

## RESEARCH REPORT

**Heavy metal induced biomolecule and genotoxic changes in earthworm *Eisenia fetida*****G. Muthukaruppan***Department of Biotechnology, School of Bio-Engineering, SRM University, Kattankulathur 603203, Tamilnadu, India**Accepted September 16, 2015***Abstract**

A vibrational [Fourier transform infrared (FTIR)] spectroscopic method was used for the structural and compositional analysis of earthworm *Eisenia fetida* by monitoring of metal binding and further transformations in live cells. The FTIR analyses for metals taken up by the *E. fetida* will be useful for analyzing the impact of the heavy metal stress on the worm metabolism. The epigeic earthworm *E. fetida* were exposed to 100 %, 75 %, 50 %, 30 %, 25 %, 15 % and 5 % of dried automobile service station waste mud. All the earthworms exposed in the 100 %, 75 % and 50 % concentrations didn't survived within 10 days. Further experiments were conducted with 25 %, 15 % and 5 % concentration of wastes. Each concentration level was tested with three replicates using 10 animals and the metabolic response after exposure to the heavy metal containing service station waste mud was assessed by FTIR. Furthermore we also emphasized that DNA damage was confirmed with the use of other biomarker like comet assay. The peaks at 1045, 1080, 1236  $\text{cm}^{-1}$  and 1650  $\text{cm}^{-1}$  represented the overall susceptibility of nucleotides, phospholipids, DNA and RNA. Nucleic acids and proteins were modified due to heavy metal accumulation. In flow-through, single cell gel electrophoresis revealed the degradation nuclear DNA. Heavy metals accumulation in the worms was measured and it was found that lead, zinc and copper accumulation increased in the treatment group. Without the use of biomarkers for identifying ecological risks of land contamination, traditional assessment would be difficult to interpret. This new FTIR based biomolecules study revealed a clear molecule shift in the exposed worms, due to heavy metal accumulation.

**Key words:** earthworm; *Eisenia fetida*; FTIR; DNA fragmentation; heavy metals and biomolecules

**Introduction**

Heavy metal pollution of soil is widespread across the globe and has caused biological problems, leading to potential toxicity to living organisms. Recent research found that the atmospheric input of heavy metals to agricultural systems also significantly contributed to metal loading in soil (Vidovic *et al.*, 2005). Given the surge in passenger vehicle usage it has become difficult to avoid exposure to the metals existing in our surroundings. The determination of the toxicity of metals is difficult because of the complex nature of their interactions with biological systems.

Earthworms, among the many kinds of soil organisms are considered potential bioindicators proven their usefulness in the evaluation of metal contamination in soil. Significant positive correlations have been observed between the metal concentrations in the earthworm and the cadmium (Cd), copper (Cu), lead (Pb) and zinc (Zn)

concentrations in the soil (Morgan and Morgan, 1988).

Earthworm celomocytes possess the celomic fluid harboring cells, which are similar to mammalian leucocytes, are relatively easy to obtain, and may be useful to perform both bioassays on the same biological samples (Burch *et al.*, 1999; Weeks and Svendsen, 1996).

The metabolic profile of healthy organisms can be compared to that of treated organisms and the variations noted could be used to identify the response to the stress. In fact, as the interaction of toxic agents with living cells causes molecular modifications in the organisms (Salman *et al.*, 2002; Argov *et al.*, 2004; Beleits *et al.*, 2005), other approaches based on Fourier transform infrared (FTIR) spectroscopy could become helpful to study the molecular modifications observed in living cells after exposure to pollutants (Saxena *et al.*, 2005). Since DNA damage may result in severe consequences for individuals, species, and ecosystems, it is regarded as an important indicator to be used in the assessment of earthworm health (Reinecke and Reinecke, 2004; Casabé *et al.*, 2007). In the current study, we analyzed heavy metal induced bio-molecule changes in *E. fetida* reflected by FTIR spectroscopic features with

*Corresponding author:*  
Gobi Muthukaruppan  
Department of Biotechnology  
School of Bio-Engineering  
SRM University  
Kattankulathur 603203, Tamilnadu, India  
E-mail: gobicc@gmail.com

attenuated total reflectance (ATR) mode and which, to the best of our knowledge, have not previously been reported. Furthermore, we aimed to identify and quantify the DNA fragmentation of exposed worms.

