

Research Article

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## Coelomic fluid protein profiling and heavy metal accumulation of three earthworms after exposure to pesticide and metal stress

V. Jeyanthi<sup>a</sup>, J. Arockia John Paul<sup>b\*</sup>, B. Karunai Selvi<sup>c</sup>, M. Biruntha<sup>d</sup> and N. Karmegam<sup>e</sup>

<sup>a</sup>Department of Biotechnology, Sri Kaliswari College, Sivakasi - 626 130, Tamil Nadu, India

<sup>b</sup>Department of Zoology, Arumugam Pillai Seethai Ammal College, Tiruppattur- 630 211. Tamil Nadu, India.

<sup>c</sup>Department of Botany, V.V. Vanniaperumal College for Women, Virudhunagar – 626 001, Tamil Nadu, India.

<sup>d</sup>Department of Animal Health and Management, Alagappa University, Karaikudi – 630 003, Tamil Nadu, India.

<sup>e</sup>Department of Botany, Government Arts College, Salem - 636 007, Tamil Nadu, India.

\*Corresponding Author: [jajpaul@gmail.com](mailto:jajpaul@gmail.com)

### Abstract

#### Keywords

Carbaryl,  
lead,  
Protein profiling,  
*Perionyx ceylanensis*.

The coelomic fluid protein profiling studies were performed under pesticide and metal stress condition in three species of earthworms namely *Eudrilus eugeniae*, *Perionyx excavatus* and *Perionyx ceylanensis*. The carbaryl (12, 25 and 50 mg/kg) and lead (70, 150 and 300 mg/kg) were used as an agent to measure the changes in the coelomic fluid profile. The changes in protein concentration were identified by SDS-PAGE. The concentration of proteins expressed gets varied between normal, pesticide and metal induced earthworms. The metal (Pb) accumulation in earthworms was estimated in Atomic Absorbance Spectrophotometer (AAS). Maximum lead accumulation was observed in the earthworm, *E.eugeniae* on day 14 at 300mg/kg of Pb contaminated beds i.e.,  $17.9821 \pm 2.4$  ppm.

### 1. Introduction

Earthworms are important components of natural agriculture ecosystems, functioning in the process of large quantities of organic materials and comprise key links in food chains. They are found in a wide range of habitats throughout the world, having adapted to many different soil types as well as lakes and streams. They provide bait for fishing, a source of protein for food and most importantly they play a unique and important role in conditioning the soil (Lee, 1985). The bodies of earthworms are extremely nutrient rich from minerals to amino acids, proteins and vitamins. When earthworms die, these nutrients are released into the soil (Edwards and Bohlen, 1996).

Among terrestrial invertebrates, earthworms are priority test organisms for soil contamination surveys. Fitzpatrick *et al.* (1996) used the earthworms in immuno toxicological studies because they are sufficiently complex for use as surrogates in immunotoxicity based research aimed at assessing the immunotoxic potential of chemicals in higher animals because

their immunoactive cells exhibit functions as analogous or homologous to those of vertebrates.

The proteins were isolated from coelomic fluid of earthworm, *Lumbricus terrestris* and it was analyzed by SDS-PAGE. The proteomic profile expressed in earthworm coelomic fluid, a component of the earthworm's immune system should prove to be worthwhile in advancing immunotoxicity biomarker based assays (Geoffrey, 2006).

Heavy metals endanger all types of terrestrial life of which earthworm is an important member, playing a major role in the development and maintenance of soil structure. It is also used as a source of food for other organisms. Earthworms have the ability to tolerate many kinds of chemical contaminants including heavy metals (Corp and Morgan, 1991). This tolerance may be a positive attribute for assessing bioaccumulation (or) toxicity studies of severely contaminated sites. In both standard laboratory and field tests, earthworms

