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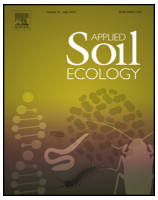

Earthworm metallothionein production as biomarker of heavy metal pollution in abattoir soil

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ABSTRACT

The direct response of animals to environmental challenges, such as the production of biomarkers, is a better tool to assess environmental pollution than the conventional methods. In this study, the production of metallothionein (MT) in earthworms (*Libyodrilus violaceous, Eudrilus eugeniae* and *Alma millsoni*) was measured as tool for assessing heavy metal pollution in abattoir soil. Earthworm and abattoir soil samples were collected from three abattoir sites (Lafenwa, Gbonogun and Madojutimi) and a control site located beside an undisturbed stream located in Abeokuta, Ogun State, in South-western Nigeria. Heavy metal (Cu, Zn, Pb, Cd, Co, Cr, Ni and Mn) and MT concentrations were measured in the earthworm tissue and abattoir soil using standard methods. The concentrations of Cu, Zn, Pb, Cd and Mn were highest in the tissue of earthworms obtained from Lafenwa abattoir. The Bioaccumulation Factors (BAFs) for all the metals tested for were less than unity, except for Cd which had a $BAF > 1$. The MT concentrations recorded in the earthworm samples from the Gbonogun and Lafenwa abattoir sites were significantly higher ($p \leq 0.05$) than in earthworms from Madojutimi. The lowest MT concentration was recorded in earthworms from the control. Significant ($p \leq 0.05$) positive correlations were observed between MT and heavy metal concentrations in all earthworm species indicating that MT concentrations can be used as biomarker of heavy metal pollution in abattoir soils.

1. Introduction

Pollution has become a global problem that exists in various dimensions and it becomes worse when it is difficult to determine when, how and to what extent the level of pollution is hazardous in a particular environment (Asonye et al., 2007). There has been an enormous increase in soil pollution due to industrial activities, urban wastes, atmospheric deposition and intensive use of biocides and fertilizers in agricultural soil (Criel et al., 2008), leading to the release of metals into the soil, which over the years becomes a sink for these metals. This invariably resulted in decreased soil fertility, alteration of soil structure, disturbance of the balance between flora and fauna residing in the soil, contamination of crops, and contamination of groundwater, constituting a threat to living organisms (Bezchlebova et al., 2007).

Heavy metals have been reported to be one of the most diffusive chemicals in the soil (Lanno et al., 2004) and the reported sources of metal pollution include natural sources (Miranda et al., 2009; Hobbelen et al., 2006), ore mining or metal smelting (Haimi and Mätäsniemi, 2002), municipal waste, industrial effluents, application of sewage sludge and animal manure on agricultural land (Salehi and Tabari, 2008), and aerial deposition of particulates from vehicular emission (Ward and Savage, 1994).

High protein demand has led to a significant increase in meat production with the resultant establishment of more slaughtering (abattoir) and meat processing facilities. These facilities produce solid, liquid and gaseous wastes which tend to be worrisome due to the high content of putrescible organic matter. This can lead to the depletion of oxygen and an impairment or disruption of water and soil eco-functionality and a preponderance of disease-causing organisms. The meat processing wastes come from stockyards, abattoirs and packing plants, etc., all containing blood, fats, protein, gut contents, heavy metals, antibodies, hormones and other substances (Itodo and Awulu, 1991). In Nigeria, many abattoirs dispose their waste directly into streams or rivers and also use water from the same source to wash slaughtered meat (Adelegan, 2002). The situation is not any different in Ghana where

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most liquid and gaseous wastes are released into the immediate environment of the abattoir (Weobong, 2001).

Coker et al. (2001) reported contamination by heavy metals, particularly lead and zinc, in many abattoir areas. Due to the increasing concern about chemical contamination of soil by abattoir wastes, there is a need for soil pollution monitoring and assessment in the vicinity of these facilities. Biological approaches to soil monitoring and assessments, such as the measurement of biochemical and cellular responses to pollutants (i.e. biomarkers) in organisms living in the soil (bioindicators), have become of major importance for the assessment of the quality of this environmental compartment (Kammenga et al., 2000).

