02	6.594±0.181 (2.755)	4.514±0.033 (2.348^c)	2.810±0.032 (1.952)
6	Co-0.04	6.666±0.164 (2.768)	4.272±0.012 (2.296^b)	2.354±0.022 (1.831^b)
7	Co-0.06	6.836±0.099 (2.799)	3.854±0.010 (2.203^a)	1.740±0.016 (1.655^a)
8	Hg-0.01 + Co-0.01	6.769±0.287 (2.785)	4.476±0.024 (2.340^c)	2.560±0.019 (1.887)
9	Hg-0.02 + Co-0.02	6.562±0.185 (2.749)	4.034±0.039 (2.244)	2.086±0.021 (1.757)
10	Hg-0.03 + Co-0.03	6.878±0.111 (2.806)	3.590±0.015 (2.142)	1.390±0.021 (1.546)
	SE(m)± CD (p=0.05)	**0.188 NS**	**0.029 0.017**	**0.029 0.031**

***Values along the column having same subscript do not differ significantly**
****Figures in parenthesis are square root transformation**

4.2.2.2 Changes in number of Granulocytes

The coelomocytes which contain large number of granules in the cytoplasm are called granulocytes. They constitute 28% of the coelomocytes. They are spherical with centrally located nuclei. Two forms can be distinguished among the granulocytes G I and G II (Fig. 1c & d). G II differ from G I in having characteristic vesicular structure on their surface.These vesicles vary from spherical to club shaped.

Granulocytes- I

The total number of Granulocytes- I (table 8) was almost same initially which decreases with a very fast rate with increasing concentration of heavy metals (Hg-0.06ppm).

CD (P=0.05) for transformed value was found to be 0.015 and 0.020 at day30 and 60. In case of earthworm reared in soil containing Hg-0.06ppm the number of Granulocytes- I was found to be 3.048 $x10^4$, 1.550 $x10^4$ and 0.534 $x10^4$ at day 1, 30 and 60 respectively. In Co-0.06ppm toxicity the total number of coelomocytes per body weight were 3.132 $x10^4$ at day 1, 1.784 $x10^4$ at day 30 and 0.795 $x10^4$ at day 60. When combination of both the heavy metals were sprayed on the soil at 0.03 ppm each the total number of coelomocytes per body weight were 3.158 $x10^4$, 1.646 $x10^4$ and 0.634 $x10^4$ at day 1, 30 and 60. The number of Granulocytes- I of earthworm at day 60 treated with Co-0.04ppm was found significantly par with the number of coelomocytes per body weight treated by Hg-0.02ppm whereas Co-0.06 was found significantly par with Hg-0.04.

Table 8: Effects of different concentration of heavy metals on total number of Granulocytes- I

Sr. No.	Treatment (ppm)	Total number of Granulocytes I (10^4)		
		Day 1	**Day 30**	**Day 60**
1	Control	3.098±0.071 (2.024)	3.036±0.0019 (2.009)	3.328±0.080 (2.080)
2	Hg-0.02	3.136±0.032 (2.034)	1.962±0.012 (1.721^b)	1.040±0.010 (1.428^b)
3	Hg-0.04	3.178±0.110 (2.043)	1.782±0.009 (1.668^a)	0.774±0.010 (1.332^a)
4	**Hg-0.06**	**3.048±0.098 (2.011)**	**1.550±0.014 (1.597)**	**0.534±0.00**8 (1.238)
5	Co-0.02	3.032±0.095 (2.007)	2.078±0.017 (1.754^c)	1.292±0.011 (1.514)
6	Co-0.04	3.054±0.054 (2.013)	1.968±0.020 (1.723^b)	1.082±0.007 (1.443^b)
7	Co-0.06	3.132±0.040 (2.033)	1.784±0.025 (1.668^a)	0.795±0.006 (1.340^a)
8	Hg-0.01 + Co-0.01	3.090±0.121 (2.021)	2.056±0.010 (1.748^c)	1.165±0.011 (1.471)
9	Hg-0.02 + Co-0.02	3.036±0.106 (2.008)	1.852±0.012 (1.689)	0.959±0.010 (1.400)
10	Hg-0.03 + Co-0.03	3.158±0.076 (2.039)	1.646±0.025 (1.627)	0.634±0.010 (1.278)
	SE(m)± CD (p=0.05)	**0.085 NS**	**0.017 0.015**	**0.027 0.020**

***Values along the column having same subscript do not differ significantly**
****Figures in parenthesis are square root transformation**

Granulocytes II

Table 9 indicates the effect of heavy metal concentrations on total number of Granulocytes II. In case of mercury toxicity at 0.06 ppm in soil leads to changes in total number of Granulocytes II which were 3.256 $x10^4$ at day 1, which reduced to 0.564 $x10^4$ at

last day of two month of experiment. In another case of cobalt's highest concentration i.e. 0.06 ppm, number of Granulocytes II were not that much affected as in case of mercury which were found to be 3.370 $x10^4$, 1.914 $x10^4$and 0.847 $x10^4$ at day 1, 30 and 60 respectively. In case of combine toxicity of both the heavy metals at 0.03 ppm each was found to be more toxic to earthworm in terms of Granulocytes II as compare to cobalt alone in this soil containing at day 1 (3.360 $x10^4$) as compared to day 60 (0.676 $x10^4$).

Table 9: Effects of different concentration of heavy metals on total number of Granulocytes- II

Sr. No.	Treatment (ppm)	Total number of Granulocytes II (10^4)		
		Day 1	**Day 30**	**Day 60**
1	Control	3.310±0.077 (2.076)	3.234±0.044 (2.058)	3.522±0.113 (2.126)
2	Hg-0.02	3.340±0.077 (2.083)	2.102±0.018 (1.761^b)	1.112±0.007 (1.453^{bc})
3	Hg-0.04	3.426±0.108 (2.103)	1.912±0.021 (1.706^a)	0.818±0.007 (1.348^a)
4	**Hg-0.06**	**3.256±0.100 (2.062)**	**1.652±0.011 (1.628)**	**0.564±0.006 (1.251)**
5	Co-0.02	3.262±0.107 (2.064)	2.230±0.032 (1.797^c)	1.370±0.013 (1.539)
6	Co-0.04	3.272±0.095 (2.066)	2.098±0.014 (1.760^b)	1.148±0.019 (1.466^c)
7	Co-0.06	3.370±0.061 (2.090)	1.914±0.009 (1.707^a)	0.847±0.007 (1.359^a)
8	Hg-0.01 + Co-0.01	3.310±0.139 (2.075)	2.204±0.023 (1.790^c)	1.254±0.011 (1.501)
9	Hg-0.02 + Co-0.02	3.236±0.083 (2.058)	1.984±0.012 (1.727)	1.028±0.014 (1.424^b)
10	Hg-0.03 + Co-0.03	3.360±0.079 (2.088)	1.750±0.016 (1.658)	0.676±0.016 (1.295)
	SE(m)± CD (P=0.05)	**0.095 NS**	**0.022 0.017**	**0.038 0.027**