are regarded as bioindicators of soil contaminated with heavy metals, pesticides and other organic pollutants (Booth *et al.*, 2000). After entering the body, lead disturbs almost every metabolic function in body chemistry (David *et al.*, 1976). Heavy metals have been shown to cause lysosomal membrane instability, changes in gene expression, oxidative stress, to reduce growth, cocoon production and hatchability, slow sexual development, affect the population size, abundance and species diversity of earthworms (Spurgeon *et al.*, 2005). This study is aimed to determine the protein concentration and characterization of expressed proteins by SDS-PAGE and to determine the accumulated lead concentration in the body mass of earthworms. Many investigators have used *E. fetida* as the standard test species for ecotoxicity studies due to its tolerance to high concentration of pesticides (Ribera *et al.* 2001). Only few reports are available on toxicity assessment studies in *E. eugeniae* and *P. excavatus* but no reports are available for *P. ceylanensis* except Jeyanthi *et al.*(2016). This is the first report on the comparative study of proteomic analysis and lead accumulation in three earthworm species under normal and stress induced conditions.

## **2. Materials and Methods**

### **2.1.1 Experimental animals**

The pre-clitellate earthworms of *Eudrilus eugeniae* (African night crawler), *Perionyx excavatus* (Asiatic species) and recently explored epigeic species, *Perionyx ceylanensis* (Indigenous species) (Paul *et al.* 2011) were selected as the test animals for the present study. Earthworms were obtained from the Department of Biology, Gandhigram Rural University, Gandhigram. Animals were carefully transported to the laboratory and mass multiplied in culture tanks by mixing cowdung powder with water and maintained at a temperature of  $28 \pm 2$  °C.

### **2.1.2 Preparation of substrate for earthworm**

All the experiments were carried out under laboratory conditions. The earthworms were exposed to increasing concentrations of carbaryl (12, 25 and 50 mg kg<sup>-1</sup> artificial soil) and lead acetate (75, 150 and 300 mg kg<sup>-1</sup> artificial soil) for different periods of time (5 and 10 days) and (2, 7 and 14 days) using the standard soil test method (Maity *et al.*, 2010a and 2010b; Ribera *et al.*, 2001). For each species, six replicates of eighteen sets of artificial soil substrate were prepared in plastic container by mixing cowdung and soil (1:1) with water at a ratio of 1:1(W/V) in plastic container, covered with nylon net to prevent escape of earthworms. Three types of artificial soil substrate were prepared as follows: (i) control (soil + cowdung powder + 10 earthworms) (ii) Metal treated (soil + cowdung powder + lead acetate +10 earthworms) (iii) Pesticide treated (soil + cowdung powder + carbaryl + 10 earthworms). The cowdung powder was amended with appropriate amount of lead acetate and carbaryl solution made in distilled water. The same procedure using distilled water was applied to

prepare a set of three control substrates without Pb and carbaryl. The soil was mixed thoroughly to ensure a homogenous mixture. The average height of each substrate was 10 cm and initial pH of the substrate was  $6.8 \pm 0.05$ . Temperature was maintained at  $28 \pm 2$  °C throughout the study period.

### **2.1.3 Collection of earthworm coelomic fluid**

After 7 and 14 days of exposure to metal and 5 and 10 days of exposure to pesticide stress and from control earthworms, coelomic fluid was obtained by puncturing post clitellum segments of the coelomic cavity with a Pasteur micropipette and kept at 4°C. Coelomic fluid suspension from each treatment of earthworms was pooled and centrifuged (500g, 10 min, 4°C). Supernatant was centrifuged again (7000g, 10 min, 4°C), and stored at -20°C (Kohlerova *et al.*, 2004).

## **2.2 Qualitative amino acid analysis**

Chemical tests were performed to identify the both essential and non essential amino acids present in the coelomic fluid of earthworms (Jayaraman, 1968).

## **2.3 Determination of coelomic fluid protein concentration**

Total crude coelomic fluid protein concentration for samples collected from control and experimental organisms was determined according to the Lowry's method (Lowry *et al.*, 1951).

## **2.4 Protein Profiling**

### **2.4.1. SDS-PAGE**

The protein separation was accomplished using 10% SDS run at 100V for 2 hours followed by staining and destaining (Plummer, 1977).