Kammenga et al. (2000) further pointed out that soil invertebrates such as earthworms, may represent good sentinel

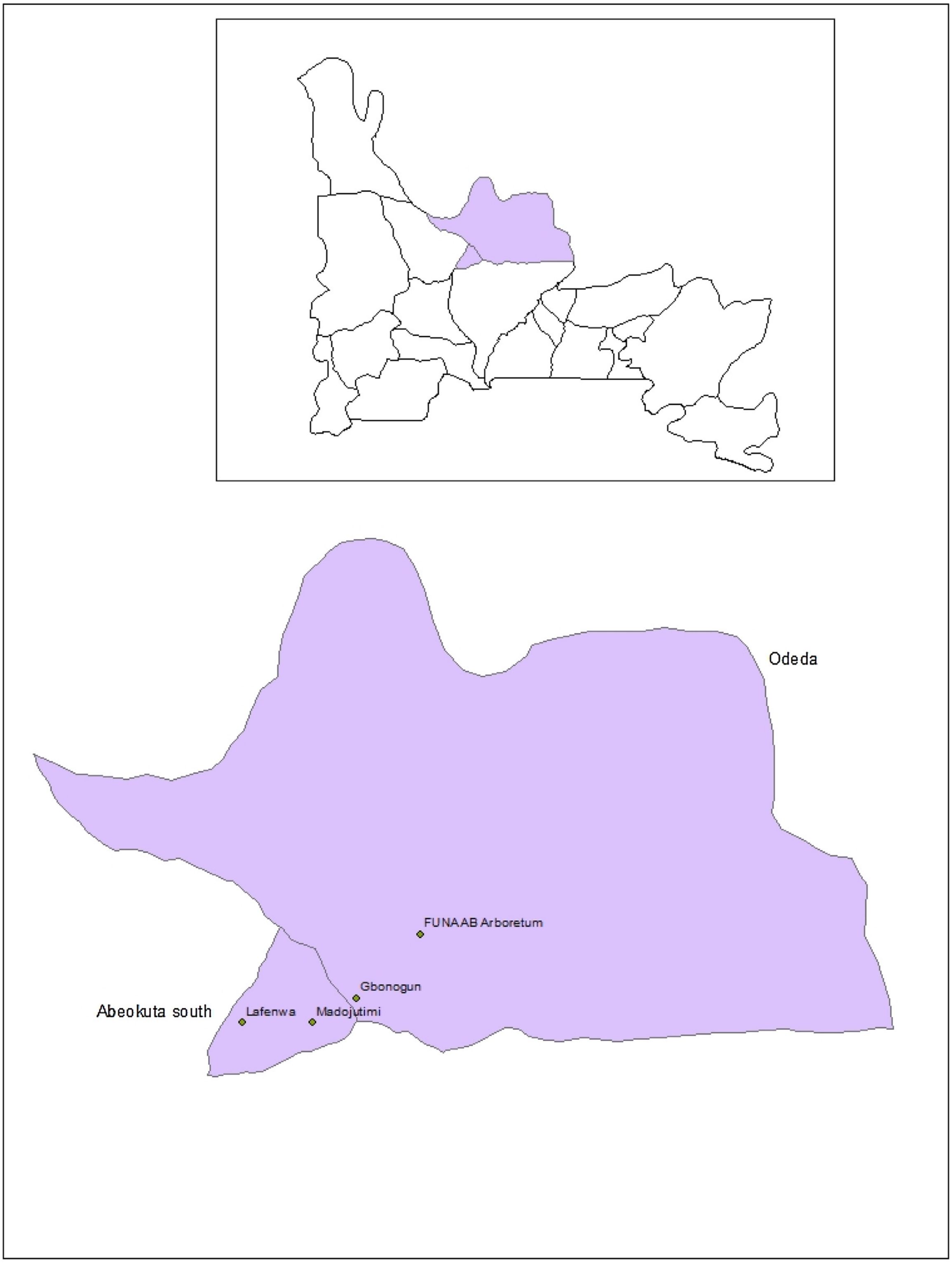

Fig. 1. Map showing the study sites, including abattoirs and a control site in Abeokuta, Ogun State, in South-western Nigeria.

organisms of soil chemical pollution because they are in direct contact with soil pore water, in contrast to many vertebrates that are indirectly exposed through the food chain. Earthworms are frequently exposed to heavy metals, pesticides, microbes and other pollutants in the environment and therefore their survival is dependent on efficient detoxification systems by eliciting certain molecules which can serve as biomarkers of soil pollution. One of these biomarkers is the metal-binding protein metallothionein, which may be a good indicator of metal exposure in earthworms (Kammenga et al., 2000; Homa et al., 2005; Suriya et al., 2012). This study therefore is aimed at evaluating metallothionein concentrations in indigenous earthworms as tools for heavy metal pollution assessment in abattoir soil in Ogun State, Nigeria.