***Values along the column having same subscript do not differ significantly**
****Figures in parenthesis are square root transformation**

4.2.2.3 Changes in number of Amoebocytes

Amoebocytes constituting the major population of coelomocytes are polymorphic in shape which varies from oval to horse shoe shape. The nuclei are located centrally or peripherally. The cytoplasm contains few granules. The amoebocytes are classified as A I and A II depending on number and shape of cytoplasmic processes. Type A I amoebocytes (Fig. 1a) form number of pseudopodia regularly distributed on the cell periphery and have a form of short

lobopodia. Type A II amoebocytes (Fig. 1b) form irregularly distributed pseudopodia, most often concentrated on one pole of the cell and having the shape of long filipodia. Usually type A I amoebocytes are observed dispersed and type A II amoebocytes are observed in groups *in vivo* preparations. The A II amoebocytes are small when compared to A I amoebocytes.

Amoebocytes I

The effect of heavy metal concentrations on total number of Amoebocytes I (Table-10). In case of mercury toxicity at 0.06 ppm in soil leads to changes in total number of Amoebocytes I which were 4.558 $x10^4$ at day 1, which reduced to 0.802 $x10^4$ at last day of two month of experiment. The CD (P=0.05) at day 30 was 0.017 and at day 60 it was 0.010. In another case of cobalt's highest concentration i.e. 0.06 ppm, number of Amoebocytes I were not that much affected as in case of mercury which were found to be 4.724 $x10^4$, 2.688 $x10^4$ and 1.200 $x10^4$ at day 1, 30 and 60 respectively. In case of combine toxicity of both the heavy metals at 0.03 ppm each was found to be more toxic to earthworm in terms of Amoebocytes I as compare to cobalt alone in this soil containing at day 1 ($4.756x10^4$) as compared to day 60 (0.960 $x10^4$).

Table 10: Effects of different concentration of heavy metals on total number of Amoebocytes I

Sr. No.	Treatment (ppm)	Total number of Amoebocytes I (10^4)		
		Day 1	Day 30	Day 60
1	Control	4.680±0.106 (2.383)	4.604±0.059 (2.367)	5.020±0.093 (2.453)
2	Hg-0.02	4.736±0.081 (2.395)	2.942±0.021 (1.985^b)	1.580±0.013 (1.606)
3	Hg-0.04	4.762±0.176 (2.399)	2.694±0.012 (1.922^a)	1.166±0.015 (1.472)
4	**Hg-0.06**	**4.558±0.152 (2.357)**	**2.330±0.021 (1.825)**	**0.802±0.012 (1.342)**
5	Co-0.02	4.598±0.128 (2.365)	3.148±0.010 (2.035^c)	1.958±0.024 (1.720)
6	Co-0.04	4.614±0.071 (2.369)	2.962±0.017 (1.990^b)	1.622±0.014 (1.619)
7	Co-0.06	4.724±0.072 (2.392)	2.688±0.012 (1.920^a)	1.200±0.012 (1.483)
8	Hg-0.01 + Co-0.01	4.688±0.217 (2.383)	3.090±0.020 (2.022^c)	1.776±0.009 (1.666)
9	Hg-0.02 + Co-0.02	4.590±0.129 (2.364)	2.812±0.022 (1.952)	1.456±0.010 (1.567)
10	Hg-0.03 + Co-0.03	4.756±0.094 (2.399)	2.494±0.018 (1.869)	0.960±0.011 (1.400)
	SE(m)± CD(P=0.05)	**0.131 NS**	**0.025 0.017**	**0.032 0.010**

***Values along the column having same subscript do not differ significantly**
****Figures in parenthesis are square root transformation**

Amoebocytes II

Table 11 showed effect of heavy metal concentrations on total number of Amoebocytes II. At 0.06 ppm mercury toxicity in soil leads to changes in total number of Amoebocytes II which were 4.786 $x10^4$ at day 1, which reduced to 0.836 $x10^4$ at last day of two month of experiment. In another case of cobalt's highest concentration i.e. 0.06 ppm, number of Amoebocytes II were not that much affected as in case of mercury which were found to be 4.956 $x10^4$, 2.820 $x10^4$ and 1.252 $x10^4$ at day 1, 30 and 60 respectively. In case of combine toxicity of both the heavy metals at 0.03 ppm each was found to be more toxic to earthworm in terms of Amoebocytes II as compare to cobalt alone in this soil containing at day 1 ($4.780x10^4$) as compared to day 60 (1.006 $x10^4$). The CD (P=0.05) at day 30 was 0.019 and at day 60 it was 0.027.

Table 11: Effects of different concentration of heavy metals on total number of Amoebocytes II

Sr. No.	Treatment (ppm)	Total number of Amoebocytes II (10^4)		
		Day 1	**Day 30**	**Day 60**
1	Control	4.860±0.125 (2.420)	4.786±0.067 (2.405)	5.250±0.132 (2.499)
2	Hg-0.02	4.916±0.057 (2.432)	3.106±0.019 (2.026^b)	1.650±0.005 (1.628^b)
3	Hg-0.04	5.014±0.166 (2.451)	2.820±0.018 (1.954^a)	1.210±0.019 (1.487^a)
4	**Hg-0.06**	**4.786±0.168 (2.404)**	**2.440±0.019 (1.855)**	**0.836±0.012 (1.355)**
5	Co-0.02	4.798±0.134 (2.407)	3.298±0.029 (2.073^c)	2.040±0.011 (1.744)
6	Co-0.04	4.864±0.100 (2.421)	3.118±0.011 (2.029^b)	1.710±0.007 (1.646^b)
7	Co-0.06	4.956±0.062 (2.440)	2.820±0.008 (1.954)	1.252±0.012 (1.501^a)
8	Hg-0.01 + Co-0.01	4.862±0.172 (2.420)	3.228±0.031 (2.056^c)	1.862±0.019 (1.692)
9	Hg-0.02 + Co-0.02	4.802±0.142 (2.408)	2.926±0.026 (1.981^a)	1.524±0.012 (1.589)
10	Hg-0.03 + Co-0.03	4.780±0.187 (2.403)	2.606±0.017 (1.899)	1.006±0.017 (1.416)
	SE(m)± CD(P=0.05)	**0.138 NS**	**0.029 0.019**	**0.044 0.027**

