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RESEARCH REPORT

Effect of heavy metals on four different earthworm's species specific autofluorescing eleocytes

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Abstract

Earthworm is key bio-indicator of soil milieu to assess heavy metal contaminations. The celomic fluid of earthworm plays a significant role in the storage of riboflavin within the celomic cavity thereby maintaining its homeostasis. So the measurement of these autofluorescent 'self-marking' eleocytes will give more information about chloragocyte derived cells and species. The present study is to investigate the percentage of autofluorescing cells, riboflavin content, elemental and heavy analysis of immunologically significant eleocytes. The present study is focused on four different earthworms namely *Lampito mauritii, Octochaetona serrata, Eudrilus eugeniae and Eisenia fetida,* to characterize immune factors such as cellular and riboflavin content exposed to various heavy metal concentrations under laboratory conditions. Four different earthworms were exposed to three different concentrations (63.5, 112.4 and 207.2 µg/mL) of heavy metals (Cu, Cd, and Pb) over 96 h. The celomic fluid were subjected for FACS analysis to find out the percentage difference of autofluorescent eleocytes cells between control and exposed species. The discrepancies between riboflavin content of control and heavy metal exposed worms were analyzed by spectrofluorescence. The low and negligible percentage of autofluorescent cells were recorded in *E. eugeniae* and *L. mauritii* but large numbers of autofluorescent cells were recorded in *E. fetida* and *O. serrata*. The experimental results show that riboflavin content and autofluroscence cells of the heavy metal exposed worms displays significant decrease in the celomic fluid. The present study clearly demonstrated that investigated species possess the significant population of celomocytes, but they differ considerably in the number of cells per body mass and by species. This non-invasive technique proves stable cellular biomarkers in earthworm for toxicological and biological soil monitoring studies.

Key Words: earthworms; autofluorescence; heavy metals; riboflavin; FACS

Introduction

Earthworms are ubiquitous and used as a model organism for many eco-toxicological studies. The interaction between earthworm and soil leads to increase in soil fertility and mobilization of heavy metals from soil to terrestrial ecosystem. The homeostasis properties of earthworm play a vital role/ significant role in maintaining the elemental concentration in the coelomic fluid. When the heavy metal concentration increases in the soil, it affects the homeostasis of the earthworm which leads to the excretion of heavy metals in the celomic fluid. The riboflavin storage is universal in earthworm species, as it was detected both in attached chloragocytes forming the chloragogen tissue and in chloragocyte-derived eleocytes (Mazur *et al*., 2011). According to (Ottaviani, 2011), the celomocytes of all earthworm species contain amebocytes. Immune system parameters may be used as a sensitive sub-lethal endpoint to assess the toxicity of atmospheric deposition to earthworms. The earthworm immune system is composed mainly of celomocytes, *i.e*., cells found within the fluids in the worm celomic cavity (Stein *et al.,* 1977). It has been demonstrated that many chemicals, including various trace elements, can adversely affect the immune system (Fournier *et al.,* 2000). The immunodeficiency of exposed species is interpreted as an indication of toxic effects of environmental contaminants (Dales and Kalac, 1992; Fournier *et al.,* 2000). The celomic fluid exhibits "molecules of many biological functions" (antimicrobial peptides and lysozymes) in annelids which provides effective

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protection mechanisms against invaders. The composition of celomocytes in earthworm are species-specific, particularly in regard to autofluorescent eleocytes (Koziol *et al.,* 2006, Plytycz *et al.,* 2006). The chloragocyte and riboflavin inclusion in the earthworm are species specific and depends on the soil metal quality in Lumbricidae (Verdrengh and Tarkowski, 2005; Iwanaga *et al.,* 2007). There was no previous report for celomocytes community of *Lampito, Octochaetona* and *Eudrilus* worms.

The aim of this work was to characterize the selected immune factors like cellular and riboflavin content. It was of interest to address the differences between immunological features of these four species, because although they share many similarities, their natural environment varies considerably. Therefore, it was expected to discover some discrepancies between the immunological features of the four species. The differences of cellular levels and riboflavin content of control and heavy metal exposed worms were analyzed by FACS and spectrofluorescence. Furthermore, the variations of elements were quantitively measured by AAS.

Materials and Methods

Collection and identification of earthworm

Four different species of earthworms *Lampito mauritii*, *Octochaetona serrata*,(two anecics worms) *Eudrilus eugeniae* and *Eisenia fetida* (two epigeic)were mass cultured at our vermiary (400 - 800 lux light) and then these earthworms were identified based on the morphological characters.

Gut cleaning

Collected earthworms were washed with distilled water after which 50 ml jars were filled with 30 ml of 1.5 % agar gel prepared with deionised water. After getting cooled and solidified in the jars, the gel was taken out and cut into small pieces. After which earthworms were transferred separately into jars and kept for 96hrs at room temperature (400 - 800 lux light) to remove all the soil from their gut (Pokarzherskii *et al*., 2000).

Harvesting of celomic fluid

After removing the gut contents, diverse species of earthworms were cranked for 30 sec with a 4.5 V electric current to expel celomic fluid with suspended celomocytes through the dorsal pores, as described previously (Plytycz *et al*., 2006). Briefly, earthworms were placed individually in petri dishes containing 3ml of extrusion fluid (phosphate-buffered saline, PBS, supplemented with 2.5 g/L ethylenediamine tetraacetic acid, EDTA). Freshly prepared 2 mL suspensions were used for spectrofluorimetry and Flow cytometry analysis. Further, celomic fluid was collected by above said method without PBS and EDTA.