2. Materials and methods

2.1. Study sites

This work was carried out in Abeokuta, Ogun State, 7°00′N, 3°35′E, in South-western Nigeria. It borders Lagos State to the south, Oyo and Osun states to the north, Ondo State to the east and the Republic of Benin to the west. The total area of the state is 16,762 km^2. Earthworm and soil samples were collected from three different abbatoir sites and a control site within two local governments in the Abeokuta metropolis in Ogun state. The sampling sites were Lafenwa Odo-Eran abattoir (07.163°N 003.328°E), Gbonogun abattoir (07.179°N 003.406°E) and Odo-Eran Asejere Madojutimi abattoir (07.163°N 003.376°E). The Control site was the Arboretum in which no manure application was allowed (Nursery for *Gmelina arborea* at the Federal University of Agriculture, Abeokuta) (07.224°N 003.450°E). Lafenwa abattoir is the oldest (60 years) followed by Gbonogun abattoir (25 years) and Madojutimi abattoir (8 years) (Fig. 1).

2.2. Sample collection

Earthworm and soil samples were collected by digging and hand sorting (Owa, 1992). Samples were taken at a depth of 0–20 cm from five 0.5 m^2 quadrats at each study site. The quadrats were placed at1 m intervals along the gradient of the sites. Sampling was conducted once a week for five weeks.

2.3. Heavy metal analysis

Sample digestion and determination of the heavy metal concentration in the digested samples was carried out by the method of the Association of Analytical Chemists (2000). Earthworm and soil samples were ashed at 600 °C for 24 h and the ash of each of the sample was weighed. To extract metals, 2 mL sulphuric acid, 4 mL perchloric acid and 20 mL nitric acid were added to 0.2 g of the ashed sample. The mixture was then boiled on a heating mantle until the mixture turned colourless. After cooling, the mixture was diluted with distilled water to 100 mL, and subsequently analyzed by Atomic Absorption Spectrophotometry (PerkinElmer Model 403, North Chicago USA.) to determine the concentrations of Pb, Zn, Cu, Cr, Cd, Co and Ni.

2.4. Bioaccumulation factor (BAF)

The metal accumulation by earthworms is usually quantified as BAF, which is the ratio of the concentations of metals in earthworms and the soil substrate (Mountouris et al., 2002). If the metal concentration in earthworms is higher than that of the soil substrate, the BAF is higher than 1, indicating bioaccumulation.

2.5. Determination of metallothionein (MT)

Metallothionein (MT) concentrations were measured using the method of Suriya et al. (2012). The earthworms (4 worms/sample) collected for MT measurements were rinsed with distilled water and placed in containers with moistened filter paper to purge their gut contents for 3 days; the filter paper was changed every day. The earthworms were subsequently immobilized in ice-cold 20 % (V/V) glycerol solution for 3 min, and the guts were separated.

The worms were placed in 3 mL ice-cold Tris-HCl 25 mmol buffer (pH 7.2 at 20 °C), 0.1 mmol phenylmethylsulfonyl fluoride