***Values along the column having same subscript do not differ significantly**
****Figures in parenthesis are square root transformation**

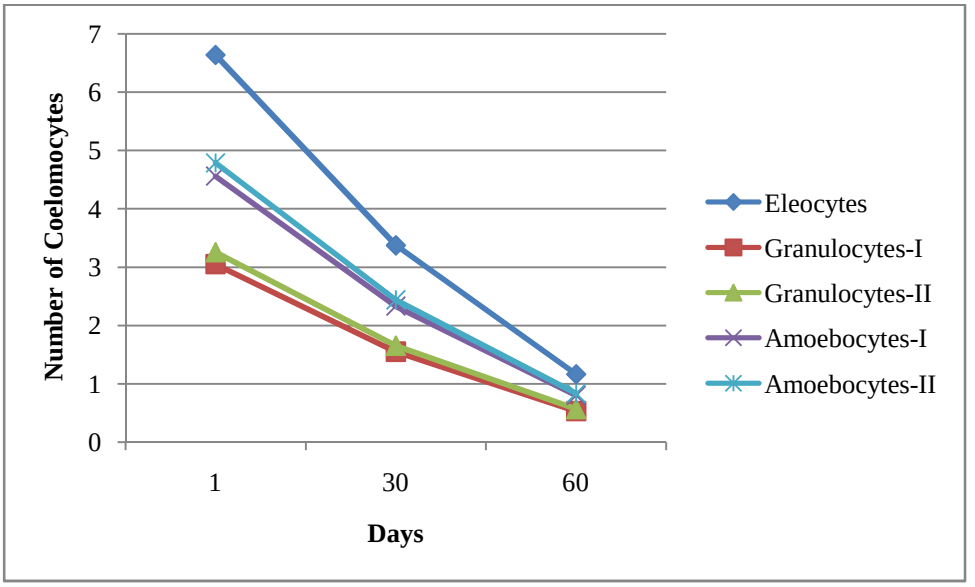

Figure 3: Change in number of types of coelomocytes by Hg-0.06 toxicity

4.3. Effect of toxicity on antioxidant activity

The comparison of antioxidant activity of redworm extracts by rearing earthworms in different heavy metals toxicity is shown in Table 12-14. There is great variation in antioxidant activity was seen in the experiment in the experiment of 60 days at an interval of 15 days. The inhibitory activity of *E. fetida* extracts in 80% methanol (38%) was very much less as compared to extract using 80% ethanol (47%) and 75% methanol (47%). The great variation was seen in the inhibitory activity from day 1 to day 15. Initially there was sharp increase in inhibitory activity but latter on it tend to become almost stable.

Among the various toxicity used in the experiment mercury showed highly effectiveness as compared to cobalt and combination of both.

4.3.1 Effect of heavy metals on antioxidant activity of earthworm using 75% methanol

Antioxidant activity of earthworm *E. fetida* increases with increase in duration of exposure to heavy metal in soil. In case of mercury given in the soil at 0.06 ppm there is sharp increase in inhibition activity from 47.732% at day 1 to 59.242% at day 15. Even after day 15, the increase in inhibition activity was measured, but it was almost non- significant which were 60.212% at day 30, 61.930%at day 45 and 62.212% at day 60 (figure 3). The CD (P=0.05) at day 15 was found to be 0.083, 0.402, 0.067 and 0.070 at day 15, 30, 45 and 60. In case of cobalt toxicity at its maximum concentration according to this experiment antioxidant activity using 75% methanol as solvent was 47.734%, 51.722%, 52.622%, 53.282% and 54.040% at day 1, 15, 30, 45 and 60 respectively. In combination of these two metal toxicant at the concentration of 0.03 ppm showed inhibition activity of 47.762%, 56.140%, 56.636%, 57.130% and 57.736% at day 1, 15, 30, 45 and 60 respectively.

Table 12: Effect of heavy metal concentration on the Antioxidant activity of Earthworm using 75% methanol as solvent

Sr. No.	Treatment (ppm)	Antioxidant activity of Earthworm using 75 % methanol as solvent (%)				
		Day 1	Day 15	Day 30	Day 45	Day 60
1	Control	47.822±0.057	47.784±0.044	47.078±0.129	47.618±0.039	49.114±0.036
2	Hg-0.02	47.790±0.052	54.172±0.013[a]	56.112±0.420	56.600±0.018	57.112±0.028
3	Hg-0.04	47.736±0.062	56.026±0.023	57.918±0.031	58.716±0.019	59.236±0.016
4	**Hg-0.06**	**47.732±0.057**	**59.242±0.025**	**60.212±0.014**	**61.930±0.028**	**62.122±0.033**
5	Co-0.02	47.752±0.083	49.524±0.019	50.216±0.022	51.726±0.013	52.142±0.021
6	Co-0.04	47.704±0.054	50.110±0.031	51.416±0.009	52.536±0.021	53.240±0.018
7	Co-0.06	47.734±0.068	51.722±0.034	52.622±0.017[a]	53.282±0.012	54.040±0.034
8	Hg-0.01+Co-0.01	47.684±0.116	52.058±0.035	52.900±0.029[a]	53.166±0.020	53.706±0.012
9	Hg-0.02+Co-0.02	47.744±0.075	54.126±0.033[a]	54.700±0.013	55.042±0.022	55.426±0.013
10	Hg-0.03+Co-0.03	47.762±0.070	56.140±0.021	56.636±0.019	57.130±0.031	57.726±0.011
	SE(m)±	**0.072**	**0.029**	**0.140**	**0.024**	**0.025**
	CD (P=0.05)	**NS**	**0.083**	**0.402**	**0.067**	**0.070**

***Values along the column having same subscript do not differ significantly**

4.3.2 Effect of heavy metals on antioxidant activity of earthworm using 80% Ethanol

Eisenia fetida antioxidant activity increases with increase in duration of exposure to heavy metal in soil. In case of mercury given in the soil at 0.06 ppm there is sharp increase in inhibition activity from 47.232% at day 1 to 57.264% at day 15. Even after day 15, the increase in inhibition activity was measured, but it was almost non- significant which were 58.466% at day 30, 59.082%at day 45 and 60.718% at day 60 (figure 3). In case of cobalt toxicity at its maximum concentration according to this experiment antioxidant activity using 80% Ethanol as solvent was 47.916%, 52.092%, 53.258%, 54.478% and 55.380% at day 1, 15, 30, 45 and 60 respectively. In combination of these two metal toxicant at the concentration of 0.03 ppm showed inhibition activity of 47.338%, 55.192%, 56.394%, 57.492% and 58.736% at day 1, 15, 30, 45 and 60 respectively. The CD (P=0.05) at day 15 was found to be 0.074, 0.069, 0.198 and 0.060 at day 15, 30, 45 and 60.