Cell counting and viability

Cells viability were assessed by Trypan blue exclusion test, mixing an equal volume of celomic fluid and 0.4 % Trypan blue solution. Cell viability should always exceed 90 %. Collected celomic fluid was smeared on a clean glass slide, to observe the autofluorescence of celomic fluid cells.

Flow cytometric measurements and analysis

A thin smear was prepared on a clean glass slide using collected celomic fluids and examined under Olympus inverted fluorescence microscope. Samples of celomocytes were analyzed with a BD FACSCalibur flow cytometer system. During scientific experiments, 10,000 threshold events per worm sample were collected and analyzed by their forward scatter (FS) (for cell size) and sideward scatter (SS) (cell complexity) properties. Fluorescence FL1-H (emission 530 nm; excitation 488 nm) was recorded and resulting data were analyzed using WinMDI 2.8 software, by producing dot plots of cell size versus FL1 autofluorescence.

Table 1 shows the species-specific characteristics of celomocytes in four earthworm species

Species	N	BW (g)	TC (X 10^5)	TC/BW (X 10^5/g)	% AFC	AFC/ BW (X 10^5/g)
Eisenia fetida	4	0.16 ± 0.00	3.4 ± 0.4	21.2	55.1 ± 0.07	344.3
Lampito mauritii	4	0.54 ± 0.02	3.06 ± 0.3	5.6	18.8 ± 1.25	34.8
Octochaetona serrata	4	0.51 ± 0.01	4.4 ±0.23	8.6	42.4 ± 0.97	83.1
Eudrilus eugeniae	4	1.2 ± 0.07	1.6± 0.3	1.3	0.18 ± 0.09	0.15

Means ± SD; N = number of individuals; BW = body weight; TC = total celomocytes; TC/BW = celomocytes /gram of body weight; % AFC = percentage of autofluorescent celomocytes; AFC/BW = percentage of autofluorescent celomocytes/gram body weight.

Estimation of riboflavin by spectrofluorometer

Spectrofluorometric analyses were performed using the celomocyte lysate. Different species of the earthworm celomocyte suspension lysates with 2 % Triton. The supernatant was collected, and it was subjected to spectrofluorometric analyses with standard riboflavin (HiMedia Laboratories). Excitation spectra were recorded at 300 and 520 nm (excitation at λ = 525 nm) while emission spectra were recorded at 380 and 700 nm using excitation at λ = 370 nm. The spectrofluorometric signatures of unbound riboflavin were characterized by two maxima (at 370 nm and 450 nm) in the excitation spectrum, and an emission spectrum maximum at 525 nm. Arbitrary units (AU) of fluorescence were recorded using Microsoft Excel v. 2007.

Metal exposure

Metal chlorides (Cu, Cd, Pb) were dissolved in double distilled water to a final 1mM metal concentration being equivalent of 63.5, 112.4 and 207.2 µg/mL respectively. Dissolved metal chlorides were incorporated in 1.5 % agar gel, after cooling and solidifying this gel in the jars, it was taken out and cut into small pieces. The earthworms were then transferred to jars containing agar pieces and kept for 96 h and these agar pieces were made possible to consume easily by the earthworm. One group served as a control, and other three groups were exposed to a concentration of 63.5, 112.4 and 207.2 µg/mL, respectively. Each level was tested in three replicates using ten animals.

Heavy metal analysis

The concentration of heavy metals was analyzed in acid digested samples of earthworm by atomic absorption spectrometer as described by AQAC (1999). Heavy metal bioavailability to earthworm evaluated both in terms of relative toxicity and bioaccumulation factor (BAF) (Saxe *et al*., 2001). The BAF was calculated as per equation; BAF = [Metal] Earthworm/[Metal] Agar, where [metal] EARTHWORM represents the total metal concentration of earthworm (mg kg), and [metal] Agar represents the total metal concentration of sludge (mg kg^{-1}).

Statistical analysis

Results were expressed as mean ± standard deviations. Further data were analyzed using the one-way ANOVA with post hoc Tukey's test. The level of statistical significance was set at $p < 0.05$.

Results

Celomocytes

The celomycytes of four different earthworms were calculated as per its body weight as shown in Table 1. The low and negligible percentage of autofluorescent cells were recorded in *E. eugeniae* and *L. mauritii*. The high rate and the large numbers of autofluorescent cells were recorded in *E. fetida* and *O. serrata*. The celomocytes number per gram of body weight was found to be maximum in *E. fetida* ($4.6x10^5$/g), and it was recorded as $2.2x10^5$/g in *O. serrata*, but it was $1.27x10^5$/g, $3.0x10^5$/g in *L. mauritii* and *E. eugeniae* respectively. It indicates the absence of a simple correlation between the body size/ weight and the number of celomocytes inhabiting the celomic cavity.