### **2.4.2. MALDI-TOF Analysis**

Identification of protein expressed in earthworm coelomic fluid from control and earthworms exposed to pesticide and metal stress was carried out by MALDI-TOF/TOF-MS and database searching analysis at IIT, Chennai (Wang *et al.*, 2010b)

## **2.5. Amino acid sequencing**

The earthworm *P. ceylanensis* DNA was isolated and sent to the Chromous Biotech Lab, Chennai for DNA sequencing. The 630bp of Cytochrome Oxidase I (COI) gene was obtained and was subjected to ExPASy translate tool to obtain amino acid sequencing (Joskova *et al.*, 2009).

## **2.6. AAS (Atomic Absorption Spectrophotometer) analysis**

The earthworms gut contents were evacuated and their muscles were kept in a hot air oven to get dried. For analysis, the dried residues were dissolved in 0.7M HNO<sub>3</sub>

and this dissolved solution was used to estimate the metal (lead) concentration in Atomic Absorption Spectrophotometer (Welz and Sperling, 1999).

### 3. Results and Discussion

#### 3.1 The qualitative analysis of amino acids

A total of twenty amino acids consisting of ten essential amino acids namely arginine, valine, histidine, isoleucine,

leucine, lysine, methionine, phenylalanine, threonine, tryptophan and ten non-essential amino acids namely aspartic acid, serine, glutamic acid, proline, glycine, alanine, tyrosine, asparagine, cysteine, cystine were recorded in this study from each species of earthworm (Table 1). Notable changes in the concentrations of amino acids were observed between the control and stress conditions. Similar observations were made for four species of earthworms from Nigeria namely *E. eugeniae*, *H. africanus*, *A. millsoni* and *L. violaceus* by Dedeke *et al.* (2010).

Table 1. Qualitative analysis of amino acids in the earthworm coelomic fluid under pesticide and metal stress conditions.

Amino acids	<i>E. eugeniae</i>			<i>P. excavatus</i>			<i>P. ceylanensis</i>		
	Control	Pesticide stress	Metal stress	Control	Pesticide Stress	Metal stress	Control	Pesticide stress	Metal stress
<b>Essential amino acids</b>									
Arginine	++	++	++	+	++	++	+	+	+
Valine	++	++	+++	+	++	++	+	++	+
Histidine	+	+	+	+	++	+++	++	+	++
Isoleucine	+	+	+	+	+	+	++	+++	++
Leucine	++	+++	++	++	+++	+	+	+++	+
Lysine	+	+	++	+	++	+++	++	++	+++
Methionine	+	+	+	+	+	++	+++	++	++
Phenylalanine	++	++	+++	++	++	++	+	++	+
Threonine	+	++	++	+	++	+++	+	+	+
Tryptophan	+	+++	+	++	++	++	+	+	++
<b>Non-essential amino acids</b>									
Aspartic acid	++	+	++	+	+	++	++	++	+
Serine	+	+	+	++	++	++	+	+	++
Glutamic acid	+	++	++	+	++	+++	+	++	++
Proline	+	++	+	+	+++	+	++	+++	++
Glycine	++	+++	++	+	+	++	++	++	+++
Alanine	+	+	+++	+	+	+	++	++	++
Cystine	++	++	++	+	++	++	+	+	+++
Tyrosine	+	+++	+	++	++	++	+	+	++
Asparagine	+	++	++	+	+	++	+	++	+
Cysteine	++	+	++	++	++	+++	+	++	+

(+ + +) = highly present; (+ +) = moderately present; (+) = least present

#### 3.2 Protein analysis of earthworm coelomic fluid

Percentage increase in protein concentration of the coelomic fluid in three species of earthworm after pesticide and metal stress are given in Table 2. In all the three species with the increase in the concentration of metal and pesticide stress, the expression of protein is also increased when compared with the respective control. Fig.1 and 2 represents the SDS-PAGE showing protein expression profile of coelomic fluid from control, pesticide and metal stressed earthworms of