## Materials and Methods

### *Earthworm species exposed and experimental setup*

In the present study, automobile waste mud was collected from Madurai, Tamilnadu, India. The experimental beds were prepared with cow dung and automobile waste at 100 %, 75 %, 50 %, 25 %, 30 %, 15 % and 5 % levels in rectangular plastic tubs (of 12"×17"×51" size) in triplicates and control (cow dung only) was maintained. All the earthworms exposed in the 100 %, 75 % and 50 % concentrations didn't survived within 10 days. Further experiments were conducted with 25 %, 15 % and 5 % concentration of wastes. Tests were conducted using laboratory cultured adult specimens of *Eisenia fetida*, each concentration level was tested with 10 worms and tests were run for a period of 35 days. After completion of 35 days, earthworms from each concentration were taken and washed with distilled water. Then, (Pokarzheritskii *et al.*, 2000) the earthworm's gut was cleaned and then these earthworm samples were lyophilized and powdered, further these lyophilized samples were subjected for FTIR analysis. For FTIR in the absorbance mode, samples were mixed with KBr (Merck) or, for diffuse reflectance infrared Fourier transform (Shimadzu FTIR-8400S) measurements, used as dry finely ground powder in a micro sampling cup. FTIR ATR spectra were collected at 4 cm<sup>-1</sup> of spectral resolution after 250 scans in the 400 - 4000 cm<sup>-1</sup> range. Then spectra were baseline corrected and normalized using the absorption band at 1650 cm<sup>-1</sup> belonging to the C–O group of protein structures. Second derivative spectra were also collected for enhancing peak resolution to identify some low resolved infrared bands.

### *Comet assay*

Earthworm coelomocytes were obtained using the modified protocol of Reinecke and Reinecke (2004). The extrusion fluid containing cells was centrifuged and the supernatant removed. The cell pellet was suspended and washed three times in Phosphate Buffer Solution (PBS), using microcentrifugation, for 3 min at 380g. The concentration of cells in the final suspension was determined using the trypan blue exclusion method and dilutions calculated to be used for the exposures (*in vitro*) and for the comet assay, described below. The comet assay was conducted under yellow light, to prevent UV-induced DNA damage, and performed Nogueira *et al.* (2006), with a few minor modifications: normal microscope slides, not fully frosted slides, were used; the slides, were covered with the first agarose layer and left to dry to enable the adherence of the gel layer to the slides; only two layers of agarose were used (the first dried layer and the layer with the cells). Visual scoring of cellular DNA on each slide was based on

**Table 1** Physico-chemical parameters of automobile service station waste mud was analyzed and given below

Parameters	Values
Colour	Brownish black
Texture	Sandy clay loam
pH	7.0-7.6
Electrical conductivity(mS/cm)	0.4-0.8
Moisture content (%)	40-50
Bulk density (m/m <sup>3</sup> )	1.21- 1.32
Porosity (%)	37.8-39.2
N (%)	8.4- 8.9
P (%)	1.45 -1.67
K (%)	12.0- 12.9
Cu (ppm)	47.35-50.06
Pb(ppm)	40.25-42.64
Cd (ppm)	86.7-89.36
Zn(ppm)	98.2-101.0
Fe(ppm)	5796.7-5800.1

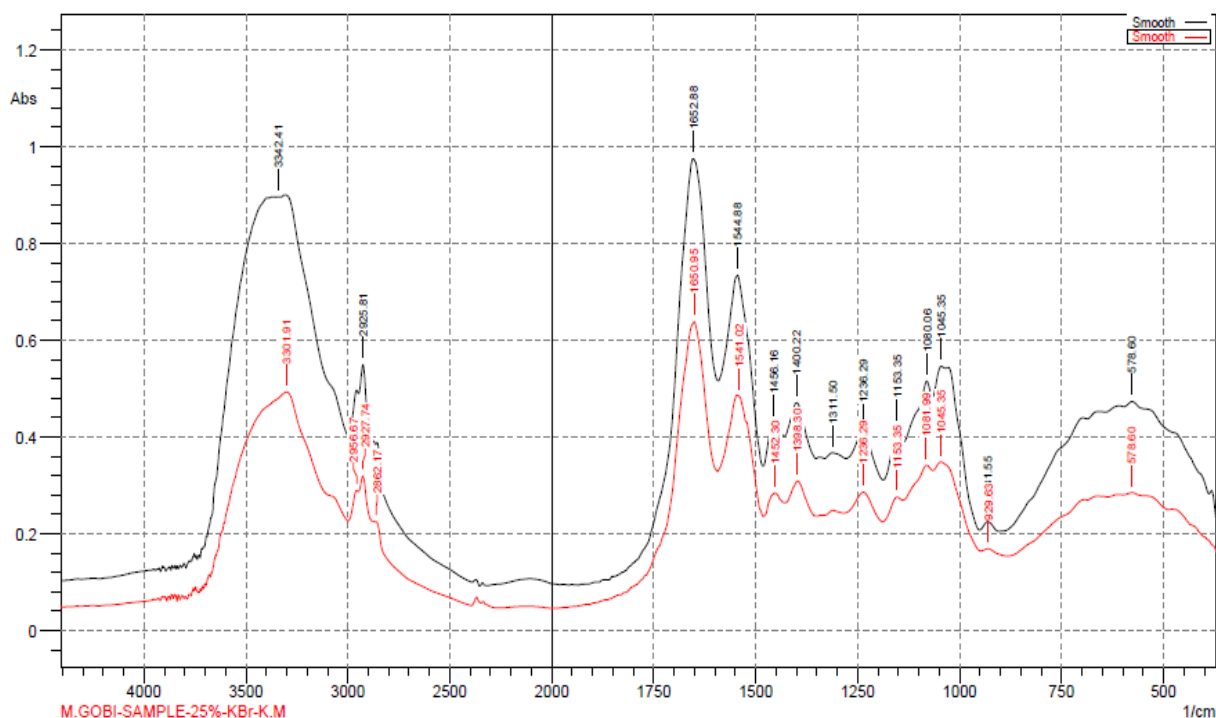
the categorization of 100 randomly-selected cells. Four specimens per dose were used along with the negative control. For positive control, the cells were treated with *ex vivo* with 100 µM H<sub>2</sub>O<sub>2</sub> for 7 min at 0 °C. Two slides per specimen were prepared and 100 cells per slides were scored by using autocomet software.

