HEAVY METAL ACCUMULATION AND BIOMARKER RESPONSES IN THE EARTHWORM (*Lumbricus terrestris*) COLLECTED FROM KOLO CREEK, BAYELSA STATE, NIGERIA

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Abstract: In this study, heavy metals (HMs) accumulation and biomarker responses in *Lumbricus terrestris* (*LT*) collected from Kolo Creek, Bayelsa State Nigeria, were evaluated. Composite soil and *LT* samples were collected from one control (Otuoke) and two experimental (Imiringi and Kolo Creek) sites, and analysed using standard methods for HMs (Lead (Pb), Copper (Cu), Zinc (Zn), Manganese (Mn) and Iron (Fe). Biochemical analyses were also performed on tissue supernatant of *LT*. Concentrations (mg/kg) of HMs in soil (*LT* in parenthesis) ranged as follows: Cu: 0.95-2.77 (0.79-2.23), Fe: 6.71-68.55 (0.35-0.43), Pb: 2.28-4.47 (0.80-11.60), Mn: 7.86-12.67 (3.57-8.25) and Zn: 0.63-4.40 (.98-6.56). Experimental sites had higher concentration of HMs than the control. High accumulations of Cu, Zn and Pb in *LT* was observed in all sites. Superoxide dismutase and catalase activities were reduced ($p<0.05$) while high ($p<0.05$) concentration of reduced glutathione (GSH) with conconmitant increase in the activities of glutathione s- transferase (GST) and glutathione peroxidase (GP_X) were observed in *LT* in experimental sites compared to the control. Other antioxidant enzyme activities (aldehyde, xanthine, sulphide and monoamine oxidases) and acetylcholinesterase were comparable in *LT* from all sites. This study thus confirms the presence of HMs in soil of Kolo Creek and environs. The accumulation of these metals in soft tissues of *LT* could have led to possible changes in oxidative stress enzymes, lipid peroxidation indices and glutathione-related enzyme system, which could be used to interpret risk level arising from HMs contamination, and present *LT* as a good bioindicator organism for soil pollution.

Keywords: Heavy metals, *Lumbricus terrestris*, Kolo Creek, oxidative stress enzymes

Introduction

Pollution is currently a global problem that exists in various dimensions (Asonye *et al*., 2007). It results from the negative impact of human activities, which causes pollution of the environment and its biodiversity (Ogamba *et al*., 2016). These impacts include transmission of diseases by water borne pathogens, eutrophication of natural water bodies and accumulation of toxic or recalcitrant chemicals in the soil. Toxic chemicals in the soil leads to decrease in soil fertility, alteration of soil structure, disturbance of balance between flora and fauna and contamination of crops and groundwater (Dawood *et al*., 2017). Heavy metals, pesticides and petroleum are the most diffusive chemical occurring in soil and exposure to these heavy metal contaminations can interfere with physiological as well as biochemical activities through oxidative stress in an organism (Verlecar *et al*., 2007).

The measurement of these biochemical and physiological responses with respect to the exposure to, and effects of contaminants (referred as biomarkers) in field exposure of organisms can be effectively employed for soil pollution monitoring. These cellular responses (including oxidative stress markers, biotransformation phase II enzyme and lipid peroxidation indices) to pollutants which provide insights into both the causal factors of the hazard and its ecological consequences can be measured in the body or its products of the organism inhabiting that environment. Earthworm has been reported as a novel organism for assessing effects of chemicals on terrestrial saprotrophic invertebrates (Spurgeon *et al*., 2003).

The earthworm *Lumbricus terrestris* originally traced to be native to Western Europe but it is now globally distributed in temprate to mild boreal climates is the most abundant invertebrates in the soils of temperate regions and are extremely important for soil formation (Edwards, 2004). Apart from permanently being in close contact with soil particles and participation in nutrient cycle in terrestrial ecosysytem, *Lumbricus terrestris* are significantly affected by pollutants that reach the soil system and are thus well suited for the monitoring of soil contamination.