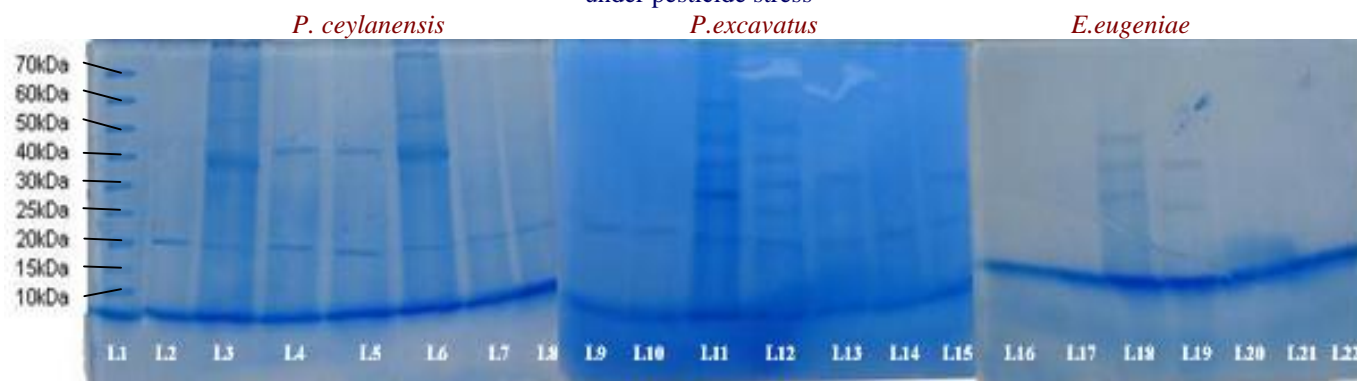
three species. The number of proteins expressed gets varied between normal and stress induced earthworms. The proteomic analysis was done on earthworm *Eisenia fetida* during Cadmium exposure (Wang *et al.*, 2010a) and *E.coli* 0157:H7 stress (Wang *et al.*, 2010b). The study of protein expression profile of coelomic fluid in earthworm *Lumbricus terrestris* was also done with bacteria, *Aeromonas* (Geoffrey, 2006) and copper sulphate (Herring, 2010) challenged earthworm.

Table 2. Percentage increase in protein concentration of earthworm coelomic fluid after pesticide and metal stress

Groups	<i>E. eugeniae</i> *		<i>P. excavatus</i> *		<i>P. ceylanensis</i> *	
	After 5 days	After 10 days	After 5 days	After 10 days	After 5 days	After 10 days
<b>Pesticide stress</b>						
12mg	5.41	7.37	2.27	9.39	8.57	13.33
25mg	8.85	16.19	6.52	12.94	11.11	16.67
50mg	10.26	21.78	10.42	14.62	16.52	18.75
<b>Metal stress</b>	<b>After 7 days</b>	<b>After 14 days</b>	<b>After 7 days</b>	<b>After 14 days</b>	<b>After 7 days</b>	<b>After 14 days</b>
75mg	7.69	20.83	2.75	2.69	15.74	21.37
150mg	10.00	22.45	4.62	4.53	22.66	22.26
300mg	14.29	24.00	8.15	9.64	29.29	27.21