Table 13: Effect of heavy metal concentration on the Antioxidant activity of Earthworm using 80% ethanol as solvent

Sr. No.	Treatment (ppm)	Antioxidant activity of Earthworm using 80% methanol as solvent(%)				
		Day 1	Day 15	Day 30	Day 45	Day 60
1	Control	47.726±0.022	47.668±0.025	47.718±0.016	47.650±0.022	48.074±0.033
2	Hg-0.02	47.518±0.015[a]	53.146±0.024	54.394±0.026	55.778±0.018	56.158±0.021
3	Hg-0.04	47.630±0.018[b]	55.114±0.022[b]	54.844±0.016	57.098±0.017	58.248±0.022
4	**Hg-0.06**	**47.232±0.021**	**57.264±0.039**	**58.466±0.029**	**59.082±0.013**	**60.718±0.022**
5	Co-0.02	47.450±0.021	49.380±0.025	50.552±0.020	51.234±0.018	52.302±0.018
6	Co-0.04	47.808±0.018	51.430±0.020	52.656±0.020	53.778±0.026	54.248±0.018
7	Co-0.06	47.916±0.021	52.092±0.025[a]	53.258±0.021	54.478±0.018	55.380±0.017
8	Hg-0.01+Co-0.01	47.536±0.020[a]	52.150±0.019[a]	53.364±0.032	54.550±0.022[a]	55.770±0.019
9	Hg-0.02+Co-0.02	47.620±0.011[b]	53.446±0.028	54.274±0.032	55.440±0.021[a]	56.826±0.026
10	Hg-0.03+Co-0.03	47.338±0.024	55.192±0.026[b]	56.394±0.019	57.492±0.022	58.736±0.013
	SE(m)± **CD (P=0.05)**	**0.019** **0.056**	**0.026** **0.074**	**0.024** **0.069**	**0.069** **0.198**	**0.021** **0.060**

***Values along the column having same subscript do not differ significantly**

4.3.3 Effect of heavy metals on antioxidant activity of earthworm using 80% methanol

Antioxidant activity of earthworm *E. fetida* increases with increase in duration of exposure to heavy metal in soil. In case of mercury given in the soil at 0.06 ppm, there is sharp increase in inhibition activity from 38.084% at day 1 to 48.170% at day 15 even after day 15, the increase in inhibition activity was measured, but it was almost non- significant which were 50.238% at day 30, 51.708% at day 45 and 52.820% at day 60 (figure 4). The CD (P=0.05) at day 15 was found to be 0.078, 0.064, 0.023 and 0.052 at day 15, 30, 45 and 60. In combination of these two metal toxicant at the concentration of 0.03 ppm showed inhibition activity of 38.306%, 46.536%, 48.170%, 49.574% and 50.400% at day 1, 15, 30, 45 and 60 respectively. In case of cobalt toxicity at its maximum concentration according to this experiment antioxidant activity using 80% methanol as solvent was 38.282%, 43.264%, 45.528%, 46.242% and 47.392% at day 1, 15, 30, 45 and 60 respectively.

Table 14: Effect of heavy metal concentration on the Antioxidant activity of Earthworm using 80% methanol as solvent

Sr. No.	Treatment (ppm)	Antioxidant activity of Earthworm using 80% methanol as solvent (%)				
		Day 1	Day 15	Day 30	Day 45	Day 60
1	Control	38.258±0.029	38.482±0.020	38.150±0.026	38.350±0.023	38.524±0.014
2	Hg-0.02	38.470±0.020	44.324±0.013	46.160±0.035	47.708±0.015	48.232±0.018[a]
3	Hg-0.04	38.548±0.022	46.054±0.024	47.824±0.011	48.048±0.074	50.212±0.028
4	**Hg-0.06**	**38.084±0.021**	**48.170±0.032**	**50.238±0.021**	**51.708±0.017**	**52.820±0.014**
5	Co-0.02	38.184±0.051	41.544±0.039	42.340±0.017	44.074±0.027	45.324±0.020
6	Co-0.04	38.320±0.035	42.256±0.038	44.440±0.019	45.724±0.014	46.560±0.018
7	Co-0.06	38.282±0.021	43.464±0.030	45.528±0.022	46.242±0.020	47.392±0.015
8	Hg-0.01 + Co-0.01	38.616±0.013	43.724±0.022	44.918±0.021	46.206±0.016	47.472±0.020
9	Hg-0.02 + Co-0.02	38.164±0.028	44.636±0.018	46.094±0.018	47.7440±0.019	48.224±0.020[a]
10	Hg-0.03 + Co-0.03	38.306±0.015	46.536±0.021	48.170±0.025	49.574±0.022	50.400±0.013
	SE(m)±	**0.028**	**0.027**	**0.022**	**0.067**	**0.018**
	CD (P=0.05)	**0.079**	**0.078**	**0.064**	**0.023**	**0.052**

***Values along the column having same subscript do not differ significantly**

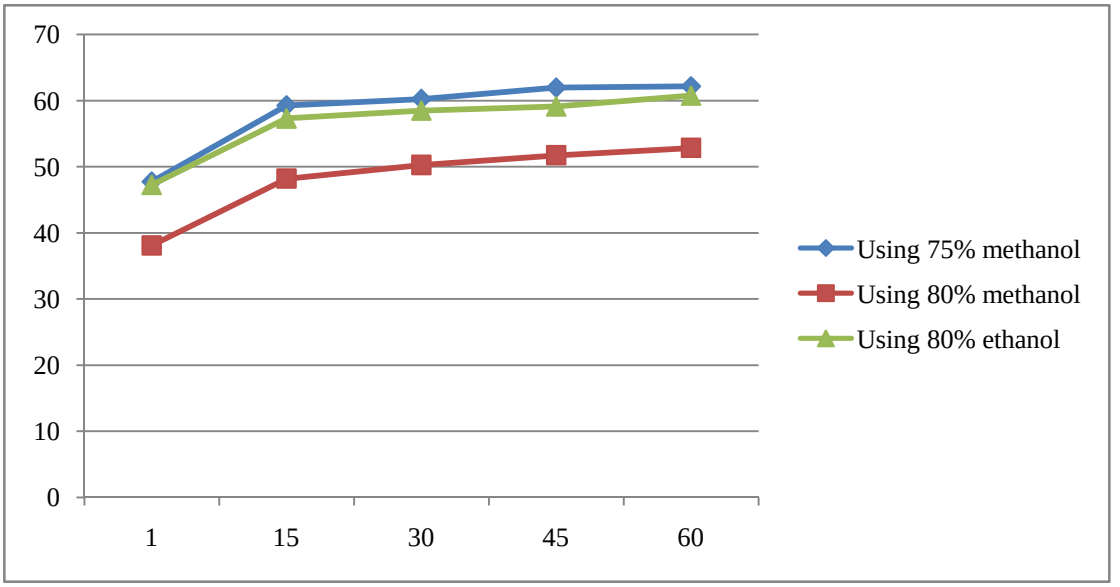

Figure 4: Changes in inhibition activity of earthworm treated with most effective concentration of heavy metal

CHAPTER-V

DISCUSSION

The process of vermicomposting allows waste materials to be remediated and the compost used subsequently for use in growing human food without the risk of accumulating heavy metals in crops. The worm's digestive system is capable of detaching heavy metal ions from the complex aggregates between these ions and humic substances in the waste as it rots. Various enzyme-driven process then seem to lead to assimilation of the metal ions by the worms so that they are locked up in the organism's tissues rather than being released back into the compost as worm casts.