Pollution caused by crude oil is the most prevalent problem in the Niger Delta Area of Nigeria. Soil, plant, animals and water resources are adversely affected because of the effect of oil (Kinako, 1997). Many environmental pollutants are capable of inducing oxidative stress in organisms and petroleum hydrocarbon can act as mediator in free radical generation in fish (Achuba and Osakwe, 2003).

High concentration of total hydrocarbon and heavy metal contaminants in Kolo Creek due to the presence of oil and gas infrastructure has been reported by various researchers (Inengite *et al*., 2010; Oluwu *et al*., 2010; Aghoghovwia and Chijoke 2012; Ebenezer and Eremasi, 2012; Ogamba and Ebere, 2017). The effect of these pollutants on *Lumbricus terrestris* has not been reported, hence, this present study investigates some oxidative stress indicators (superoxide dismutase, catalase, reduced glutathione, glutathione –S-transferase and glutathione peroxidase) and non – microsomal enzymes (Xanthine oxidase, Aldehyde oxidase, Sulphide oxidase and Monoamine oxidase) activities of *Lumbricus terrestris* collected from Kolo creek, Bayelsa State, Nigeria with a view of employing these changes as biomarkers of environmental pollution in environmental monitoring programme and thus, present *Lumbricus terrestris* as a bio-indicator organism in ecotoxicological studies.

Materials and Methods

Sampling sites

Lumbricus terrestris were collected from three (3) different locations in Ogbia Local Government Area, Bayelsa State, Nigeria and were labeled accordingly as A- Otuoke; B – Imiringi and C – Kolo Creek. Crude oil exploration activities take place in Kolo Creek and Imiringi while Otuoke is not having such oil facilities and this qualifies it as control site for this study.

Collection of Lumbricus terrestris

Samples of *Lumbricus terrestris* were collected from the three different locations using spade and forceps to dig beneath the

earth, then handpicked into a sterile plastic universal container and labeled accordingly. For each site, three determinations were obtained by pooling together ten (10) worms (i.e. n= 3 per site).

Collection of soil sample

The soil samples were obtained from the specific sites where the *Lumbricus terrestris* were collected into a polyethene bag and labeled accordingly.

Digestion of earthworm samples (Csuros and Csuros, 2002)

Exactly 2.00 g of *Lumbricus terrestris* was placed in a 250 mL conical flask and 10 mL of aqua regia (1:3: HNO_3:HCl) was added, followed by 5 mL of perchloric acid and covered with a dish. The mixture was heated in a digestion block at 150ºC until acid mixture reduced to about 5 mL. This was allowed to cool, then filtered using Whatman No. 4 filter paper. The filtrate was transferred to a 100 mL volumetric flask and made up to mark with 1M HNO_3. The filtrates were analyzed using an Atomic Absorption spectrophotometer (Thermo Jarrell Ash A.A. 12E) for the following metals: Pb, Cu, Zn, Mn and Fe.

Digestion of soil samples (Csuros and Csuros, 2002)

Sample of pulverized soil (3.00 g) was placed in a 250 mL conical flask and 10 mL of aqua regia (1:3; HNO_3:HCl) was added, followed by 5 mL of perchloric acid and covered with a dish. The mixture was heated in a digestion block at 150ºC until acid mixture reduced to about 5 mL. This was allowed to cool, then filtered using Whatman No. 4 filter paper. The filtrate was transferred to a 100 mL volumetric flask and made up to mark with 1M HNO_3. The filtrates were analyzed using an Atomic Absorption spectrophotometer (Thermo Jarrell Ash A.A. 12E) for the following metals: Pb, Cu, Zn, Mn and Fe.