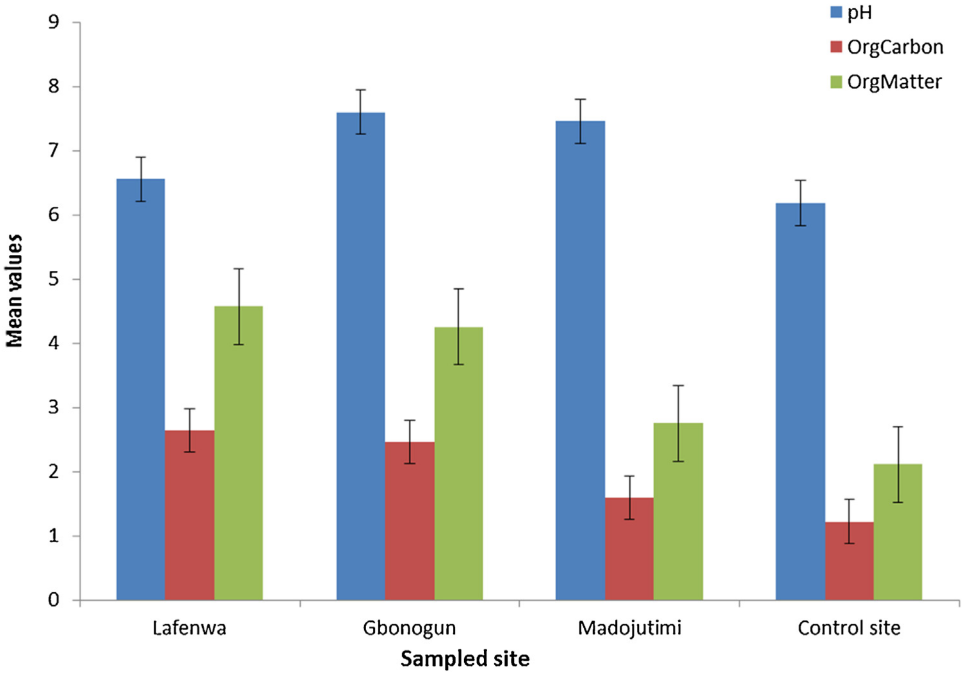

Fig. 2. pH and organic matter contents of the soils of Lafenwa, Gbonogun, Madojutimi abattoir and control site in Abeokuta, Ogun State, in South-western Nigeria. (The Error bar indicate Standard deviation; n = 5).

(PMSF) as antiprotease and 0.5 mmol dithiothreitol (DTT) as the sulfydryl-protecting agent to prevent the MT from oxidation, then homogenized with distilled water. The homogenate was centrifuged at 10,000g for 20 min at 4 °C in individual 1.5 mL microtubes. To precipitate non-MT proteins, 1 mL of the supernatant was heated at 100 °C for 10 min. Then the heat-treated homogenates were cooled on ice for 5 min and centrifuged at 10,000g for 20 min at 4 °C. The MT concentration in the tissue samples was estimated with the mercury-saturation assay, with small modifications. Blanks were prepared concurrently by adding 200 μL Tris-HCl (20 mmol, pH 7.2) to replace the heat-treated sample.

Aliquots of the heat-treated homogenate were fractionated by gel-permeation chromatography [Sephadex G 75 (26 mm × 60 cm)]. The elution was made with Tris-HCl 25 mmol, pH 7.2 containing 0.5 mmol DTT. For each fraction, the absorbance at 254 nm was measured. The total protein was determined from the above supernatant without heat treatment by the Bradford (1976) method using bovine serum albumin as a standard.

2.6. Data analysis

All the data are presented as the mean ± standard deviation (SD). Statistical analyses for all measurements were performed by Statistical Package for Social Sciences (SPSS) version 16.0. Analysis of Variance (ANOVA) and Correlation coefficient were conducted. Post Hoc test was also conducted using Duncan Multiple Range Test (DMRT). $P < 0.05$ was considered to be statistically significant.

3. Results

At Lafenwa and Gbonogun abattoirs, we found and collected *Eudrilus eugeniae* (an epigeic species) and *Libyodrilus violaceus*. *Alma millsoni* and *L. violaceus* (both endogeic limicolous species) were found and collected from Madojutimi abattoir while *A. millsoni* was the only species found and collected at the control site. The pH and organic matter contents of the abattoir soils are shown in Fig. 2; Lafenwa, Gbonogun and Madojutimi soils had significantly ($p < 0.05$) higher concentrations of organic matter and carbon than the control site.

Cu, Pb, Cd and Co concentrations in the soil samples from all the abattoir locations were significantly different ($p < 0.05$). The Lafenwa abattoir recorded the highest concentrations of Zn, Pb, Cd and Ni in its soil and rumen waste. The Gbonogun abattoir recorded the highest concentrations of Cu, Co and Mn in its soil, while the lowest concentrations of all the heavy metals were recorded for the soil from the control site (Table 1).

The concentrations of heavy metals in the tissues of all earthworm species obtained from the abattoir locations were significantly lower ($p < 0.05$) than their respective abattoir soil except for cadmium, for which concentrations in the earthworms were significantly higher than in the soil ($p < 0.05$) (Table 1). Mean concentrations of Cu, Zn, Pb, Cd and Mn were highest in the earthworms from the Lafenwa abattoir, followed by Gbonogun and Madojutimi abattoir, while the earthworms from the control site had the lowest mean heavy metal concentrations. Mean Ni concentration was also highest in the earthworms from the Lafenwa abattoir (Table 1). There were no significant differences ($P > 0.05$) between the Mn, Cr, Co and Zn concentrations in earthworms from all the abattoir locations, but these were significantly higher ($p < 0.05$) than the values obtained for the control site. The BAFs for all the metals tested for were less than unity except for Cd which had a BAF>1 (Table 2).