### *Residual content analysis*

Earthworms from each concentration were taken and washed with distilled water. Using above said method Pokarzheritskii *et al.* (2000) earthworm's gut was cleaned and then these earthworms were used for further extraction. Then to assess the total metal content of samples, soil and earthworm samples from all the treatments were acid digested to determine the total amount of selected metals. Substrates used for acid digestion were dried for 48 h at 70 °C and weighed. After samples were digested in nitric acid and perchloric acid, the metal dry weight concentration was determined by using atomic absorption spectroscopy. The heavy metals concentration in medium, earthworm over 35 days and DNA damage scores at percentage of cells in each damage class in coelomocytes of *E. fetida* after heavy metals exposure over 35days were subjected to suitable statistical transformations and the transformed values were evaluated by one-way analysis of variance in the Microsoft Excel statistical package.

## Results

Table 1 summarizes physico-chemical properties of automobile service station waste mud and it was found (47.35 - 50.06 ppm Cu, 40.25 - 42.64 ppm Pb, 86.7 - 89.36ppm Cd, 98.2 - 101.0 ppm Zn and 5796.7 - 5800.1 ppm Fe) that highest range of heavy metals.



**Fig. 1** FTIR-ATR overlapped spectra of *E. fetida* both control and 25% concentration spectra with molecular modifications are reported by a red line while control spectra are reported by black line.

#### Metabolic action of *E. fetida* against heavy metals investigated by FTIR spectroscopy

FTIR analysis offers excellent information on the nature of the bonds present on the earthworm surface and allows identification of different functional groups on the cell surface which are capable of interacting with metal ions. Changes in band frequency can also be used to estimate the relative importance of the various surface functionalities in metal sorption. The FTIR spectra of *E. fetida* are as shown in Figs 1 - 3 and the list of FTIR band assignment is reported in Table 2. FTIR spectroscopy shows many bands belonging to the different functional groups of worm's biomolecules which were resulted by the structural modifications caused by the presence of pollutants. As clearly evident from Figs 1- 3, all the bands were modified by the exposure to the heavy metals containing waste.

The absorbance bands at  $2925.81\text{ cm}^{-1}$  correspond respectively to the  $\text{CH}_2$  asymmetric and symmetric stretching of methylene group, which mainly monitor lipids. In the present study, the asymmetric band shift from  $2925.81$  to  $2927.74$  in 25 % levels respectively, due to heavy metal exposure. The sharp band observed at  $1652.88\text{ cm}^{-1}$  and  $1544.32\text{ cm}^{-1}$  corresponding to amide I and amide II vibration of structural proteins, respectively, (Casado *et al.*, 2007). Identified Amide - I bands absorption was at  $1652.88\text{ cm}^{-1}$  shifted to  $1650.95\text{ cm}^{-1}$  in 25 %,  $1654.81\text{ cm}^{-1}$  in 15 % and  $1652.88\text{ cm}^{-1}$  in 5 % level and it was due to zinc exposure. According to peak assignment (the peaks