Preparation of tissue supernatant of Lumbricus terrestris

Earthworm sample (0.5 g) was homogenized in ice cold 50 mM phosphate buffer (pH 7.2). The resulting homogenate was centrifuge at 4000 rpm for 10 min and supernatant decanted into a 2 mL sterilized plain container. This was frozen and stored at -30ºC until required.

Biochemical analysis

Total protein concentration in the tissue supernatant obtained was determined using the method of Doumas *et al*. (1981). Superoxide dismutase activity was determined by measuring the inhibition of autoxidation of epinephrine at pH 10.2 as described by Misra and Fridovich, (1972). Catalase activity was estimated from the rate of consumption of hydrogen peroxide levels (Kaplan and Groves, 1972). Lipid peroxidation indices was determined in terms of thiobarbituric acid reacting substances (TBARS) using Malondiadehyde (MDA) as standard (Buege and Aust, 1978). Tissue reduced glutathione (GSH) was determined by the method of Ellman, (1959) while, the activity of glutathion S-transferase was determined by Habig *et al*., (1974) using 1 – chloro – 2, 4-dinitrobenzene (CDNB) as substrate. The activities of glutathione peroxidase and Acetylcholinesterase were determined by the methods of Moin, (1986) and Ellman, (1961), respectively. The activities of xanthine oxidase was estimated using the method of Stripe and Della Corte, (1969), Aldehyde oxidase (John, 1967), Sulphite oxidase (Macleod *et al*., 1961) and Monoamine oxidase, Tabor *et al*. (1954).

Bioaccumulation factor (BAF)

The Bioaccumulation Factor was evaluated as the concentration of heavy metals in the soft tissues of *Lumbricus terrestris* using the method of Cortet *et al*. (1999) as indicated in the equation below:

$BAF_{(Heavy\ metals)} = HME/HMS$

Where: $BAF_{(Heavy\ metals)}$ = Bioaccumulation factor for heavy metals analysed; HME = Heavy metal concentration in *Lumbricus terrestris* (mg/kg); HMS = Heavy metal concentration in soil (mg/kg)

Metal pollution index (MPI)

The metal pollution index (MPI) according to Usero *et al*. (1997) was used to compare the total metal content at the different sampling sites:

$MPI = (Cf_1 \times Cf_2 \ldots\ldots Cf_n)^{1/n}$

Where: Cf_n = concentration of the metal n in the sample

Quality control measures

All laboratory glass wares used were initially washed with detergent and tap water; then, soaked in 5% Nitric acid for 24 h, washed and rinsed with deionized water. Samples were prepared and analysed in triplicates to check for precision of the results obtained. Reagents blanks were also included in analysis.

Statistical analysis

Mean ± Standard deviation of replicate experiments with triplicate sampling were taken for each analysis. Significant differences of results were established by one way Analysis of Variance (ANOVA) and differences between/within groups at $p < 0.05$ were carried out by Duncan Multiple Range Test. All statistical analysis was carried out using SPSS version 21.0.

Results and Discussion

The results of heavy metal concentrations in soil and *Lumbricus terrestris* from experimental sites are depicted in Table 1. Copper concentration in soil from Otuoke (A, 2.47±0.11 mg/kg) and Imiringi (B, 2.77±0.62 mg/kg) are comparable ($p < 0.05$) but higher than that obtained for Kolo Creek (C, 0.95±0.41 mg/kg). Soil from site C showed the highest ($p <0.05$) concentration of Fe, while site B the lowest. Lead (Pb), Manganese (Mn) and Zinc (Zn) concentrations in soil sample from site B is significantly higher than that of sites A and C which have comparable ($p > 0.05$) concentrations.