Concentration of MT (Fig. 3) was significantly higher ($p < 0.05$) in the earthworms from all the abattoir sites than in control animals. Earthworms from Gbonogun abattoir had higher MT concentration than earthworms from the other abattoir locations, followed by the earthworms from Lafenwa and Madojutimi abattoir soils. The lowest MT concentration (0.26 ± 0.09 μM/mg protein) was recorded in earthworms from the control site. There was no significant difference ($p > 0.05$) between the MT concentrations recorded in earthworms from Lafenwa and Gbonogun abattoir soils while those of Madojutimi and the control site were

Table 1
Comparison of heavy metal concentrations (mg/kg dry weight) in the soil and earthworm species collected from sampling sites at abattoirs in Abeokuta, Ogun State, in South-western Nigeria.

Heavy Metals	Control (N = 5)		Lafenwa (N = 5)			Gbonagun (N = 5)			Madojutimi (N = 5)		
	Soil	*Alma millsoni*	Soil	*Libyodrilus violaceus*	*Eudrilus eugeniae*	Soil	*Libyodrilus violaceus*	*Eudrilus eugeniae*	Soil	*Libyodrilus violaceus*	*Alma millsoni*
Cu (mg/kg)	16.4 ± 0.21^{a}	7.82 ± 0.86^{b}	39.0 ± 0.47^{a}	22.6 ± 0.75^{b}	21.5 ± 0.28^{b}	40.6 ± 0.62^{a}	21.8 ± 0.15^{b}	21.5 ± 0.77^{b}	36.5 ± 0.56^{a}	23.1 ± 0.76^{b}	20.0 ± 0.73^{c}
Zn (mg/kg)	5.30 ± 0.13^{a}	3.47 ± 0.30^{b}	28.1 ± 0.30^{a}	12.5 ± 0.54^{b}	12.2 ± 0.63^{b}	27.0 ± 0.22^{a}	11.8 ± 0.90^{b}	11.1 ± 0.17^{b}	26.7 ± 0.87^{a}	11.5 ± 0.24^{b}	10.2 ± 0.11^{c}
Pb (mg/kg)	10.7 ± 0.28^{a}	2.31 ± 0.20^{b}	30.7 ± 0.35^{a}	10.1 ± 1.38^{b}	9.46 ± 1.16^{a}	27.6 ± 0.68^{a}	7.49 ± 0.54^{b}	6.35 ± 0.34^{b}	24.6 ± 0.57^{a}	8.08 ± 0.56^{b}	5.32 ± 0.43^{c}
Cd (mg/kg)	2.54 ± 0.27^{a}	2.37 ± 0.35^{a}	10.8 ± 0.19^{a}	14.9 ± 0.77^{b}	14.4 ± 0.37^{b}	9.79 ± 0.27^{a}	12.3 ± 0.29^{b}	11.6 ± 0.43^{c}	7.10 ± 0.79^{a}	11.8 ± 0.57^{b}	10.4 ± 0.24^{c}
Co (mg/kg)	1.58 ± 0.23^{a}	0.78 ± 0.15^{b}	6.46 ± 0.17^{a}	5.02 ± 0.10^{b}	4.59 ± 0.38^{b}	8.56 ± 0.50^{a}	5.89 ± 0.71^{b}	4.98 ± 0.77^{b}	4.78 ± 0.88^{a}	6.01 ± 0.35^{b}	5.28 ± 0.41^{c}
Ni (mg/kg)	10.5 ± 0.13^{a}	7.61 ± 0.28^{b}	25.8 ± 0.18^{a}	24.0 ± 0.30^{b}	23.1 ± 0.28^{c}	23.9 ± 0.30^{a}	20.7 ± 0.51^{b}	20.4 ± 0.44^{b}	23.7 ± 0.44^{a}	22.5 ± 0.82^{a}	20.1 ± 0.19^{ab}
Cr (mg/kg)	0.17 ± 0.05^{a}	0.12 ± 0.08^{a}	2.87 ± 0.30^{a}	2.24 ± 0.42^{a}	1.37 ± 0.18^{b}	2.48 ± 0.46^{a}	1.67 ± 0.21^{b}	1.55 ± 0.22^{b}	2.69 ± 0.51^{a}	2.22 ± 0.34^{a}	1.51 ± 0.26^{b}
Mn (mg/kg)	1.69 ± 0.23^{a}	0.16 ± 0.05^{b}	3.67 ± 0.07^{a}	3.67 ± 0.47^{a}	3.32 ± 0.37^{a}	4.12 ± 0.39^{a}	2.75 ± 0.58^{b}	2.40 ± 0.40^{b}	3.73 ± 0.69^{a}	2.76 ± 0.09^{a}	2.35 ± 0.30^{ab}