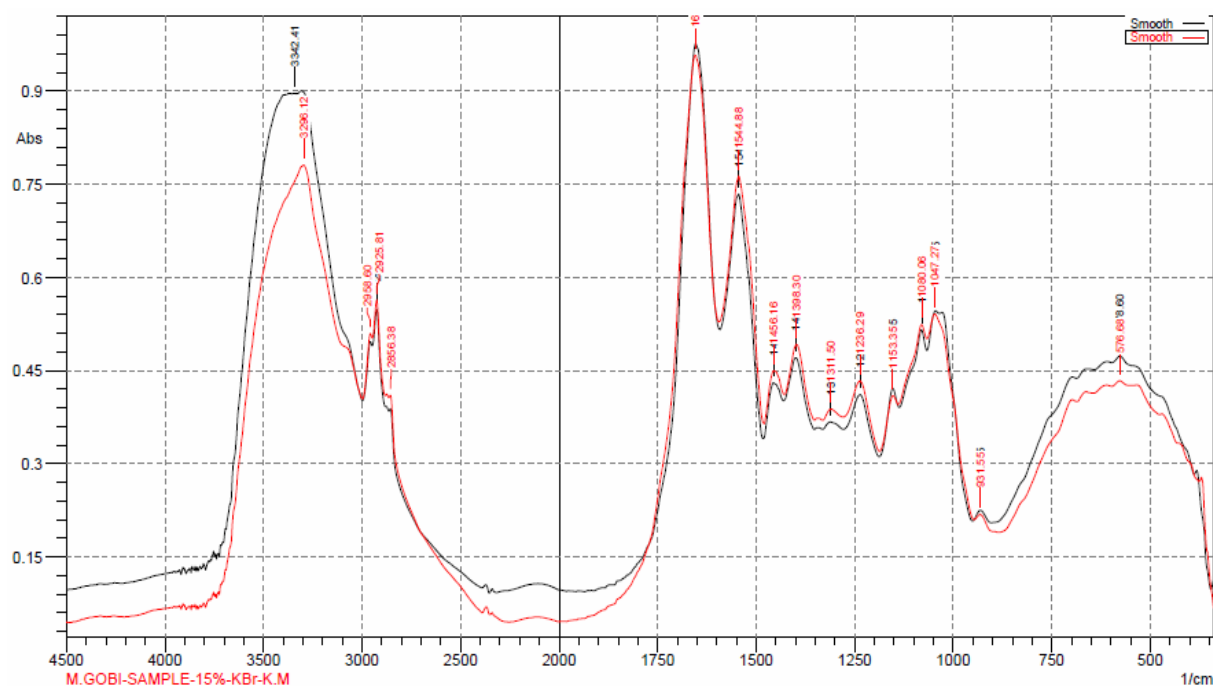
at  $1045$ ,  $1080$ ,  $1236\text{ cm}^{-1}$  and  $1650\text{ cm}^{-1}$ ), these peaks represent the nucleotides and phospholipids. There were shifts in these regions, which mean that heavy metals strongly affect nucleic acids. Furthermore we also emphasized that this approach should be confirmed (nucleic acid) with the use of other biomarker like comet assay.

#### Comet assay

Figures 4 and 5 show DNA damage scores at percentage of cells in each damage class in coelomocytes of *E. fetida*, after heavy metals exposure over 35 days at 25 %, 15 % and 5 % concentration. After 35 days of exposure the DNA damage, evaluated with the parameter tail DNA percentage and tail moment in the coelomocytes of *E. fetida* exposed to the heavy metals containing automobile service station waste. These findings clearly indicate a connection between accessible or rather available metal content and highest DNA damage in the celomocytes of *E. fetida*. A significant concentration-related increase in the percentage of damaged cells was observed ( $4.91 \pm 5.3$  in 5 %,  $8.49 \pm 10.2$  in 15 %,  $12.42 \pm 12.72$  in 25 % and  $7.57 \pm 10.7$  in positive control). This percentage differed significantly when compared with controls, at all assayed heavy metal concentrations.

#### Heavy metal accumulation

The earthworms collected from different treatments groups, showed considerable accumulation of metals in their tissues. Statistically



**Fig. 2** FTIR-ATR overlapped spectra of *E. fetida* both control and 15 % concentration spectra with molecular modifications are reported by a red line while control spectra are reported by black line.