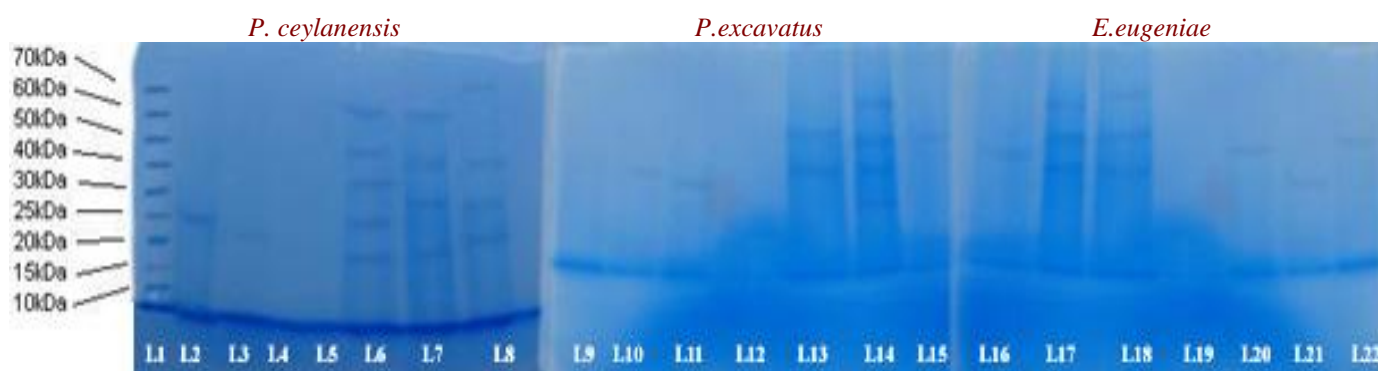
\*Percentage increase over control

Fig. 1. SDS – PAGE gel showing protein expression profile of coelomic fluid from three species of earthworms under pesticide stress



Lane 1- Standard protein marker; Lane 2, 9 and 16- Respective control; Lane 3, 10 and 17- Pesticide stress (12mg/kg) after 5 days  
 Lane 4, 11 and 18- Pesticide stress (25mg/kg) after 5 days; Lane 5, 12 and 19- Pesticide stress (50mg/kg) after 5 days; Lane 6, 13 and 20- Pesticide stress (12mg/kg) after 10 days; Lane 7, 14 and 21- Pesticide stress (25mg/kg) after 10 days; Lane 8, 15 and 22- Pesticide stress (50mg/kg) after 10 days

Fig. 2. SDS – PAGE gel showing protein expression profile of coelomic fluid from three species of earthworms under metal stress



Lane 1- Standard protein marker; Lane 2, 9 and 16- Respective control; Lane 3, 10 and 17- Metal stress (75mg/kg) after 7 days  
 Lane 4, 11 and 18- Metal stress (150mg/kg) after 7 days; Lane 5, 12 and 19- Metal stress (300mg/kg) after 7 days; Lane 6, 13 and 20- Metal stress (75mg/kg) after 14 days; Lane 7, 14 and 21- Metal stress (150mg/kg) after 14 days; Lane 8, 15 and 22- Metal stress (300mg/kg) after 14 days

By using MALDI-TOF/TOF-MS and database searching analysis, total number of proteins expressed in earthworm coelomic fluid from control and earthworms exposed to pesticide and metal stress is identified and given in Table 3. Compared with control eight new proteins in *E. eugeniae* and *P. ceylanensis* and nine new proteins in *P. excavatus* were expressed under stress condition as a result of increased immunomodulatory activity. In the present study, a higher number of different proteins (8) were recorded in the coelomic fluid of *P. excavatus* in carbaryl stress (trypsin

inhibitor, -lactoglobulin, carbonic anhydrase II, trypsinogen, carbonic anhydrase I, fetidin, glutamate dehydrogenase, coelomiccytolytic factor) and *P. ceylanensis* in lead stress (trypsinogen, myoglobin, lactate dehydrogenase, coelomic cytolytic factor, coelomic mitogenic factor, fetidin, -lactoglobulin and heat shock protein) respectively. A high number of proteins (56) were identified by Wang *et al.* (2010a) from *E. fetida* living in a cadmium-polluted environment

Table 3. Total number of proteins expressed in earthworm coelomic fluid from control and earthworms exposed to pesticide and metal stress.

Earthworm species	Treatments		
	Control	Pesticide stress	Metal stress
<i>E. eugeniae</i>	Calreticulin	Trypsin inhibitor, Carbonic anhydrase II, Fetidin, Carbonic anhydrase I	Lactate dehydrogenase, Coelomic cytolytic factor, Glutamate dehydrogenase, Heat shock protein, Carbonic anhydrase I, Calreticulin, Fetidin
<i>P. excavatus</i>	Trypsin inhibitor	Trypsin inhibitor, -lactoglobulin, Carbonic anhydrase II, Trypsinogen, Carbonic anhydrase I, Fetidin, Glutamate dehydrogenase, Coelomic cytolytic factor	Fetidin, Cleavage stimulating factor subunit 1, Lysenin, Carbonic anhydrase II, Glutamate dehydrogenase
<i>P. ceylanensis</i>	Trypsin inhibitor, Trypsinogen, Cytochrome oxidase I	Trypsin inhibitor, Fetidin, Heat shock protein, Cleavage stimulating factor subunit 1, Coelomic cytolytic factor	Trypsinogen, Myoglobin, Lactate dehydrogenase, Coelomic cytolytic factor, Coelomic mitogenic factor, Fetidin, -lactoglobulin, Heat shock protein