Means with the same superscripts in a row are not significantly different ($p \leq 0.05$).

Table 2
Bioaccumulation Factors (BAFs; n = 5) for the uptake of metals in earthworm species across sampling sites, including abattoir soils and a control site in Abeokuta, Ogun State, in South-western Nigeria.

Heavy Metals	Control	Lafenwa		Gbonagun		Madojutimi	
	Alma millsoni	*Libyodrilus violaceus*	*Eudrilus eugeniae*	*Libyodrilus violaceus*	*Eudrilus eugeniae*	*Libyodrilus violaceus*	*Alma millsoni*
Cu	0.48	0.55	0.58	0.53	**0.54**	**0.55**	0.63
Zn	0.65	0.44	0.44	0.41	0.44	0.38	0.43
Pb	0.22	0.31	0.33	0.23	0.27	0.22	0.33
Cd	0.93	1.33[a]	1.39[a]	1.19[a]	1.26[a]	1.46[a]	1.66[a]
Co	0.49	0.71	0.78	0.58	0.69	0.10	0.26
Ni	0.73	0.04	0.93	0.85	0.87	0.85	0.95
Cr	0.71	0.48	0.78	0.63	0.67	0.56	0.83
M)	0.09	0.90	1.00	0.58	0.67	0.63	0.74

[a] BAF above unity.

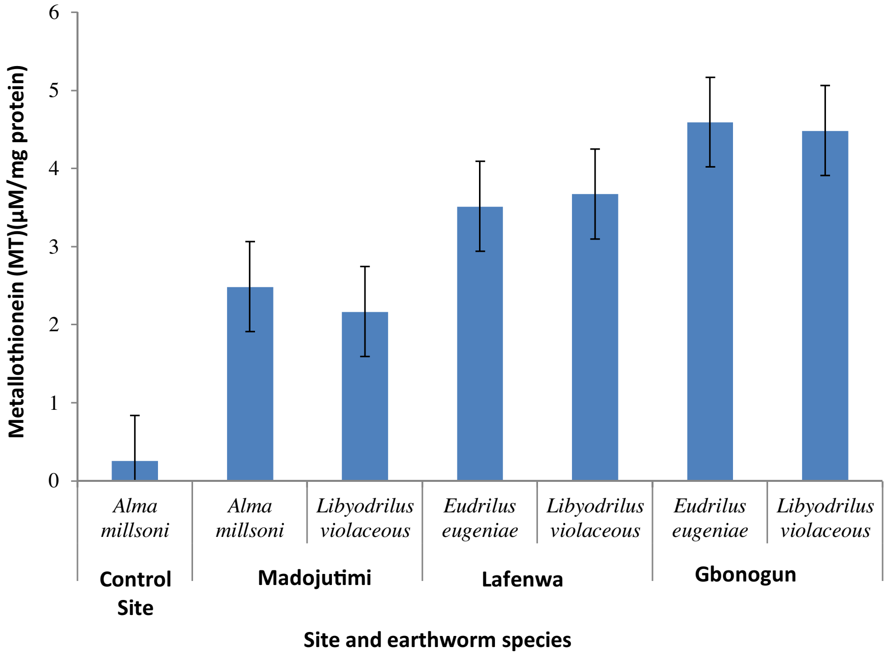

Fig. 3. Metallothionein (MT) concentrations (µM/mg protein) in earthworm species obtained from the abattoirs and control site in Abeokuta, Ogun State, in South-western Nigeria. (The Error bar indicate Standard deviation; n = 5).