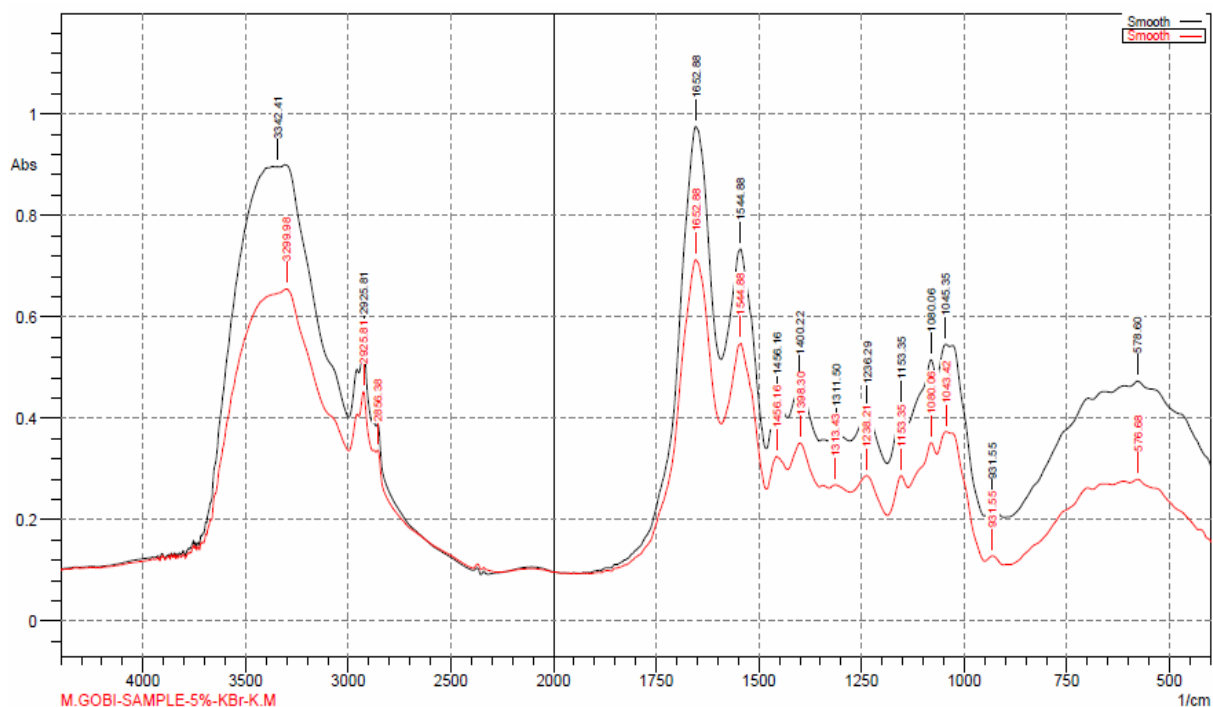
(ANOVA), the difference among treatments for contents of metals in earthworms was significant. Table 3 depicts the heavy metal contents of the automobile service station mud and the mean copper concentration of 25 %, 15 % and 5 % were  $22.13 \pm 0.32$ ,  $15.13 \pm 0.007$  and  $5.34 \pm 0.14$  ppm, respectively. The Pb concentration was  $19.4 \pm 0.36$ ,  $12.48 \pm 0.08$ ,  $7.06 \pm 0.11$ ,  $30.96 \pm 0.15$ ,  $24.6 \pm 2.27$  and Cd concentration was  $12.7 \pm 0.2$  ppm, respectively. Zn and Fe content in the automobile waste was  $43.03 \pm 0.29$ ,  $29.59 \pm 0.04$ ,  $10.4 \pm 0.01$ ,  $924.23 \pm 11.92$ ,  $404.57 \pm 7.3$  and  $320.78 \pm 3.65$  (ppm), respectively. Differences in the mean heavy metal concentrations between the lower and higher concentrations were significant at 0.05 %. Table 2 shows that the accumulated heavy metal concentration in earthworm on 35<sup>th</sup> day, the Cu and Pb content in 25 %, 15 % and 5 % earthworm was  $1.369 \pm 0.042$ ,  $1.023 \pm 0.04$ ,  $0.09 \pm 0.001$ ,  $6.60 \pm 0.095$ ,  $4.306 \pm 0.02$  and  $2.2 \pm 0.017$  ppm, respectively. Similarly, the Cd, Zn and Fe content were  $0.082 \pm 0.004$ ,  $0.063 \pm 0.006$ ,  $0.021 \pm 0.001$ ,  $3.613 \pm 0.006$ ,  $3.101 \pm 0.003$ ,  $1.997 \pm 0.013$ ,  $2.907 \pm 0.004$ ,  $1.242 \pm 0.3$  and  $0.007 \pm 0.002$ , respectively. When compared to all heavy metals, Fe accumulated more in earthworm tissues. This could have been the result of both heavy metal accumulation with time as well as the increased service station waste mud concentration of the substrate with feed. The difference in waste concentrations in the body tissues of the earthworms between the three groups of exposure was significant ( $p < 0.005$ ) at the end of the experiment.