Metal contaminated land is found in many parts of the world due to atmospheric emissions from smelters and other metallurgic processes and this pollution has drastic effects on the impacted ecosystems. The effects of metals (especially copper (Cu), zinc (Zn), cadmium (Cd), nickel (Ni) and lead (Pb)) on soil organisms have been much studied since the early 1970s because surface litter and humus are the principal metal sinks in the soil (Bengtsson and Rundgren, 1988; Hopkin, 1989). Several field and laboratory investigations have shown that the density of soil invertebrates in many cases is severely reduced in metal polluted soils up to several km away from the emission source (Bengtsson and Rundgren, 1982; Bengtsson, *et al*., 1983; Spurgeon, *et al*., 1994; Spurgeon and Hopkin, 1995, 1999; Haimi and Siira-Pietikäinen, 1996). On the other hand, there is also evidence that soil invertebrates can genetically adapt to metal stress, thus modifying metal toxicity and promote persistence in contaminated soils (Posthuma and Van Straalen, 1993; Van Straalen and Timmermans, 2002; Timmermans, *et al*., 2005). Thus, soil invertebrates are able to detoxify and store excess metal ions in membrane enclosed cellular granules or in metallothionein complexes (Ireland and Richards, 1977; Posthuma and Van Straalen, 1993; Stürzenbaum, *et al*., 2001; Köhler, 2002; Schill and Köhler, 2004; Vijver, *et al*., 2004; Timmermans, *et al*., 2005). These heavy metals have deleterious effect on the body of earthworm. So it seemed worthwhile to check the effect of heavy metals on biomolecular concentration and on immune system of earthworm.

5.1. Biochemical Alteration

The effects of Mercury and cobalt alone and in combinations were studied to estimate changes in total body carbohydrate, protein and lipid levels. These data clearly reveal that when the concentrations of metals were increased, the body tissue carbohydrate and lipid levels were reduced as compared to the control. The carbohydrate level decreased in metal treated earthworm, due to the earthworm under stress and this may induce an increase in metabolic rate resulting in increased utility of carbohydrates as energy source. This decrease

might be a counteract mechanism to fight and survive under toxic conditions. Depleted levels of carbohydrates in tissues indicated the possibility of active glycogenolysis (Gill and Pant, 1981). Tissues acidosis due to reduced oxygen transport must have also favoured the process of glycogenolysis in tissues.

The impact of heavy metal on carbohydrates was studied and study revealed the maximum decrease of carbohydrates was observed in treatment of Hg 0.06 ppm followed by Hg 0.03ppm + Co 0.03ppm and then Zn at the concentration of 0.06ppm. Similar result was seen in case of lipid content of earthworm which decreases gradually with increase in concentration of heavy metal contamination. The maximum detrimental effect was seen in case of Hg (0.06ppm).

The heavy metal exposure showed great decrease in the protein content 29.81% in the earthworm treated with mercury at the concentration of 0.06ppm. The decline effect was seen in following order: Hg 0.06ppm followed by Hg 0.03ppm + Co 0.03ppm and then Co 0.03ppm. The decrease in protein content may be attributed either to increased turnover rate of protein or interference of the toxicant at certain site during protein synthesis (Rao, *et al.*, 2003).

It is established fact that proteins are used as alternate energy source especially under stress conditions. It is expected that *Eisenia fetida* and *Perionyx excavatus* also must have utilized protein for the production of energy to mitigate the stress caused by heavy metals. These results are supported by several investigators who reported decline in protein content in different organisms under the influence of heavy metals (Sturzenbaum, *et al*., 2001).

The decrease in protein content may be attributed either to increased turnover rate of protein or interference of the toxicant at certain site during protein synthesis (Rao, *et al.*, 2003).

Among terrestrial invertebrates, earthworms are priority test organisms for soil contamination surveys (Reinecke and Reinecke, 2004; Ricketts, *et al*., 2004) because they are easy to handle, widespread in their terrestrial distribution and have the capacity to accumulate and concentrate large quantities of inorganic and organic pollutants (Fitzpatrick, *et al*., 1996; Saint-Denis *et al*., 1999; Spurgeon and Hopkin, 1999a; O'Halloran, *et al*.,1999; Booth, *et al*., 2000; Scott- Fordsmand and Weeks, 2000; Rao, *et al*., 2003) with acquired adaptive resistance to the toxicity of these pollutants including heavy metals (Spurgeon and Hopkin, 1996b). They also play an important role in the decomposition of organic matter, nutrient mineralization and primary production in terrestrial ecosystem (Edwards and Bohlen, 1996). Earthworms are frequently exposed to heavy metals, pesticides and other pollutants in the environment and are therefore dependent on efficient detoxification systems for their survival.

The diversity of earthworms have been frequently studied for developing bioindicators of soil health associated with environmental pollution and ecosystem

degradation under impacts of pesticides, heavy metals, and soil degradation. There has been increasing attention paid to the changes in the physiological and biological property of earthworms under environmental stresses. Garcia (2004) investigated the effect of heavy metal pollution on *Pheretima califonica'*s stomach intestinal mucosa.

Xue *et al*. (2009) reported a study on the toxicity of a dimethoate pesticide on earthworms via the respiratory activity. Some recent works have shown the significance in developing bioindicators of the changes in contents and composition of bioactive components such as amino acids and special proteins from earthworms living in the soil body.

A study by Hua *et al*. (2003) showed a response of earthworm amino acid composition to spiked rare earth in soils and demonstrated an induction of Hsp-70 in earthworm blood when exposed to organic pollutants and spiked heavy metals (Pb, Cd, Cu, and Hg) in soils, suggesting a possible biomarker for monitoring soil pollution. However, little knowledge is available on the effect of different fertilizations on earthworm populations and on the biomarker of living components.