Flow cytometric analysis of eleocyte

Fluorescence microscopic observation of celomocytes from representative adults of four different species of earthworm revealed autofluorescent eleocytes and some adherent amebocytes which are devoid of autofluorescence. Moreover, flow cytometry and fluorescent microscopy revealed the significant proportion of eleocytes in *Octochaetanea* and *E. fetida*, while autofluorescent cells were very lower in *Lampito* and *Eudrilus*. Flow cytometric analysis showed the high proportion of amebocytes in *Lampito* and *Eudrilus*. Figure 1 shows the percentage of granular

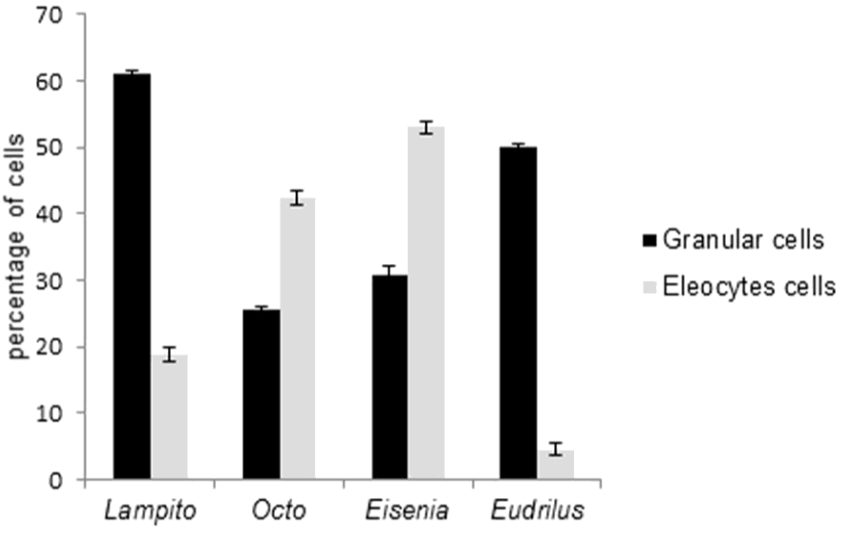
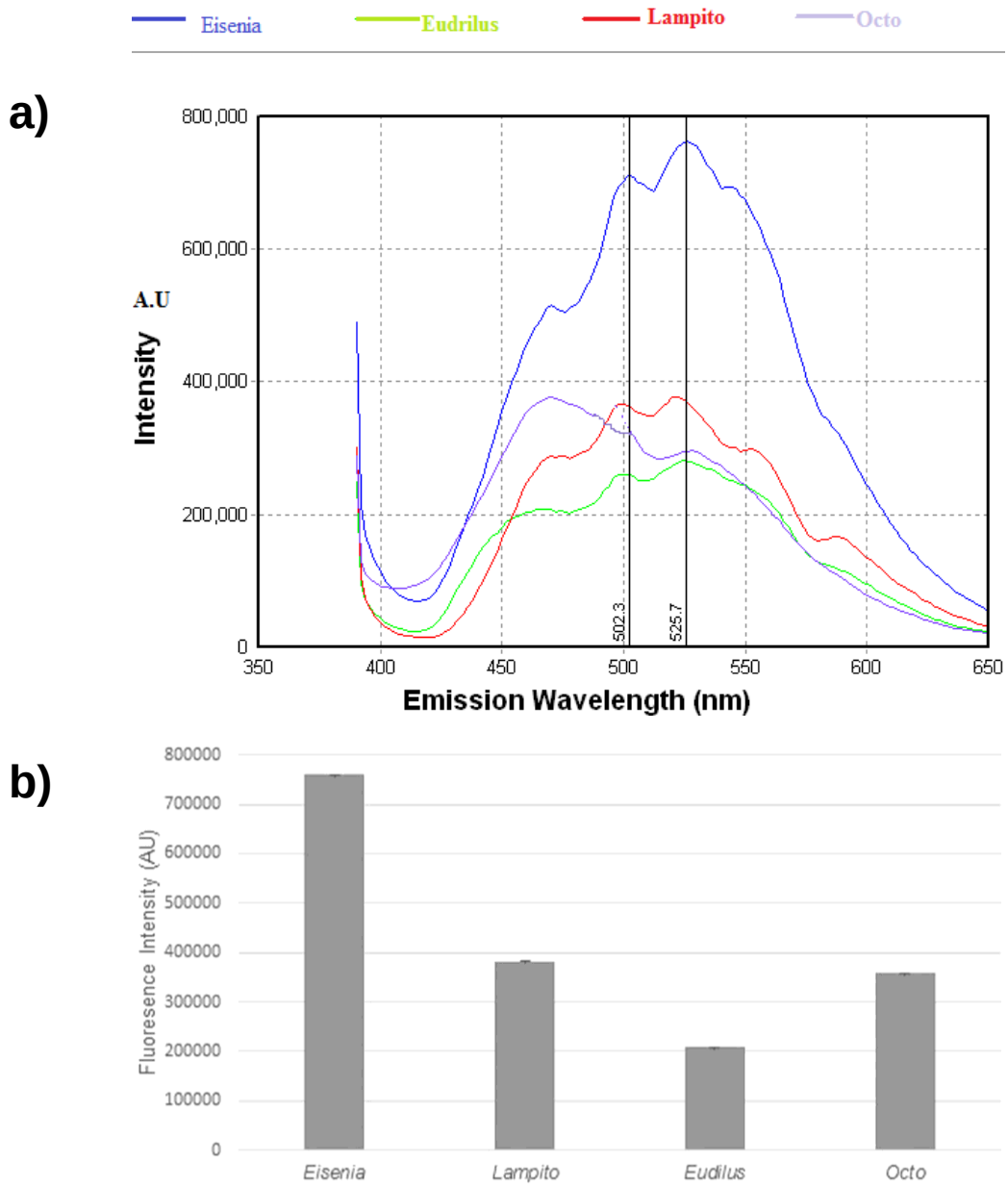


Fig. 1 Percentage of granular and eleocytes in four different species of earthworm at flow cytometric analysis. Mean ± SD; Significant $p < 0.01$.