Results in Table 1 also indicated that Fe concentration in *Lumbricus terrestris* from all three sites were comparable ($p > 0.05$); however, Pb content in earthworm from Otuoke is higher ($p < 0.05$) than that of Imiringi and Kolo Creek. Again, from Table 1, copper concentrations in earthworm from the three experimental sites were significantly different from one another with Site A> Site B> Site C. *Lumbricus terrestris* from Site B showed a significant high ($p < 0.05$) Mn concentration as compared with that of Site A and Site C. The zinc content of earthworm from Otuoke is higher ($p<0.05$) than that of Imiringi and Kolo Creek which have comparable concentrations. The results in Table 1 also indicated that the total metal content as expressed by the Metal Pollution Index is in the following ascending order: A < C < B.

Table 1: Concentrations of heavy metals in soil samples and earthworm (*Lumbricus terrestis*) from experimental sites

Parameters (mg/kg)	Soil			Earthworm		
	A	B	C	A	B	C
Copper (Cu)	2.47 ±0.11 ^{a}	2.77±0.62 ^{a}	0.95±0.41^b	2.23±0.35^a	0.79±0.04^b	1.61±0.09^c
Iron (Fe)	16.59±0.62 ^{a}	6.71±1.44 ^{b}	68.55±0.44^c	0.36±0.03 ^{a}	0.35±0.06^a	0.43±0.07^a
Lead (Pb)	2.28±0.63^a	4.47±0.74 ^{b}	2.67±0.06^a	11.60±0.40 ^{a}	7.80±1.63^b	0.80±0.05^c
Manganese (Mn)	7.86±1.29 ^{a}	12.67±0.34^b	9.78±0.38^a	3.57±0.82 ^{a}	8.25±0.25^b	6.58±1.38^b
Zinc (Zn)	1.38±0.18 ^{a}	4.40±1.67^b	0.63±0.45^a	6.56±0.75 ^{a}	5.17±0.93^b	3.98±0.22^b
MPI	3.99	5.41	4.04			

Values are expressed as Mean ±SD of triplicate determinations; Means not showing the same superscript alphabet on same row differ significantly at P<0.05; A = Otuoke; B= Imiringi; C = Kolo Greek. MPI = Metal Pollution Index

Results in Table 2 indicated that *Lumbricus terrestris* accumulates zinc more in Otuoke and Kolo Creek as compared with all other heavy metals analysed. It was also observed from the results presented in Table 3 that earthworms from Imiringi and Kolo Creek accumulate copper more than worms obtained from Otuoke soil. Pb accumulation for earthworms from Otuoke and Imiringi were greater than unity while that of Kolo Creek is < 1. Iron and Manganese were not accumulated by *Lumbricus terrestris* from all three experimental sites.

Table 2: Bioaccumulation Factors of *Lumbricus terrestris* from experimental sites

Heavy metals (mg/kg)	A	B	C
Copper (Cu)	1.00	2.04	1.25
Iron (Fe)	0.02	0.05	0.01
Lead (Pb)	1.30	1.08	0.04
Manganese(Mn)	0.41	0.65	0.78
Zinc (Zn)	4.68	1.00	3.88

A = Otuoke; B = Imiringi; C = Kolo Creek

Changes in biomarker responses in *Lumbricus terrestris* from sampling sites are indicated in Table 3. Earthworm from three sites (A, B, and C) under investigation showed comparable ($p > 0.05$) concentrations. Activities of superoxide dismutase and catalase are significantly higher ($p < 0.05$) in *Lumbricus terrestris* from Otuoke (site A) as compared that of Imiringi and Kolo Creek, although, SOD activities of Otuoke earthworm is comparable ($p > 0.05$) to that of Imiringi. Lipid peroxidation indices measured as malondiadehyde is significantly ($p < 0.05$) higher in earthworm from Kolo Creek as compared to the two other sites (A & B). Results from Table 3 also indicated that the levels of reduced glutathione (GSH) are comparable ($p > 0.05$) between sites B & C, but higher ($p < 0.05$) than that of Otuoke (site A). The activities of glutathione S – transferase (GST) is higher ($p < 0.05$) in tissue of earthworm from Kolo Creek as compared with the two other sites (A & B), with earthworms from Otuoke expressing the lowest GST activity.