Table 3
Correlation between Metallothionein (MT) and heavy metal concentrations in earthworms collected from abattoir soils and a control site in Abeokuta, Ogun State, in South-western Nigeria.

Heavy Metals	MT	
	r value	*p value*
Cu	0.723	0.000**
Zn	0.765	0.000**
Pb	0.605	0.004**
Cd	0.760	0.000**
Co	0.677	0.001**
Ni	0.705	0.000**
Cr	0.537	0.012*
Mn	0.631	0.002**

** r value is significant at 0.01 level.
* r value is significant at 0.05 level.

significantly different ($p < 0.05$). There were significant positive correlations between MT and metal concentrations in the earthworms from all study sites (Table 3).

4. Discussion

Indigenous earthworms such as *L. violaceus*, *A. millsoni* and *E. eugeniae* in this study bioaccumulated heavy metals in their tissues from the soil substrates as reported for various earthworm species by other studies. Holmstrup et al. (2010) demonstrated that Cd, Pb and Cu were accumulated to high concentrations in *Dendrobaena octaedra*. This, according to Edwards and Bohlen (1996), was due to the chemical and microbial changes that ingested substrates undergo during their passage through the alimentary canal of the earthworm. It was pointed out that a considerable proportion of the organic fraction is converted into soluble forms that are more available to the organisms. These also include the heavy metal fractions chelated by these organic fractions, hence, explaining their accumulation in the tissues of the earthworms.

Furthermore, *L. violaceus* was observed to bioaccumulate higher metal concentrations than *E. eugeniae*, which may be attributed to individual differences in their habits. While *L. violaceus* is a limicolous (marsh dwelling) species that rarely migrates from its habitat, *E. eugeniae* by nature is gregarious, which may have prevented bioaccumulation of metals to such high concentrations as observed in *L. violaceus*. This observation agreed with the study of Hobbelen et al. (2006) which showed that the accumulation of heavy metals in earthworms varies from species to species.

The concentrations of heavy metals in the earthworms were directly dependent on their concentrations in the respective abattoir soils. *E. eugeniae* and *L. violaceus* collected from the three abattoirs in this study also contained significantly higher metal concentrations than *A. millsoni* from the Arboretum (control site). This agrees with the work of Gupta et al. (2005) who reported that earthworm tissue metal level is directly related to their proportion in a given soil. A similar pattern of metal bioaccumulation was observed by Suthar et al. (2008). The higher metal concentrations in the Lafenwa abattoir soil may be attributed to its old age and the observed larger volume of slaughtering activities which resulted in higher organic matter and carbon load in its soil. Soil organic matter had been reported by Agbaire and Emoyan (2012) to act as sink for metals, and may also affect bioavailability of the metals.

The BAFs were less than unity for all the metals except for Cd for which concentrations in the earthworms exceeded the concentration in the soil. Earthworms easily accumulate Cd and retain it in their body tissue. This confirms the findings of Liu et al. (2010) who reported higher levels of metals in worms from more polluted site and that irrespective of the degree of pollution, BAF is less than unity with the exception of a few metals such as Cd. The observed difference in BAFs could be related to the differences in specific metabolism and regulating mechanisms for essential and non-essential metals in earthworms as reported by Lukkari et al. (2009).

Significantly higher metallothionein (MT) concentrations were observed in the earthworms from the Gbonogun and Lafenwa abattoirs which corresponds to the significantly higher levels of heavy metals in these earthworms. Significant positive correlations were found between MT and heavy metal concentrations in all the earthworms, especially for Cu, Zn, Cd and Ni. This agrees with the work of Lukkari et al. (2004) who observed a significant correlation between MT activities and the metal concentrations in *Apporectodea tuberculata* exposed to Zn and Cu and conclude that MT represents an efficient biomarker for monitoring soil contamination with metals. Homa et al. (2005) also linked increased MT levels in *Eisenia fetida* to exposure to Zn, Cu, Pb and Cd.

This study showed that abattoir soils contained high levels of heavy metals due to uncontrolled dumping, leading to bioaccumulation in earthworms living in the abattoir soils. Heavy metal accumulation by the earthworms correlated with the metal concentrations in the soil. The soil pH, organic matter content and the age of the abattoirs accounted for the variations in heavy metal concentrations from one abattoir site to the other.

The high induction of MT in the earthworm species from the vicinity of the abattoirs makes it a potential useful biomarker to monitor heavy metal contamination.

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