Carbohydrates represent the principal and immediate energy precursors for earthworm exposed to stress condition while protein is spared during chronic period of pollutant stress (Umminger, 1970). The observed depletion in carbohydrates may be due to hypoxia, since hypoxia increase carbohydrate consumption (Dezwaan and Zandee, 1972). Severe environmental hypoxia caused by pollutants is responsible for rapid depletion of stores of carbohydrate (Heath and Pritchard, 1965). In the present study muscles showed moderate levels of total carbohydrate to a large extent the total carbohydrate present in muscle tissue should be glycogen, which forms the major source of reserved energy for body functions. The observed low levels of the total carbohydrates in the gill tissue suggests low glycogen synthetic potentials or these levels might be due to the presence of blood in the gills contributing to the levels of glycogen in the gill tissue. Heavy metals thus may produce damage to an organ, inhibition of enzymes activity and significant alterations in various metabolic activities

Inorganic Hg is capable of causing hormetic-like biphasic effects on earthworms and high dose of pollutants may conceal the hormesis of exposure to Hg. In an experiment lasting 17days, survival rates of exposure to mercury chloride were investigated. 7days and 14days LC_{50} of mortality were 155.97 and 143.67 mg/kg, respectively. There was a significant dose-response relationship between survival rates and Hg concentration (0, 50, 100, 150, 200 and 250 mg/kg). Survival of *E. fetida* is a stable and sensitive biomarker to dialogue adverse effects of Hg in soil environment. Sub-chronic treatment (0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, 100 mg/kg) caused a significant increase at low doses yet an inhibitory at high level in survival of earthworms over 7days. The OECD soil exposure of the mature *E. fetida* showed a hormetic stimulation of 114-132% of survival rates. (Jiang, *et al*., 2012)

In the present study, protein, carbohydrate and lipid level decreased in the tissue of earthworms after exposure to Cd and Pb. Increase in enzymatic activity with decrease in protein content has been reported earlier (Cerdeira, *et al.,* 2003). Under the stress of energy demand imposed by toxicity, alternate metabolic pathway may be utilized to obtain energy for maintenance. Yadav *et al.* (2005, 2011) have reported decrease in protein content in *Lumbricus terrestris* and *Approrectodea colginoga* under the stress of organic pesticides. Similar observations were reported in earthworms *Metaphire posthina* and *Amynthas gracils* after exposure to Cd, endosulfan and butachlor. Shukla and Kumar (2004) reported lipid content decrease after exposing endosulfan and cypermethrin in earthworms.

The animal utilized carbohydrate as a source of energy in tissue. It is therefore obvious that depletion of carbohydrate may be due to its direct utilization for energy generation demand caused by pesticide induced hypoxia. In the present study, carbohydrate contents decrease in earthworm during exposed to heavy metals. Earlier Reddy *et al.* (2005) observed a decrease in level of total carbohydrate and glycogen in *Lampito mauritti* and attributed it to mobilization of carbohydrate from muscle to coelomic fluid as a consequence of hypoxic or anoxic condition included by the pollutants. Dezwaan and Zandee (1972) reported that carbohydrate metabolism gets altered during toxic stress and decrease may be due to the prevalence of hypoxic and anoxic conditions which normally increase carbohydrate utilization.

By Subramanian ***et*** *al.* (2002) reported that when earthworm, *Lambito Mauritii* (Kinberg) were exposed to various concentrations of cadmium and mercury in soil and water medium. The 24 hours LC_{50} value of cadmium and mercury in water environment was 1.1 mg/L and 0.09 mg/L respectively and in soil medium it was found to be 800.7 mg/kg and 336.6 mg/Kg .The various behavioural changes observed were burrowing, fast movement, lifting of the body, curling and coiling. The morphological changes were rupture of the cuticle, blood accumulation, constriction, bulging, oozing out of blood and oozing out of coelomic fluid .

The activity of esterase isozymes of earthworm in polluted groups was inhibited by the heavy metals. On the contrary, the activity of peroxidase isozyme bands in serious polluted groups was promoted by the heavy metals. Obvious effects of heavy metals on earthworm cytology happened on worm body wall. In light pollution group, the cutin layer of the body wall obviously became thick as a reaction against bad environments. In serious polluted group surface ulcer appeared on some parts of the body wall. Hyperemia or hemorrhage symptom was found in the epithelial tissue of worm stomach and intestines. The perinuclear cistern of stomach epithelial cells swelled, unclear envelope broke, nucleoplasm flowed out and complasm autolysed sometimes. The special yellow cells in worm body significantly varied with levels of heavy metals pollution of soil. In natural situation without

pollution, many yellow cells appeared in body cavity in the wall of intestine and blood tube. So, there was a close relation between yellow cells and environments. Heavy metals can damage yellow cells. Changes of yellow cells in number and cytology perhaps are taken as a reliable index for monitoring soil pollution. (Zhenjun, S., 2005)

Lipids are one of the most important sources of energy and structural components in animal. In the Present study, lipid content decreased after exposed to heavy metals. Decrease in lipid content suggests that mobilization of energy rich lipids for production of energy during toxic stress caused by metals. The decrease in lipid content correlates with the increased activity of lipase, the energy responsible for the breakdown of lipids into free fatty acid and glycerol. Decrease in tissue lipid under toxicant stress could be due to formation of lipoproteins, which are used to provide energy.

5.2. Coelomocytes and heavy metals

The second objective was aimed at studying the effect of heavy metal i.e. mercury and cobalt on the immune cells i.e. Coelomocytes of earthworm *Eisenia fetida.* The result clearly depict that types of coelomocytes i.e. Eleocytes, Amoebocytes and Granulocytes decreases on increase in heavy metal contamination and on increase in concentration. An earlier studies by Scapes *et al*., (1997) showed that although cadmium and lead are bio concentrated in *Eisenia fetida* tissue, bioaccumulation is not shown for concentrations below 100 ppm for lead, individuals eliminating as much metal as they ingest or the interactions between lead and organic matter in our substratum reduce the bioavailability of lead at low concentration. The cholinesterase activity was not inhibited when individuals were exposed for 8 weeks to either 8 or 80 ppm of cadmium or 100 or 2,000 ppm of lead.

D. veneta (Kwadrans *et al*., 2008) and *Aporrectodea caliginosa* were maintained for 4 and 8 weeks, respectively, in soil samples soaked with Cd, Cu, Pb, or Ni chlorides. Body weights of *D. veneta* were unaffected by 4-week metal exposure, but eleocyte numbers and riboflavin content were increased in Pb and/or decreased in Ni exposed groups of worms. In contrast, after 8 week experiments on A. caliginosa, body weight gain was inhibited in all metal-exposed groups while coelomocyte number was significantly increased in Pb-exposed worms. This indicates that the effects of metal soil pollution on the earthworm immune system are species-specific and do not always correspond with the general condition of worms. We may assume that effects of metal exposure on immunity are rather associated with the disrupted balance between the worm immune system and microbial impact from surrounding metal-polluted soil (Salice and Roesijadi, 2002; Olchawa, *et al*., 2006).