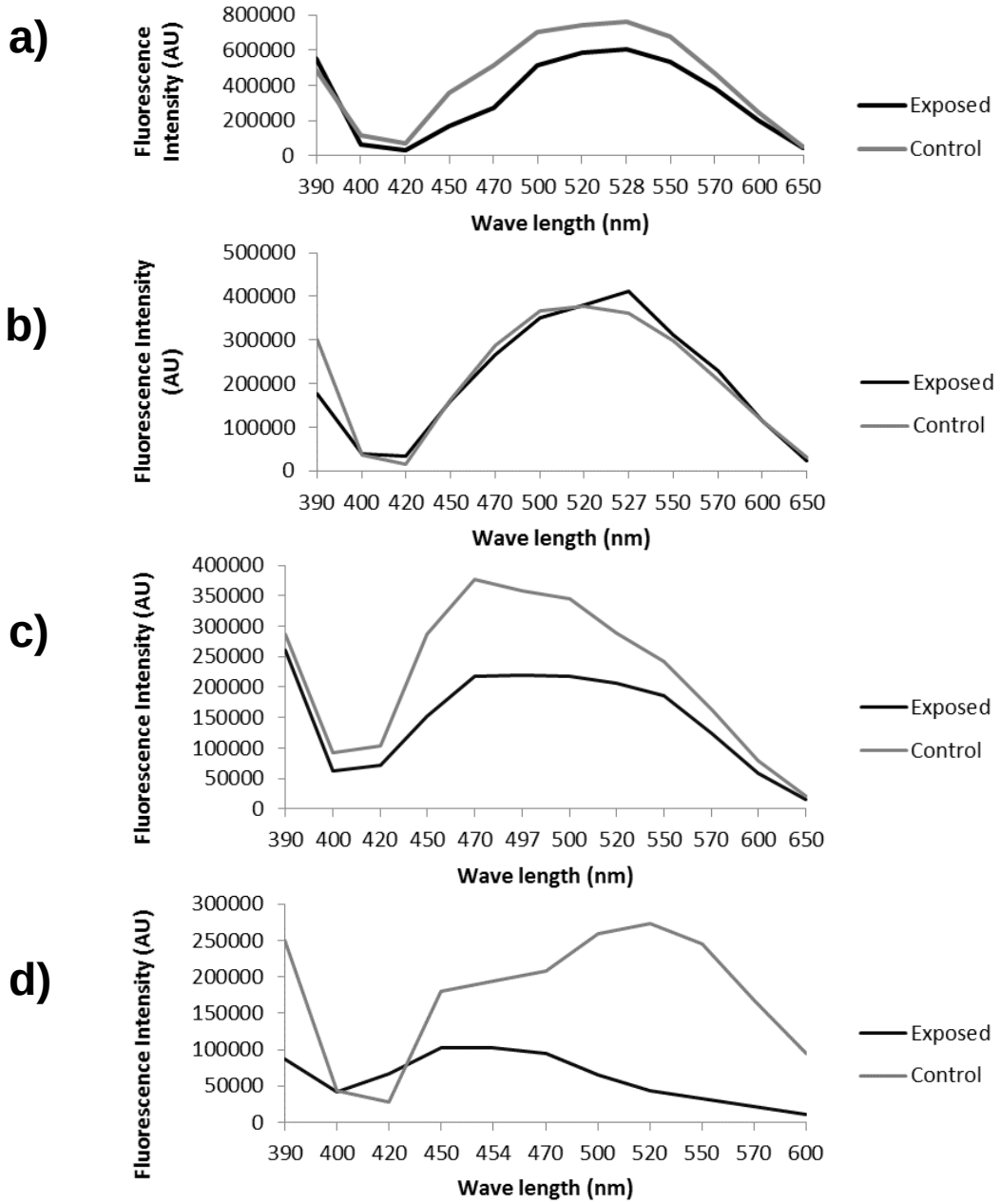


Figs 2 (**a, b)** Riboflavin derived fluorescence spectra of celomocyte lysates from four different species of earthworm, AU = Arbitrary units of fluorescence intensity. Mean ± SD; Significant $p < 0.01$.

and eleocytes of four different earthworms species. The forward scatter is an indicator of particle size (cell volume), whereas side scatter is an indicator of particle granularity (internal complexity) in the flow cytometer dot plot analysis. Both measurements are obtained uniformly in the absence of fluorochrome molecule. The fluorochrome molecule is bound to the particles and get excited in to higher energy state and followed by the release of energy as a photon of light at a longer wavelength (lower energy state) which is known as Stokes shift. Density plots of the cell size versus cell complexity show that all the four different species of earthworm possess two distinct celomocyte populations *i.e.,* agranular amebocytes and eleocytes. Density plots of SSC-H versus FL1-H show that the latter exhibit very distinct FL1-H autofluorescence, arbitrarily assessed as that more intensive than 10^2 units on X-axis. The percentage of eleocytes are differ from species to species, and the ratio of eleocytes is directly proportional to granular cells.

Riboflavin content measurement by spectrofluorometry

All earthworms *E. fetida, E. eugeniae, L. maruitii* and *O. serrata* possess riboflavin in celomocytes lysates. Figure 2a shows emission spectra of four different species of earthworm celomic fluid and the comparison of the emission spectra of evocative samples of $1x10^5$ celomocytes. The riboflavin content in the eleocytes is proportional to the peak fluorescence at 525 nm.



Figs 3 (a, b, c, d) Riboflavin-derived fluorescence spectra of control and heavy metal treated worm's celomocyte lysates. AU = arbitrary units of fluorescence intensity. Emission spectra (kex = 370 nm) with peaks at 525 nm.