Table 3: Changes in Biomarker responses in *Lumbricus terrestris* from sampling sites

Parameters	A	B	C
Total Protein (g/dl)	14.74±0.83^a	14.91±0.57^a	14.64±1.34^a
Superoxide dismutase (unit/mg/protein)	13.23±1.88^a	13.83±0.33^a	10.55±0.88^b
Catalase (unit/mg/protein)	36.63±3.15^a	30.17±0.69^b	20.32±3.15^c
Malondiadehyde (unit/ml)	1.20±0.39^a	1.40±0.09^a	3.09±0.34^b
Reduced Glutathione (unit/mg/protein)	29.95±0.53^a	41.99±0.91^b	42.71±0.89^b
Glutathion S-transferase (nmol/mg/protein)	21.19±0.36^a	24.55±1.14^b	27.04±0.52^c
Glutathione peroxidase (unit/mg/protein/min)	35.63±0.54^a	38.09±1.21^a	36.82±1.54^a
Acetylcholinesterase (nmol/mg protein/min)	0.099±0.014^a	0.095±0.002^a	0.090±0.092^a
Xanthine oxidase (unit/mg/protein)	33.61±0.05^a	33.48±0.02^a	34.11±1.24^a
Aldehyde oxidase (unit/mg/protein)	74.24±1.03^a	77.61±1.54^a	78.70±3.77^a
Sulphite oxidase(unit/mg/protein)	44.42±0.33^a	44.42±0.33^a	44.33±0.21^a
Monoamine oxidase (unit/mg/protein)	23.94±0.76^a	24.57±0.40^a	24.17±0.31^a

Values are expressed as Mean ±SD of triplicate determinations; Means not showing the same superscript alphabet on same row differ significantly at P<0.05; A = Otuoke; B= Imiringi; C = Kolo Greek

Table 3 also showed that the activities of glutathione peroxidase and acetylcholinesterase were comparable ($p > 0.05$) in earthworm from all the experimental sites. For the non – microsomal antioxidants (xanthine oxidase, aldehyde oxidase, sulphide oxidase and monoamine oxidase) their activities were comparable in all three sites under investigation.

The interference of heavy metal with physiological as well as biochemical activities through oxidative stress in an organism has been a major concern to environmental research**.** Heavy metal contamination which continues to attract the attention of environmental researchers can interfere with physiological as well as biochemical activities through oxidative stress in an organism (Shulkin *et al*., 2003; Verlecar *et al*., 2007). These contaminants (heavy metals) in soil do not decompose or disappear from soil but rather are taken up by crop plants, transfer via earthworms and other soil fauna through food chain (Wang *et al*., 2018). In this present study, significantly high ($p < 0.05$) concentration of Iron (Fe) was observed in Kolo Creek (site C) as compared to other two sites (Otuoke = A; Imiringi = B). High Fe content and other heavy metals in the soil of Niger Delta and Kolo Creek has been previously reported (Iwegbue *et al.*, 2006; Inengite *et al*., 2010; Aghoghovwia and Ayatari, 2012; Ebenezer and Eremasi, 2012; Eremasi *et al*., 2015). Lead (Pb), Manganese (Mn) and Zinc (Zn) were significantly higher ($p < 0.05$) in site B as compared to site A and C. In all, the heavy metal concentrations obtained from the three sites were found to be lower than the target values of DPR (EGASPIN, 2002). However, the total metal content of the three sites as expressed by the metal pollution index showed that Imiringi and Kolo creek have higher heavy metal content. From the results obtained (Table 1), there may be no imminent danger but the bioaccumulation of these heavy metal by organism inhabiting the environment could be of concern, since such metal can be possibly transferred through the food chain to their predators and ultimately humans (Owagboriaye *et al*., 2015) and generate reactive oxygen species (ROS) through the Fenton/Haber – Wiess reaction.