## Discussion

### *Metabolic action of E. fetida against heavy metals investigated by FTIR spectroscopy*

The aim of this work was to evaluate the effect of heavy metals on the biomolecule changes in the earthworm *E. fetida*. As FTIR spectroscopy may be more sensitive to certain functional group (e.g. including polar bonds) as compared to FT-Raman spectroscopy (Kamnev *et al.*, 2006), we attempted FTIR analyses of worms in control and in the presence of heavy metals containing medium. There are striking differences in the overall FTIR profiles between the control and heavy metal exposed worms. A most prominent feature of the metal stressed worm is the appearance of a relatively strong and well resolved CH<sub>2</sub> asymmetric and symmetric stretching of methylene group at about  $2925.81\text{cm}^{-1}$ . Lipids play an important role in the membrane fluidity. By affecting the conformation of membrane proteins, they govern exposure and diffusion of membrane component (Palaniappan *et al.*, 2010).

In the control, where the amide I at about  $1655.10\text{cm}^{-1}$  dominates, there was shift to  $1650.95\text{cm}^{-1}$  in 25 %,  $1654.81\text{cm}^{-1}$  in 15 %. The shift observed of the amide bands in the zinc exposed tissues indicates important structural alteration in the existing proteins as suggested by Toyran *et al.* (2008). Further, the area of amide II band observed at  $1544.32\text{cm}^{-1}$  in the control tissues shifted to  $1541.02\text{cm}^{-1}$  in 25 %. These changes reflect the loss of protein level in the zinc exposed tissues. This loss of protein may be due to increased protein



**Fig. 3** FTIR-ATR overlapped spectra of *E. fetida* both control and 5 % concentration spectra with molecular modifications are reported by a red line while control spectra are reported by a black line.

oxidation in the tissues with zinc exposure (Takahashi *et al.*, 1991; Cakmak *et al.*, 2006; Carpena *et al.*, 2007).

The amide I and amide II bands of cellular proteins at 1656 and 1544  $\text{cm}^{-1}$  are asymmetric and the test showing higher intensity and modified shapes with respect to the same band of the worms is the control. This is likely to reflect some partial changes in the secondary structure of the cellular proteins from dominating  $\alpha$ -helix to other possible conformations (Naumann *et al.*, 1993; Bonnina *et al.*, 1999). This reduced amide bands in the heavy metal exposed tissues indicates important structural alteration in the existing proteins as suggested by Toyran *et al.* (2008). Further, the area of amide II band observed at 1544.88  $\text{cm}^{-1}$  in the control tissues decreases and these changes reflect the loss of protein level of the heavy metal exposed worms. This loss of protein may be due to increased protein oxidation in the exposure worms (Takahashi *et al.*, 1991; Cakmak *et al.*, 2006; Akkas *et al.*, 2007).

Nucleic acids and proteins intensity change when an external perturbation (i.e. the increased pollutant concentration) is applied to the biological system (Melin *et al.*, 2004). This is due to accumulation of Pb, Zn and Cu in *E. fetida*. Some metals, such as hexavalent chromium (CrVI), manganese (Mn), and Pb, as well as Cd and arsenic (As), also reportedly inhibited the 8-oxo-Gua repair system (Bolin *et al.*, 2006; Hodges and Chipman, 2002; Lee *et al.*, 2005; Sava *et al.*, 2004; Singh *et al.*, 2009) which in turn increased the

mutation frequency. FT-IR spectroscopic technique is used to determine the biomolecular profile of micro-samples of coelomic fluid. Presence of arginine and lysine and absence of glutamic acid under toxicological condition could be considered as markers of pollutants in the environment (Joe *et al.*, 2014).

#### Comet assay

The comet assay is a genotoxicity assay for the detection of DNA single strand breaks (Singh *et al.*, 1988) in single cells. The exposure to the heavy metals containing soil seem to have caused significant DNA damages after 35 days of exposure compared with the control. The increase in DNA damages was probably caused by the production and intracellular accumulation of ROS, induced by the exposure of earthworms to heavy metals.