The peaks at 525 nm is evident in each of them, albeit of different height in the order of *E. fetida*>> *Lampito* spp>> *Octochatenea* spp. >> *Eudrilus* spp. It confirms, the richness of the riboflavin content in the eleocytes of *Eisenia, Lampito* and *Octochatenea* inhabiting the unpolluted soil. The amount of riboflavin is proportional to the intensity of the emission band measured at 525 nm (expressed in arbitrary units, AU). When compared to the control, the intenstity of riboflavin fluorescence found to be drastically reduced in *Eudrilus celomocytes.* Figures 3a, b, c, d displays the comparative riboflavin emission spectra of celomic fluids of four different species of earthworms. The experimental results show that that the emission spectrum occurs very high in *E. fetida* followed by *Lampito* and *Octochaeta*. The fluorescence spectrum observed from *E. eugeniae* is almost nonexistent, compared to control. The fluorescence emission spectrum of fluorophore characterizes the electron distribution of the molecule in the ground state. Thus, it helps to identify the structure or the nature of the emitting molecule.

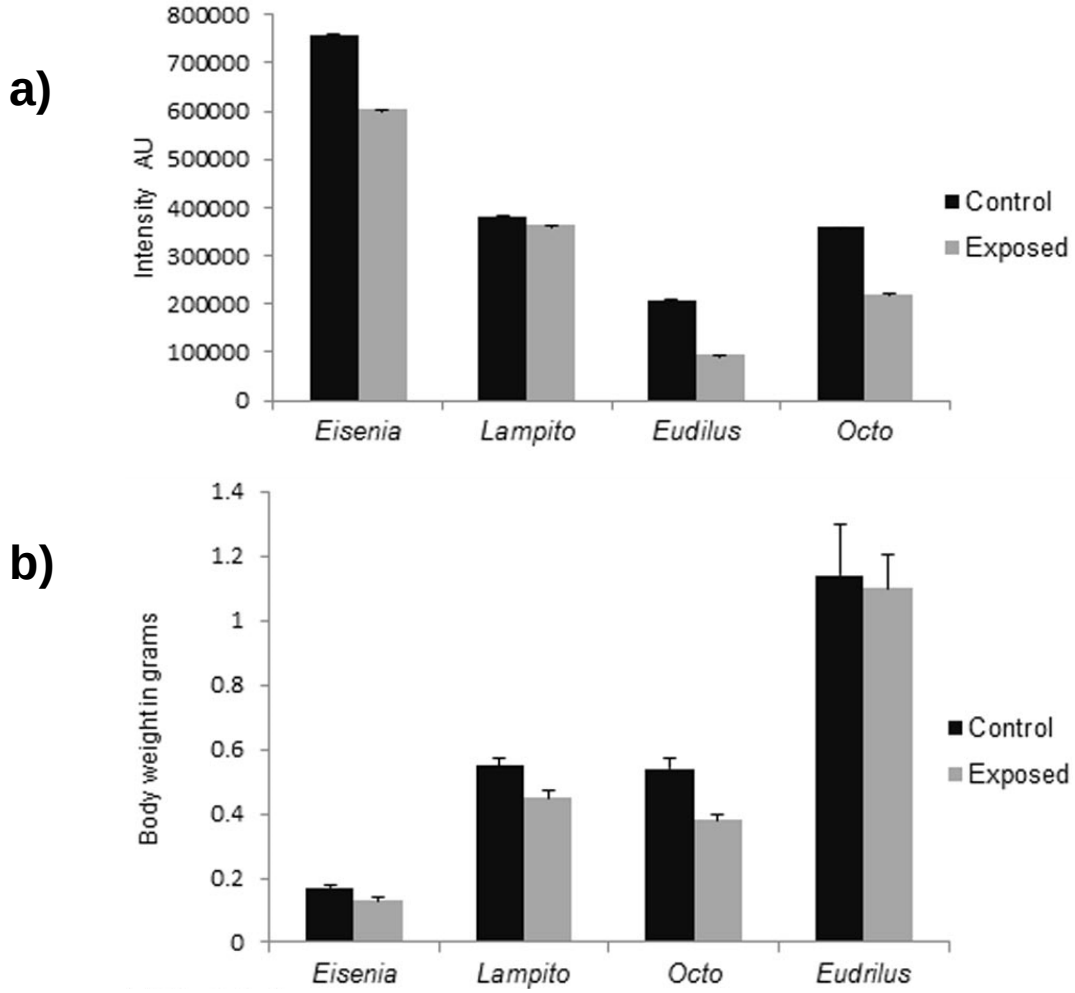


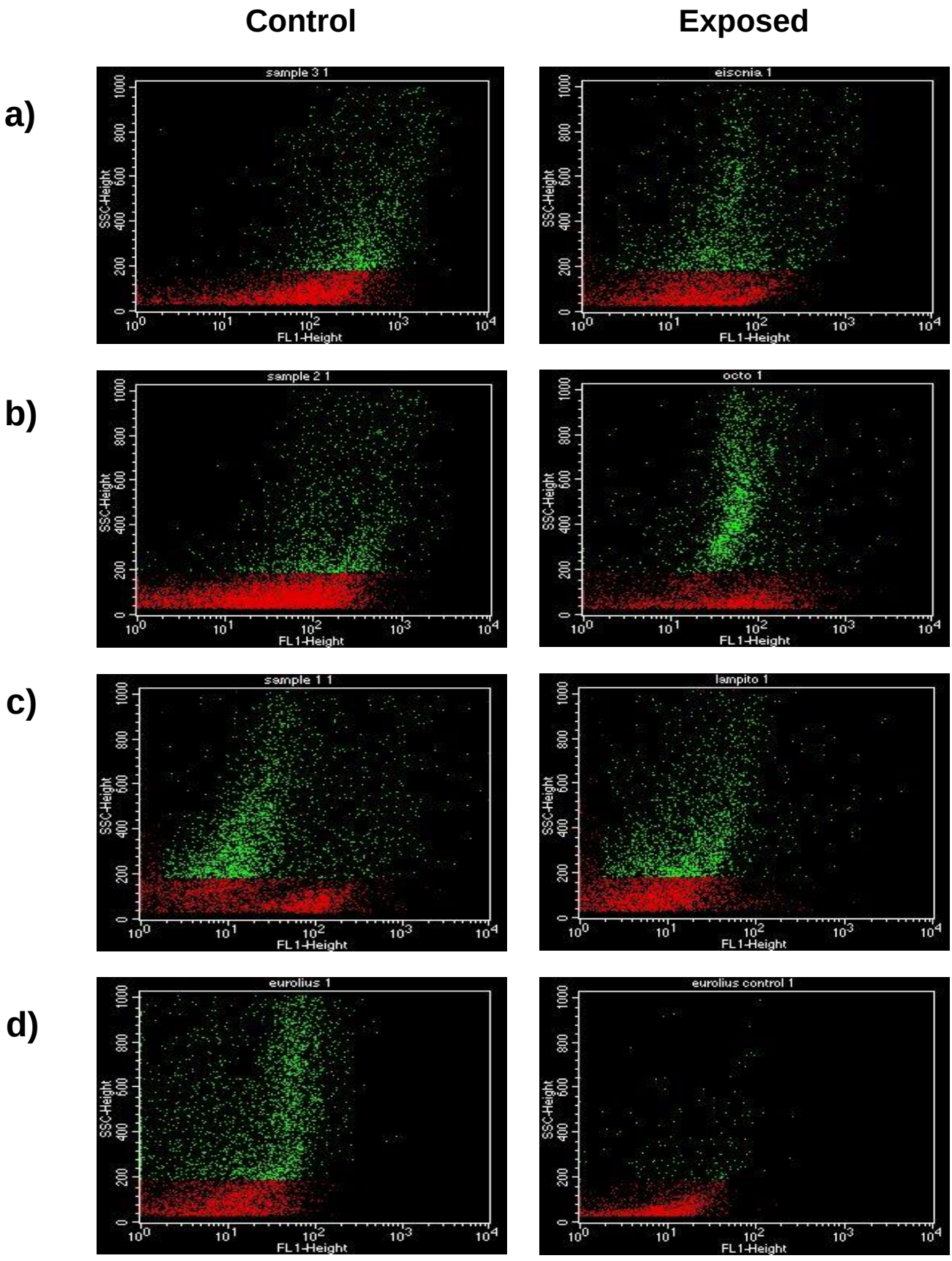
Fig. 4 Analysis of celomocytes of four different earthworms after 3 days exposure to heavy metal: **a)** comparative total riboflavin content**; b)** biomass of four different species of earthworm. Mean ± SD; Significant $p < 0.01$.

Figures 4a, b shows body weights of four different species were unaffected by the experiment days, and the mean biomass of four different worms was slightly decreased. One-way ANOVA revealed that significant effect of metals on total number of celomocytes F value = 181.4; P-value = 0.000; $p < 0.01$), percentage of eleocytes