Earthworm accumulates metals through two pathways: absorption following dermal content or through the gut tissues (Holbelem *et al*., 2006; Nannoni *et al*., 2011) and thus possess a serious risk of secondary poisoning due to bio – magnification (Kamitani and Kaneko, 2007). Result from this study showed that copper and zinc were accumulated more in *Lumbricus terrestris* than other heavy metals (Pb, Fe and Mn).

Earthworm from sites A and B also accumulated Pb. Bioaccumulation of many heavy metals by earthworm has been previously reported (Hartenstein *et al*., 1980; Gupta *et al*., 2005; Li *et al*., 2010; Nannoni *et al*., 2014; Owagboriaye *et al*., 2015; Maity *et al*., 2018).

The resultant effect of heavy metal accumulation by *Lumbricus terrestris* is the production of reactive oxygen species (ROS) through the Fenton/Haber – Wiess reactions (Aboul – Ela *et al*., 2011) which could lead to oxidative stress and subsequent cellular damage. The accumulation of ROS is counteracted by an intrinsic antioxidant defense system that includes low molecular mass molecules such as reduced glutathione and antioxidant/biotransformation enzymes. The activities of SOD and CAT were reduced ($p < 0.05$) in *Lumbricus terrestris* from Kolo Creek as compared to earthworms from the control site (Otuoke). Similar response was also observed in earthworm from Imiringi. The above observations in the activities of SOD and CAT coupled with the high malondiadehyde level indicate that *Lumbricus terrestris* from sites (B & C) could be experiencing stress arising from the accumulation of heavy metal in the environment.

Reduced glutathione (GSH) detoxifies H_2O_2 and lipid hydroperoxides through reactions catalysed by glutathione peroxidase (Ahmed, 2005) and also act as a cofactor in the phase II transformation reaction carried out by GST (Jakoby, 1985). An increase ($p< 0.05$) in the concentration of GSH observed in *Lumbricus terrestris* from sites (B & C) with the conconmitant increased in the activities of GST and GPx could indicate that the rate of GSH synthesis through the γ – glutamyl cycle was higher than the rate of consumption. This utilization of cellular GSH content together with the activation of GST and GPx, according to Maity *et al*. (2018) is required to maintain homeostasis of the organism's internal cell environment.

Other enzymes (aldehyde, xanthine and sulphite oxidases) which are molybdenum and haem containing soluble enzymes involved in the oxidation of xenobiotics (Beedham, 2005; Hille, 2005) and monoamine oxidase that is involved in the biotransformation of aromatic monoamines (Asagba, 2010) showed no significant difference in *Lumbricus terrestris* from the three sites (A, B & C). This could be due to adaptive measures by the organism of absence of stressors (contaminants) that are specific to modulate the activities of these enzymes. The activities of acetylcholinestrease (AChE) did not show any significant difference ($P > 0.05$) in the soft tissues of *Lumbricus terrestris* from the three sites (A, B & C) under investigation. This could mean that earthworms are not exposed to organophosphorus or carbamate pesticides.

In conclusion, this study confirm the presence of Cu, Fe, Pb, Mn and Zn in the soil of Kolo Creek and environs but the concentrations of these heavy metals were lower than the target values of DPR (EGASPIN, 2002). The observed accumulation of these metals in soft tissues of earthworm could have lead to the possible changes in oxidative stress enzymes and lipid peroxidation indices. Exposure to organophosphate and carbamate insecticides in earthworm was not confirmed by this study. Other enzyme involved in the oxidation of xenobiotics such as aldehyde, xanthine, sulphite and monoamine oxidases were not induced. The changes in the oxidative enzymes (SOD & CAT), lipid peroxidases indices (MDA) and glutathione related enzyme system in *Lumbricus terrestris* could be used to interpret the level of risk arising from heavy metal contamination and earthworm a good bioindicator and thus, present *Lumbricus terrestris* a good bioindicator organism for soil pollution.

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