Evaluation of metal sublethal toxicity should always include biomarkers of DNA damage because such damage may result in inappropriate gene expression and, subsequently, in more concerning genotoxic and mutagenic effects. Cadmium is of environmental interest due to its high toxicity to organisms and because it is usually mined and extracted from zinc ores (Mirsal, 2004). Zinc is an essential element, but can be highly toxic when present at high concentrations. It is possible that Cd and Zn share an uptake pathway in organisms since they have similar electron configuration, as well as chemical and physical properties (Brzóška and Moniuszko-Jakoniuk, 2001). Cadmium induces disruption of DNA repair leading to mutations that

**Table 2** List of functional groups present in FTIR spectra of *E. fetida* assigned according to the identified bands of absorption

Peaks				Identified bands
Control	25%	15%	5%	
1045.35	1045.35	1047.27	1043.42	Phospholipids, DNA and RNA
1080.06	1081.99	1080.06	1080.06	C–O carbohydrates
1153.35	1153.35	1153.35	1153.35	CO–O–C asymmetric stretching: mainly glycogen and nucleic acids
1236.29	1236.29	1236.29	1238.21	C–N peptide group of nucleic acids
1456.16	1452.3	1456.16	1456.16	CH <sub>2</sub> bending: mainly lipids with little contribution from proteins
1544.88	1541.02	1544.88	1544.88	Amide II: N–H bending and C–N stretching of the polypeptide and protein backbone
1652.88	1650.95	1654.81	1652.88	Amide I: C=O stretching of proteins
2925.81	2927.74	2925.81	2925.81	CH <sub>2</sub> asymmetric stretching: mainly lipids, with little contribution from proteins, carbohydrates, nucleic acids
3342.41	3301.91	3296.12	3299.98	Amide A: mainly N–H stretching of proteins with negligible contribution from O–H stretching of intermolecular hydrogen bonding

together with increased cell proliferation and blocked apoptosis could result in tumor formation (Waalkes 2003; Waisberg *et al.*, 2003; Hei and Filipic, 2004). Zn has an optimal intracellular range above or below which internucleosomal DNA cleavage, chromatin condensation, and nuclear fragmentation are induced (Krug, 2002). Hwang *et al.* (2004) and Seve *et al.* (2002) reported that metals such as Zn and Cd may have apoptotic and/or necrotic effects over cells of different organs.

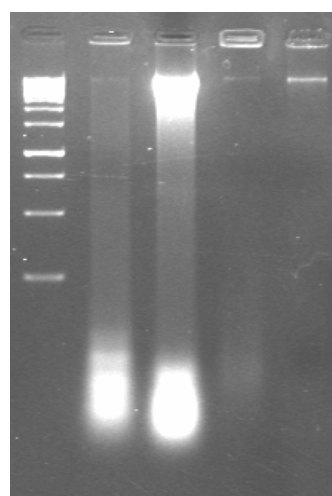
#### Heavy metal accumulation

Earthworms are known to accumulate metals from the soil efficiently as observed by various authors (Ireland, 1975a, b; Labort *et al.*, 1998; Morgan and Morgan, 1988; Wright and Stringer, 1980). The toxicity of heavy metal for earthworms increases with increasing the soil metal concentration (Marinussen *et al.*, 1997). Earthworms predominantly take up heavy metals from soluble metal fractions (Saxe *et al.*, 2001; Vijver *et al.*, 2003).

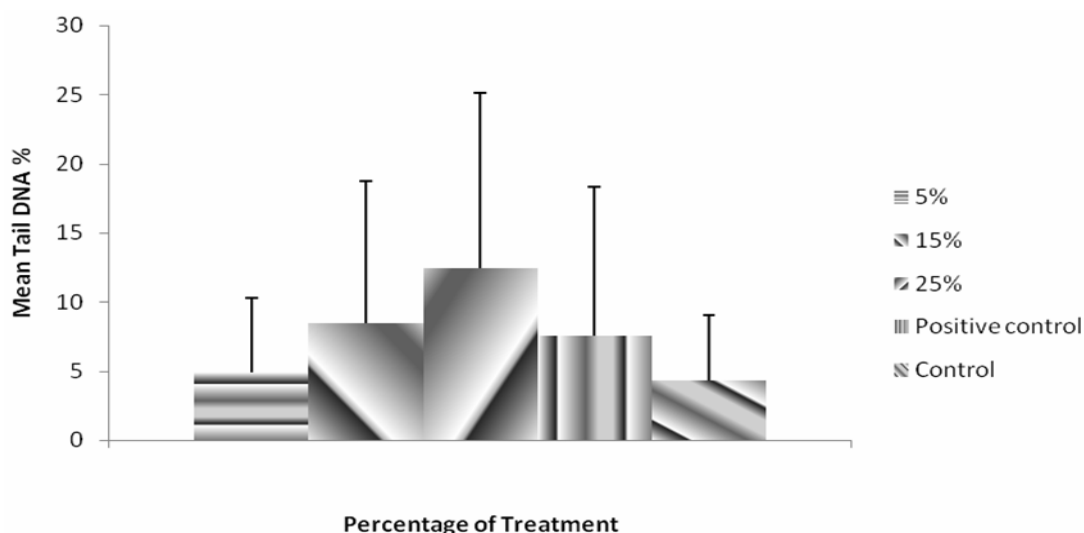
As a result, the possibility for Fe metal to be bound to ions and carbonates (i.e. more soluble fractions) increases in ingested material. As a result, the metal content reduces in digested organic material due to bioaccumulations of more soluble fractions of metals in an earthworm's gut or cutaneous tissues. In general, earthworms consume a great amount of organic waste to achieve appropriate nutrition, and during this process metals are liberated in free forms due to the enzymatic actions in their gut (Suthar, 2007). Furthermore, such available forms of metals are then absorbed by the epithelial layer of gut during the transiting of wastes through it. Bioaccumulation of high