set up by flow cytometry ($p < 0.01$) and eleocytes number ($p < 0.01$) and also riboflavin content in arbitrary units (AU) established by spectrofluorometry ($p < 0.01$). Results of post hoc t-Tukey's test revealed that all investigated parameters coelomocytes, eleocytes and riboflavin were consistently lowest in celomocytes from exposed worms.

Quantitative analysis of celomocytes

Flow cytometer analysis offers excellent information on the nature of the cells present in the earthworm celomic fluid and allows the identification of different cells structure which is capable of interacting with metal ions. All the earthworm was retaining two distinct population *i.e.,* granular amebocytes (green) and autofluorescent eleocytes (red). Predisposed eleocytes population were observed in the right of 10^2 units (Figs 5 a, b, c, d) than in control. The autofluorescent intensity increases from left to right indicating a more intensive population of eleocytes in control than in heavy metal treated worms.

Figures 6, 7 shows the autofluorescent eleocytes of *Octochaetona* was more biased towards the right than in control. But the eleocyte population is influenced toward left in heavy metal exposed worms indicating a reduction in the percentage of eleocytes. The dot plot intensity of granulocytes is more in control than in exposed worms. Similarly, flow cytometry of *Lampito* confirmed the presence of amebocytes and eleocytes, the latter exhibiting strong autofluorescence. The earthworms from control possessed a significantly higher percentage of autofluorescence value than their counterparts from the metalliferous soil. Dot plot analysis of *Eudrilus* revealed a significant reduction in the amoebocytes population in the exposed worms when compared to control.