Cytoskeletal fluorescent staining studies indicate that for filopodia to form, the actin cortical ring, present in most coelomocytes in isotonic conditions, must be disrupted. Breakdown of the actin ring by exposure to a hypertonic environment or actin disrupting drugs allows the formation of actin or tubulin-based filopodia. Kasschau, *et al.* (2006) stated

that coelomocytes from *L. terrestris* respond to an increase in environmental osmotic pressure from isotonic conditions (170 mOsm) to hypertonic conditions (715 mOsm) by changing from a round/petalloid morphology to a filopodial morphology.

Coelomocytes retrieved from the B soil survivors exhibited significant impairment of pinocytosis and plastic adherence. Perhaps impairment of immune functions contributed to the poor survival under conditions of heavily polluted B soil samples (Homa, *et al*., 2005). Scapes *et al.* (1997) showed that although cadmium and lead are bio concentrated in *Eisenia fetida* tissue, bioaccumulation is not shown for concentrations below 100 ppm for lead, individuals eliminating as much metal as they ingest or the interactions between lead and organic matter in our substratum reduce the bioavailability of lead at low concentration. The cholinesterase activity was not inhibited when individuals were exposed for 8 weeks to either 8 or 80 ppm of cadmium or 100 or 2,000 ppm of lead. Results are different from those reported in another species *Eisenia fetida andrei* (5 *E. andrei*) showing an inhibitory effect of lead on ChE activity; thus, differences in cholinesterase inhibition reflects the existence of two separate species. No effect of cadmium and lead on the activity of esterases, malate dehydrogenase, phosphoglucomutase and glutamate oxalate transferase was found in our experimental conditions, but we observed the disappearance of the fast moving band after electrophoretic separation for phosphoglucose isomerase. Earthworm *Lampito mauritii*, found throughout in India has been reported to have antimicrobial, anti-inflammatory, antioxidative and antiulceral properties (Balamurugan, *et al*., 2007; Prakash, *et al*., 2007).

Ptumporn, *et al.* (2011) reported that the LC_{10} and LC_{50} values of Cd for earthworms were 15-fold and 7-fold lower, respectively, than those of Pb, indicating that Cd was more acutely toxic to *P. peguana* than Pb. The exposure time of 48-h to Cd used in the present study was adequate to induce coelomocyte DNA damage. The same exposure time and found tissue Cd accumulation and subsequent DNA damage in earthworms exposed to a slightly higher Cd concentration in artificial soil-water medium.

Homa, *et al.*(2010) reported that at least 3 days is required for Cd to accumulate in significant concentration in coelomocytes for induction of stress response proteins in *Eisenia fetida* exposed to 1.32 $\mu g\ cm^{-2}$ Cd (and Zn, Cu, Pb) in filter-paper contact tests. Nahmani, *et al*. (2007) have shown that in *Eisenia fetida*, about 80% of cellular Cd was in the cytosol and only 20% in the cell membrane. This is in contrast to Pb where 50% of Pb was located in the cell membrane and much less in the cytosol. This may be due to the fact that Pb^{2+} has a larger ionic radius (1.19 Å) compared with Cd^{2+} (0.97 Å), and therefore a greater diffusion of Cd ions into the coelomocytes, whereas most of the Pb^{2+} binds to the cell membrane with limited transport to the coelomocyte cytosol and hence minimal toxicity to the coelomocytes.

5.3Antioxidant activity of earthworms toxicated by heavy metals

Antioxidant activity of earthworm during the experiment period of 60 days showed great variation for 15 days increase in antioxidant activity was seen which may be early warning indices of pollution in the environment. Many enzymatic activities and low molecular mass molecules, such as the glutathione of the phase II biotransformation system, have been considered as biomarkers of environmental pollution (Saint-Denis, *et al.*, 1999). These enzymes and low molecular mass molecules of living organisms possess antioxidant capabilities and can protect cells against adverse effects of reactive oxygen species (ROS) and xenobiotics (Saint-Denis, *et al*., 1998). On the other hand, very few studies on oxidative stress and antioxidant defences in earthworms have been performed.

Increase in inhibition activity of antioxidant enzymes was supported by Zang, *et al*. (2009) who validated that Cd at low concentrations induced an increase in the activity of catalase and superoxide dismutase (SOD), but high concentrations inhibited the enzymes, and this was reflected in an inverted U-shaped curve. The maximum hormetic magnitude of SOD activity was higher than that of catalase. The presence of hormesis induced by cadmium in the earthworm may be related to activation of adaptive pathways.

Sulata, *et al*.,(2008) validated the response of the GSH-GST system and antioxidant enzymes of the earthworm as a sign of adaptation to the disturbed terrestrial ecosystems. Earthworms were chosen since these have a particular intimate contact with the soil. Increase in the GSH concentration in tissues after 14 and 21 days of exposure may be due to an up regulation of the GSH synthetic pathway and may be a protective mechanism against the toxic effects of the metal. Usually GST activation leads to depletion of the GSH with a simultaneous re-synthesis of GSH for the replacement of the normal level. In the cell there is equilibrium between the reduced and oxidized forms of glutathione. However, GSH is the predominant and chemically active form. Glutathione acts as a redox buffer of the cell by maintaining the thiol disulfide status of proteins. Thus the GSH:GSSG ratio is considered as an index of the cellular redox status (Schafer and Buettner, 2001). GSTs catalyse the conjugation reaction of GSH and electrophilic xenobiotics, and thus contribute to the removal of reactive electrophiles. GSH also scavenges reactive oxygen species (ROS) non-enzymatically or in a reaction catalysed by GPx through the oxidation of two molecules of GSH to a molecule of GSSG. Thus as with GPx activity, GST activities also cause a reduction in the GSH level and a decrease in the cellular antioxidant status. GPx uses GSH as a hydrogen donor to eliminate H_2O_2 and to convert the organic hydroperoxides into alcohols. Next, the product of this reaction, GSSG, is reduced by GR in the presence of NADPH, and in this way the GSH level is restored in the cell (Halliwell and Gutteridge, 1999).