concentration of metals is well documented (Hsu *et al.*, 2006).

Heavy metal accumulation depends on the exposure duration whereas the accumulation of Fe, Zn, Cu, Cd and Pb is dependent upon the metabolic turnover. Thereafter metal concentrations remain constant throughout the entire life span. The present study clearly demonstrates that statistically



**Fig. 4** Apoptotic DNA fragment induced in *E. fetida* with mixer of heavy metals for 35 days exposure. Lane M: 1 kp marker; Lane 1: 25% treatment, Lane 2: 15 % treatment; Lane 3: 5 % treatment and Lane 4: Control.



**Fig. 5** DNA damage scores at percentage of cells in each damage class in celomocytes of *E. fetida*, after heavy metals exposure over 35 days at 25 %, 15 % and 5 % concentration. Significant at  $p < 0.05$  % level

significant accumulation of Fe, Zn, Cu, Cd and Pb in the earthworm does take place as reported earlier (Honda *et al.*, 1984). It is known that earthworm chloragosomes function as the cation exchange system capable of taking up and retaining heavy metals (Ireland, 1978; Morgan and Morgan, 1998), which are subsequently excreted by fractionation of the chloragocytes (Fischer, 1976).

### Conclusion

Without the use of biomarkers for identifying ecological risks of land contamination, traditional assessment would be difficult to interpret. Therefore, the earthworm based biomarkers used in this study to measure ecological exposure to hazardous substance have identified risks to scientifically relevant organisms and indicated bioavailability of pollutants and their effects. This new FTIR based biomolecules study revealed a

clear molecular shift in the exposed worms, due to heavy metal accumulation and this is new to earthworm toxicology. The bio-molecule modification responses of heavy metals are best assessed exploiting one of the many budding *in vivo* techniques. Besides, the short-term biomolecule assay (FTIR study) on earthworms is an early indicator of long-term toxicity, which may result in DNA damage. Further, the diverse structural changes in the DNA, (DNA fragmentation) was visualized and quantified, which provide a promising basis for the development of sensitive biomarkers for ecotoxicological study.

### Acknowledgement

This research was supported by the Department of Science and Technology (DST), New Delhi, Fast Track Young Scientist grant SR/FT/LS-007/2007. The authors would like to thank UGC-NRCBS and MKU-USIC for laboratory assistance.

**Table 3** Various concentrations of accumulated heavy metals in the medium and as well as earthworm over 35 days exposure in the three different treatments setup

Heavy metals	Heavy metals concentration in the medium at the initial stage			Heavy metals concentration in the medium after 35 days exposure			Heavy metals concentration in the earthworm after 35 days exposure		
	25%	15%	5%	25%	15%	5%	25%	15%	5%
<b>Cu</b>	22.13±0.32	15.03±0.07	5.34±0.14	16.24±1.39	11.93±0.11	4.723±0.52	1.369±0.04	1.023±0.04	0.09±0.00
<b>Pb</b>	19.4±0.36	12.48±0.08	7.06±0.11	9.21±1.00	6.94±0.15	4.176±0.16	6.606±0.09	4.306±0.02	2.2±0.01
<b>Cd</b>	30.96±0.15	24.6±2.27	12.7±0.2	27.14±1.69	18.68±1.03	10.31±1.11	0.82±0.00	0.063±0.0	0.021±0.00
<b>Zn</b>	43.03±0.29	29.59±0.04	10.4±0.01	35.55±1.08	21.0±1.91	7.207±0.41	3.613±0.00	3.101±0.0	1.997±0.01
<b>Fe</b>	924.23±11.92	404.57±7.3	320.78±3.65	546.05±20.1	224.7±12.6	134.4±8.78	2.907±0.00	1.242±0.3	0.007±0.0
<b>F value</b>	16880.8*	7567.2*	21693.7*	1993.9*	805.19*	622.09*	7072.9*	450.0*	38399*

Mean ± SD; \* significant at  $P < 0.05$

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