Figs 5 (a, b, c, d) Analysis by flow cytometry in respect of percentages of celomocytes extruded from four different species of earthworms a) *Eisenia* b) *Octochaeta* c) *Lampito* d) *Eudrilus.*

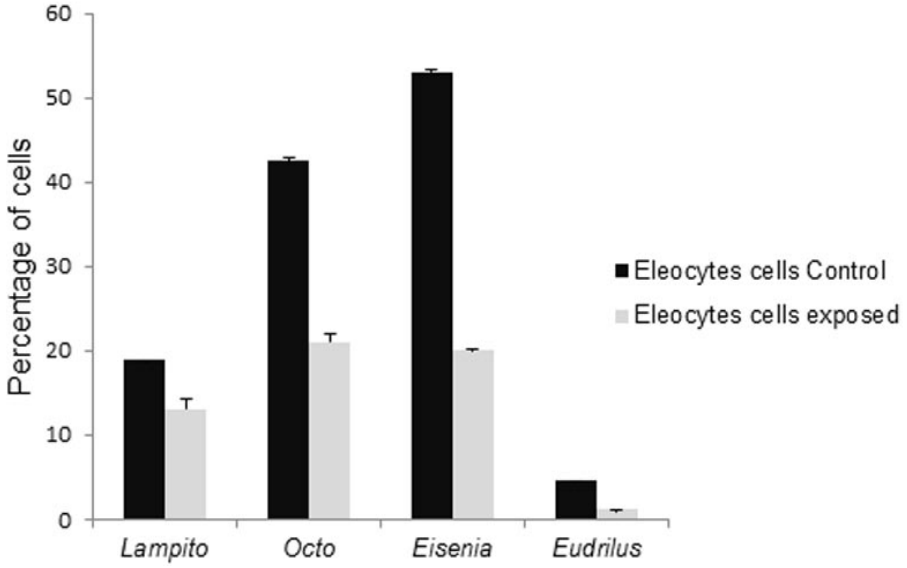


Fig. 6 Influence of heavy metals on eleocytes in four different species of earthworm. Mean ± SD; Significant $p < 0.01$.

Figures 6, 7 depicts the influence of heavy metals on eleocytes and granulocytes in four different species of earthworm. Heavy metal could, of course, have affected the riboflavin content, granulocytes and eleocytes percentage and viability of cells, which in turn could have affected the growth and reproduction indirectly. The present study also proves, cells variability depends on species-specific, and the individual differences in riboflavin content may reflect species-specific, nutritional preferences and vitamin B12 availability of the local resources. Further, present study concludes that metal contamination of the soil caused their accumulation in the earthworm body including their coelomocytes which in turn affects their health and viability.

Figure 8 depicts the accumulated heavy metal contents of four different species of earthworm. The Cr concentration was 0.36 ± 0.01, 0.34 ± 0.15, 0.27 ± 0.02, 0.23 ± 0.01 and Cd concentration was 0.76 ± 0.02, 0.56 ± 0.02, 0.63 ± 0.15, 0.0.47 ± 0.20 in *Eisenia, Lampito, Octocheta* and *Eudrilus* respectively. Their bioaccumlation Factor (BAF) values were 0.135, 0.168, 0.14 and 0.11. So the present study reveals that earthworm subjected to heavy metal (Cd, Cr and Cu) exposure exhibited a higher accumulation of the heavy metals in the coelomic fluid.

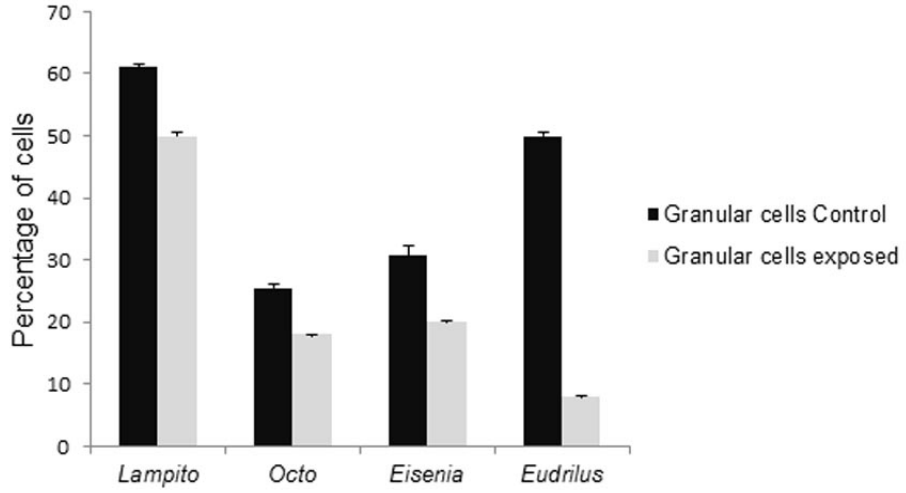
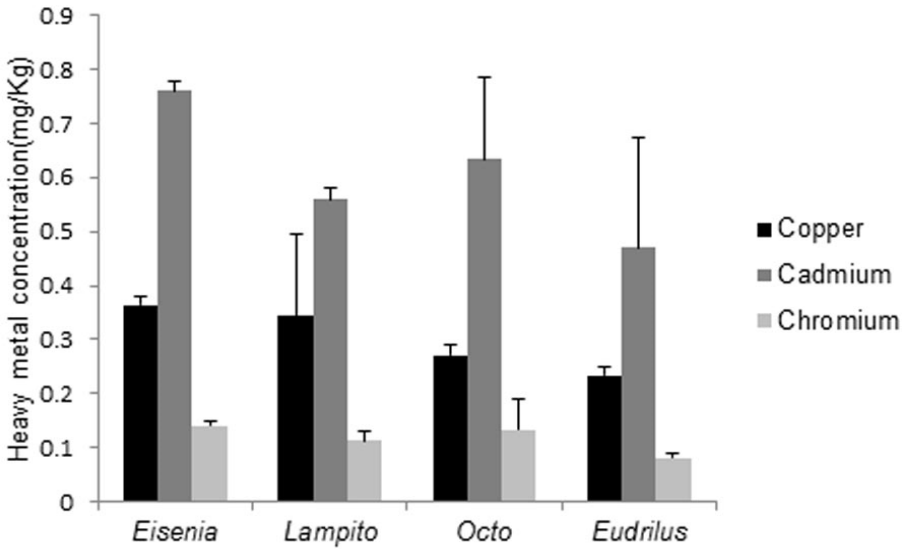


Fig. 7 Influence of heavy metals on granulocytes in four different species of earthworm. Mean ± SD; Significant $p < 0.01$.