This result corroborates earlier findings in *Eisenia fetida* andrei by Saint-Denis et al. (2001) exposed to Pb contaminated soil. It could be postulated that alteration of NADPH

availability may affect GR activity and GSH regeneration and, consequently, may promote GSSG accumulation in the cell. GSSG is a potentially damaging agent that promotes intra or intermolecular proteineprotein and proteinelipid cross linkage (Meister and Anderson, 1983)

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1, 3, 5-triazine) induces oxidative stress on antioxidant enzymes(superoxide dismutase; SOD, catalase; CAT and guaiacol peroxidase; POD) and DNA damage on earthworms and the adverse effects may be the important mechanisms of its toxicity to earthworms (West *et al.*, 1967).

Katalinic, *et al.* (2010) showed that earthworm paste has notable amounts of phenolic compounds that have significant antioxidant activity. The antioxidant activity of earthworms paste was evaluated using DPPH (1,1- diphenyl-1-2-picrylhydrazyl-hydrate) method. The extraction of phenolics with 80% ethanol in both species yielded the highest amount of phenolics i.e. 220.6 mg/L in the African night crawler extract and 247.00 mg/L in the red worm extract. Extraction with 80% methanol yielded 217.00 mg/L of phenolics in the African night crawler extract and 228.00 mg/L in the red worm extract. Extraction with 75% methanol yielded higher amounts in red worm (237.00 mg/L) than in African night crawler (207.00 mg/L). EP of the African night crawler yielded a greater amount of phenolic compounds than the red worm. DPPH assay showed that the inhibition activity of African night crawler (95.23%) was significantly higher than that of the red worm extract (38.26%) in 80% methanol, whereas, in 80% ethanol, the inhibition activity of African night crawler (48.13%) was not significantly different from that of the red worm extract (47.73%). Inhibition of antioxidant activity of African night crawler extract in 75% methanol (48.13%) was higher than that of the red worm extract (47.93%).

On the other hand, very few studies on oxidative stress and antioxidant defences in earthworm have been performed. Stenersen and Oien (1981) reported the existence of the glutathione (GSH) glutathione-S-transferase (GST) system in earthworms. Saint-Denis, *et al.* (1998) studied the activities of enzymes (catalase, glutathione peroxidise, glutathione-S-transferase and glutathione reductase) involved in antioxidant defence system in *Eisenia fetida* which are mainly localized in the cytosolic fraction. In response to organic and inorganic pollutant perturbation of glutathione concentration, the activity of glutathione-S-transferase, glutathione reductase and glutathione peroxidase has been reported in earthworms (Saint- Debis, *et al.* 2001).

CHAPTER-VI

SUMMARY AND CONCLUSION

During the present investigation, the effect of heavy metal contamination on the biomolecules and immunity parameter of *Eisenia fetida* was undertaken. The results on above aspects are summarized below:

- In the first experiment biomolecules concentration in the heavy metal contaminated earthworm was estimated which shows the decrease in protein, carbohydrate and lipid concentration.
- The maximum reduction in biomolecules i.e. 29.18% in protein 21.57% in carbohydrate and 41.25% in lipid concentration was seen in earthworms treated with Hg-0.06ppm.
- For other treatments i.e. Cobalt and combination of mercury and cobalt the results show decrease in biomolecules but decrease was almost negligible
- When the cells of coelomic fluid were calculated there was significant decrease in the total number of coelomocytes
- 82.54% in earthworms treated with Hg-0.06ppm similarly significant reduction in eleocytes, amoebocytes**I** amoebocytes**II**, granulocytes**I** and granulocytes**I** was seen.
- 74.65% decrease was seen for earthworm treated with Co-0.06ppm and reduction was 79.73% for earthworms treated with combination of both i.e. Hg-0.03 + Co-0.03 ppm.
- The antioxidant activity was measured by DPPH scavenging activity by using two solvents i.e 75% methanol, 80% methanol and 80 % ethanol.
- 75% methanol and 80% ethanol was concluded as best solvent showing 47% inhibition activity whereas for 80% methanol it shows 38 % inhibition activity.
- Antioxidant activity for different heavy metal treated earthworm was studied w.r.t. control earthworms which shows there is sharp increase in antioxidant activity in first 15 days and then become almost stable.
- Maximum increase in antioxidant activity at day 60 was seen for earthworms treated with Hg-0.06 ppm i.e. 60.718% which was 47.232% by using 80% ethanol as solvent.
- It was concluded that among two heavy metals, mercury is more toxic than cobalt and combination of both.

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ABSTRACT

Title of dissertation	:	**Effect of heavy metals on the bio-molecules and immunity parameters of earthworm, *Eisenia fetida***
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Among terrestrial invertebrates, earthworms are priority test organisms for soil contamination surveys because they are easy to handle, widespread in their terrestrial distribution and have the capacity to accumulate and concentrate large quantities of inorganic and organic pollutants with acquired adaptive resistance to the toxicity of these pollutants including heavy metals. Biochemical responses in organisms against environmental stress are regarded as early warning indices of pollution in the environment. The present study was carried out to evaluate the effect of heavy metal on the bio-molecules and immunity parameter of earthworm, *Eisenia fetida*. The results showed acute toxicity of two types of metals (mercury and cobalt) at different concentration on earthworms In the first experiment bio-molecules concentration in the heavy metal contaminated earthworm was estimated which shows the decrease in protein, carbohydrate and lipid concentration. The maximum reduction in bio-molecules i.e. 29.18% in protein 21.57% in carbohydrate and 41.25% in lipid concentration was seen in earthworms treated with Hg-0.06ppm. For other treatments i.e. Cobalt and combination of mercury and cobalt the results show decrease in bio-molecules but decrease was almost negligible. When the cells of coelomic fluid were calculated there was significant decrease in the total number of coelomocytes 82.54% in earthworms treated with Hg-0.06ppm similarly significant reduction in eleocytes, amoebocytes **I** amoebocytes **II**, granulocytes **I** and granulocytes **II** was seen 74.65% decrease was seen for earthworm treated with Co-0.06ppm and reduction was 79.73% for earthworms treated with combination of both i.e. Hg-0.03 + Co-0.03 ppm. The antioxidant activity was measured by DPPH scavenging activity by using two solvents i.e 75% methanol, 80% methanol and 80 % ethanol. 75% methanol and 80% ethanol was concluded as best solvent showing 47% inhibition activity whereas for 80% methanol it shows 38 % inhibition activity. Antioxidant activity for different heavy metal treated earthworm was studied w.r.t. control earthworms which show there is sharp increase in antioxidant activity in first 15 days and then become almost stable. Maximum increase in antioxidant activity at day 60 was seen for earthworms treated with Hg-0.06 ppm i.e. 60.718% which was 47.232% by using 80% ethanol as solvent. It was concluded that among two heavy metals, mercury is more toxic than cobalt and combination of both.

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