### 3.3. Amino acid sequencing analysis

The DNA sequence of *P. ceylanensis* was converted into amino acid sequencing using the tool of ExPASy proteome server (Fig. 3). The complete cDNA sequence for COI was determined by the Sanger method of dideoxynucleotide-mediated chain termination using primer walking. Nucleotide and amino acid sequences were compared to entries in the EMBL and Swiss-Prot databases (Lassegues *et al.*, 1997). The amino acid sequence of *E. fetida* calreticulin and calreticulin molecules of other species were aligned using CLUTALW program (Silerova *et al.*, 2007). The signal peptide of EALys (Lysozyme from *E. andrei*) was predicted using both neural network and hidden Markov model on a signal IP 3.0 server. Molecular weight and isoelectric points were predicted using Protprogram on the ExPASy server (Joskova *et al.*, 2009). The sequence derived may be useful for biomarker studies with *P. ceylanensis* and it may serve as a primary source of sequence data for this earthworm species.

### 3.4 Lead concentration in body mass of earthworms

The metal (Pb) accumulation in earthworms was estimated in Atomic Absorbance Spectrophotometer (AAS). It is

evident from Table 4, that Pb concentration in earthworms increase with increasing metal contamination. It is also apparent that tissue metal concentration in earthworms increase with a concomitant increase in exposure duration to the respective metal contaminated soil. Maximum lead accumulation was observed in the earthworm, *E. eugeniae* on day 14 at 300mg/kg of Pb contaminated beds i.e.,  $17.9821 \pm 2.40$  ppm.

Earthworms are known to accumulate metals from the soil efficiently as observed by various authors (Wright and Stringer, 1980; Labort *et al.* 1998). The toxicity of heavy metal for earthworms increases with increasing the soil metal concentration. In the present study Pb was detected in three species of earthworms exposed to Pb contaminated cowdung powder. With increasing metal contamination, Pb concentration in earthworms were also increased. This is in agreement with other studies which have clearly demonstrated that concentrations of metals in whole worms increase concomitantly with increased soil metal concentration (Morgan and Morgan, 1988). Honda *et al.* (1984) reported that accumulation of Pb, Hg, and Cd and As in the earthworm *Pheretima hilgendorfi* depends on the exposure duration whereas the accumulation of Fe, Mn, Zn, Cu, Ni and Co is dependent upon the metabolic turnover.



Thereafter metal concentrations remain constant throughout the entire life span. The present study clearly demonstrates

that statistically significant accumulation of Pb in the earthworms.

Fig. 3. Amino acid sequencing of COI gene of *Perionyx ceylanensis* using ExPASy translate tool.

### 5'3' Frame 1

GGFPRPPAARKLVPNFCPSTPGHRPRQHRHDRDHIKAV **Met** SQVQTTGASS  
SAHAGCPHVQFLQ **Stop** R **Stop** SPPDQPNCHLVKAKKQESCCRR **Met** WSTGG  
LKRSSQTRPAPLHEQAPQQDQEGNGQIQKLPFFILGRASSGAPTILGIIHLA  
RP **Met** KAGITAPT **Stop** **Stop** GRAPWSSD **Stop** KKVLHLCSRGGTFGPNHLA **Met**  
PLSQHDRVTTIRLP **Met** SLWLEK

### 5'3' Frame 2

GGSPAPRPLENWSQIFVRQHLVTAPASTG **Met** TAITLRR **Stop** **Stop** ARSRQQG  
HLLQP **Met** PGAR **Met** FNSCNDVDRPLINQIAI **Stop** **Stop** RQKNKSRVAVGVCQG  
QGGLKGPAPLGLPPPS **Met** NRLHNRIRKATGRSRNSLFSSWAGQVRAPLPS  
WGSSIWPGR **Stop** RLASRHQHDEAVHRGHLIKRCSTFAAGVELSARITW  
PCPPYHN **Met** TG **Stop** QQYACQCPCGWKK