Eisenia	*Lampito*	*Octo*	*Eudrilus*
0.168	0.135	0.14	0.11

Fig. 8 Bioaccumulation and BAF of heavy metals in earthworm celomic fluid. Mean ± SD; Significant $p < 0.01$.

Discussion

In the present study, celomocytes are collected from celomic fluid retrieved non-invasively from four different species of earthworm by electrostimulation was analyzed by spectrofluorimetry and flow cytometry. An astonishing divergence was detected in the four earthworm species with autofluorescent cells (eleocytes), and high number of celomocytes per body weight. Plytycz *et al.* (2006) concluded that amebocytes with phagocytic properties are present in all species, but the presence of autofluorescent eleocytes, derived from the chloragogenous tissue, is species specific. As Koziol *et al.* (2006) showed, the partial source of fluorescence in eleocytes is riboflavin, which is involved in immune responses in vertebrates (Verdrengh and Tarkowski, 2005), and is also considered responsible for balancing between earthworms and microorganisms present in soil (Homa *et al.,* 2010). Content and composition of celomocytes, as well as riboflavin content, may vary not only between species but also within them depending on the presence of stress factors such as heavy metals (Plytycz and Morgan, 2011). In the present study, percentages of autofluorescent eleocytes were relatively stable, as well as the amounts of riboflavin measured in celomocyte lysates samples (Plytycz *et al.,* 2012).

It is well established that riboflavin is essential for the proper functioning of the innate immunity of both animals (Powers, 2003; Verdrengh and Tarkowski, 2005) and plants (Dong and Beer, 2000; Asai *et al.,* 2010; Yoshioka *et al.,* 2011), as well as being a signaling molecule in bacterial quorum sensing (Rajamani *et al.,* 2008; Atkinson and Williams 2009). Riboflavin is considered as the chemoattractant for immunocytes in earthworms (Plytycz *et al.,* 2011) that putatively mobilizes the defense response during a disrupted balance of host and microbes/parasites induced by toxic factors (Plytycz *et al.,* 2006). The present work clearly showed that there is no correlation between body weight and riboflavin content. Furthermore, a maximum decrease of riboflavin was recorded in the *E. eugeniae* than other species while exposed to heavy metals. And also it was observed that the physical nature of the celomic fluid of the four different species shows remarkably different in color. The celomic fluid of the *E. eugeniae* is color less where as other three species are brilliant yellow in color due to the presence phosphorescence pigments. This adaptation might be connected with very efficient riboflavin metabolism, because its amount in celomocytes of *Eisenia* sp. is much higher than in another earthworm species (Plytycz *et al.,* 2006). From experimental results, it was observed that the body weight and the coelomic fluid does not correlate with respect to immunological defense point of view.

Fluorescence data shown in the present study indicates clearly that *E. fetida, L. Maruitii, O. serreta* and *E. eugeniae*, display different molecular composition in their celomic fluid and thus have different metabolisms. Therefore, interpretations of toxicological and immunological studies obtained on earthworms could be significantly different depending upon the different and the species interactions. The purpose of studying this parameter was to determine if the contaminant exposure reduced the cell defense system regarding cell survival.

Many studies concerned the effects of environmental pollution, including heavy metals on earthworm immune functions mediated by celomocytes (Scott-Fordsmand and Week, 2000; Kurek *et al.,* 2002). Fugere *et al.* (1996) found that inhibition of the phagocytic activity of coelomocytes exposed to the heavy metal solution. Plytycz *et al.* (2009) discovered that riboflavin content was reduced in the eleocytes of worms transferred from unpolluted to the metal-polluted soil. Recent findings indicate that the riboflavin/lipofuscin balance may be a sensitive indicator of soil pollution intensity (Cygal *et al.,* 2007).

According to Plytycz *et al.* (2009), states that the heavy metal pollution strongly influences the riboflavin content in the immune - competent cells of *D. rubidus*. The reduction of riboflavin content in the eleocytes cells leads to decrease in the fluorescence signal (riboflavin quenching) due to heavy metal binding. Indeed, earthworms living in stress conditions are obligated to manage their energy, because they are constantly exposed to the high cost of maintaining homeostasis. These results are concordant with previous in vitro observations (Sauve´ *et al.,* 1998) showing that the immune function of celomocytes exposed to trace elements is impaired before the onset of cell death (Brousseau, 1997). Heavy metal accumulation depends on the exposure duration whereas the accumulation of Cu, Cd and Cr is dependent upon the metabolic turnover (Gobi, 2015). The use of celomic fluid for elemental analysis in earthworm offers many advantages on the commonly used whole-body measure, particularly for in field application.

Conclusion

The present study clearly demonstrated that investigated species possess the significant population of celomocytes, but they differ considerably in the number of cells per body mass and by species. Percentage of eleocytes and riboflavin were found to be reduced drastically in the earthworms exposed to the heavy metal containing a medium and these two markers playing an important role in earthworm biology. This non-invasive technique proves stable cellular biomarkers in earthworm for toxicological and biological soil monitoring studies.

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