### 5'3' Frame 3

GVP PPPGRSKTGPKFLSVNTWSPPPPAPA **Stop** PRSH **Stop** GGDEPGPDNRG  
IFFSPCRVPACSIPA **Met** TLIAP **Stop** STKLPFSEGKKTRVVLLPAYVVNRGA  
**Stop** KVQPN SARPPP **Stop** TGSTTGSQRQADPETPFFHFGQKFGFRPYHLG  
DHPFGWDEGWHHGTNT **Met** **Met** RPTCVVI **Stop** LKKGAPPLQPGWNFRPEL  
GHAPPITT **Stop** QGNNNT PANVLVVGKK

### 3'5' Frame 1

FFSNHKDIGRRIVVTLSCCDRGG **Met** AK **Stop** FGPKVPPRLQRWSTFF **Stop** SD  
DHGARPHHG VGAV **Met** PAFIGLAKW **Met** IPK **Met** VGAPALALPR **Met** KKG SFWI  
CPLPS **Stop** SCCGACSWRGAGRVWLDLLSPPVDHIRRQQHDS CFFAFTKWQ  
FG **Stop** SGGDQRHCRN **Stop** TCGHPAWA EEDAPV VWTWLITAL **Met** **Stop** SRSC  
RCWRGR **Stop** PGVDGQKFGTSFRAAGGRGNP

### 3'5' Frame 2

FFPTTRTLGAVLLLPCHVVIGGAWPSDSGRKFHPGCKGGAPFFNQ **Met** TTV  
HGLI **Met** VLVP **Stop** CQPSSAWPNG **Stop** SPRW **Stop** GRPNLPCPG **Stop** KKG VSG  
SARCLPDPVVEPVHGGGRAEFGWTF **Stop** APLTTYAGNSTTLVFLPSLNG  
LVDQGA INVIAGIEHAGTRHGLKK **Met** PLLSGPGSSPP **Stop** CDRGHAGAGGG  
GDQVLT DKNLGPVFERP GGGGTP

### 3'5' Frame 3

FFQPQGHWQAYCCYPV **Met** L **Stop** **Stop** GGHGQVIRAESSTPAAKVEHLFLIR  
**Stop** PRCTASSWCWRDASLHRPGQ **Met** DDPQDGRGARTCPAQDEKREFLD  
LPVAFLILLWSLF **Met** EGGGPSLAGPFKPPC **Stop** PHTPATARLLFFCLH **Stop**  
**Met** AIWLIRGRSTSLQELN **Met** RAPG **Met** G **Stop** RRCPCCLDLAHHRLNVI AV **Met**  
PVLGAVTRC **Stop** RTKIWDQFSSGRGAGEPP

Table 4. Lead concentration accumulated in the body of earthworms

Sl.No.	Earthworm species	Control (ppm)	Treatments (ppm)		
			75mg/kg	150mg/kg	300mg/kg
1.	<i>E.eugeniae</i>	1.1054 ± 0.01	4.3567±0.92	7.4281±1.30	17.9821 ± 2.40
2.	<i>P.excavatus</i>	0.5268 ± 0.03	0.5794±0.02	0.6952±0.01	0.8663 ± 0.03
3.	<i>P.ceylanensis</i>	3.0907 ± 0.2	3.5273±0.03	3.9951±0.03	5.1160 ± 0.04

## 4. Conclusion

The carbaryl and lead induced stress in the earthworms, *E. eugeniae*, *P. excavatus* and *P. ceylanensis* showed twenty amino acids (ten essential amino acids-arginine, valine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan; ten non-essential

amino acids-aspartic acid, serine, glutamic acid, proline, glycine, tyrosine, asparagine, cysteine, cystine. The earthworms used in this study expressed different kinds of proteins in their coelomic fluids as a result of pesticide and metal stress. Present study clearly demonstrates the effect of carbaryl and lead stress on changes in coelomic fluid protein expression.

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