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Physiological responses of a tropical earthworm: a potential tool for biomonitoring heavy metal pollution in tropical wetlands

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ABSTRACT
Wetlands play important ecological and life-supporting roles. Therefore, appropriate and adequate wetland biomonitoring options should be available. This study investigated the burrowing and survival responses of a wetland earthworm (Libyodrilus violaceus) to heavy metal (Zn, Pb, Cd) polluted soil. The worms were exposed to soils contaminated with graduated levels of heavy metals in the laboratory and their responses evaluated following standard procedures. The median lethal concentration (LC_{50}) values for Zn, Pb, and Cd obtained from the study were 520.06 mg/kg, 1551.55 mg/kg, and 706.66 mg/kg soil respectively. The mixture of Zn and Cd gave a 14-day LC_{50} value of 626.12 mg/kg soil, while the combined action of Zn, Pb and Cd gave a 14-day LC_{50} value of 1,273.47 mg/kg soil. The species also showed delayed burrowing responses to these metals in individual and combined concentrations. These results suggest that L. violaceus could be a candidate for assessing the heavy metal state of tropical wetland soils.

Keywords: Burrowing, LC_{50}, *Libyodrilus violaceus*.

INTRODUCTION
The problem of environmental pollution in this age of industrial and technological advancements calls for proper monitoring and actions. Due to the important ecological and life-supporting roles it plays, the soil has become vulnerable to various forms of pollution, including heavy metal pollution. Sinha *et al*. (2008) reported that there are over 80,000 contaminated sites in Australia, 40,000 in United States of America (USA), 55,000 in six European countries, 7,800 in New Zealand, and about 3 million in Asia-Pacific. These polluted sites mostly contain heavy metals and organic contaminants. Soil contamination with heavy metals is a global occurrence and is of great concern because of its persistence and health consequences to humans, plants and animals (Akinola *et al*., 2008, 2011). Heavy metals may find their ways into the food chains if polluted soils are used for farming. Akinola (2008) carried out an assessment of heavy metals present in soil and *Talinum fruiticosum* grown in an industrial area in Lagos, Nigeria. The results showed higher levels of lead and cadmium in both vegetable and soil compared to those of non-industrial area. It is therefore important to employ appropriate strategy in monitoring and evaluating soil pollutants,

especially metals, and consequently proffer remediative interventions.

Earthworms have been widely used as test organisms for soil pollution monitoring and evaluation (OECD, 1984; Spurgeon and Hopkin, 2000; Smith *et al*., 2005; Shin *et al*., 2007; Hirano and Tamae, 2011). Earthworms are easy to capture and handle, they have a short life-cycle, and are globally distributed in many types of soil (Fitzpatrick *et al*., 1996). An indirect evidence of earthworms' metal tolerance is the fact that some species thrive in and can be collected from heavy metal contaminated soils (Spurgeon and Hopkin, 2000).

Quite a lot of research has been carried out on earthworms' positive roles in agro-ecosystems, environmental monitoring and sustainability (Shin *et al*., 2007; Maenpaa, 2002; Hickman and Reid, 2008; Hu *et al*., 2010; Hirano and Tamae, 2011). However, the majority of the species worked on are native to temperate region. In addition, published earthworm researches in Nigeria and West Africa region have been mainly on friable (dry) soil species (Owa and Olojo, 2003). *Libyodrilus violaceus*, together with other wetland earthworms, makes a major contribution to the productivity of many river basin agricultural projects in Nigeria (Owa and Olojo, 2003; Owa *et al*., 2010). The aim of this study, therefore, was to evaluate the burrowing and survival responses of a tropical wetland earthworm, *Libyodrilus violaceus*, to soil contaminated with varying levels of heavy metals.

MATERIALS AND METHODS

Sample collection site

The soil (sandy loamy) and earthworm (*L. violaceus*) samples used for the study were collected at the main campus of the University of Lagos, Nigeria. The university is located on longitude 3^0 24'E and latitude 6^0 27'N within the Mainland area of Lagos.

Soil collection

The soil used for the study was collected at 0.0 - 2.0 cm depth, bulked together, air dried, and passed through a 2 mm sieve. A portion, 10 kg, of the bulked soil was taken to the laboratory for physico-chemical analysis.

Earthworm collection

Libyodrilus violaceus were collected within 970 m^2 area by digging with a shovel to an average depth of 22 cm; the earthworms were manually sorted out from the soil. The earthworms were stabilized in soil collected from the same site (0.0 - 2.0 cm deep) for 24 hours before use. Clitellate adults with average live weight of 0.9 g were selected for the study. The species was authenticated by a renowned earthworm taxonomist.

Test reagents

Nitrate salts of heavy metals, namely zinc nitrate hexahydrate [$Zn(NO_3)_2$. $6H_20$], lead nitrate [$Pb(NO_3)_2$], and cadmium nitrate tetrahydrate [$Cd(NO_3)_4$. $4H_2O$] were used. Deionized water that was used to dissolve the salts was obtained from the Department of Chemistry, University of Lagos.

Experimental procedure

The burrowing and survival responses of *L. violaceus* to soil heavy metal contamination were evaluated using the protocol of the Organization for Economic Co-operation and Development, OECD, (1984) with minor modifications. Field soil was used instead of artificial soil (Laurenco *et al*., 2012), while transparent plastic containers were used in place of glass (Robidoux *et al*., 1999). Each test container measured 14 cm in length, 9 cm in breath, and 7 cm in height. *L. violaceus* samples were exposed to five combined and individual graded concentrations of Zn, Pb, and Cd. The appropriate quantities of nitrate salts of the metals were dissolved in 220 ml deionized water and used to spike the sieved soil (Spurgeon *et al*., 1994) to simulate natural contamination with Zn, Pb, and Cd metals. The choice of heavy metal concentrations was informed by the preliminary trial test carried out before the actual experiment. There were seven groups resulting from different combinations of the metals:

1. Group A – Mixture of Zn, Pb, Cd;
2. Group B – Mixture of Zn, Pb;
3. Group C – Mixture of Zn, Cd;
4. Group D – Mixture of Pb, Cd;
5. Group E – Zn;
6. Group F – Pb;
7. Group G – Cd.

Each group has five different concentrations (Table 1) in a geometric series in four replicates each. The control consisted of field soil only.

Each test container was filled with 750 g soil. The soil in each container was spiked with the appropriate quantity of nitrate salts of heavy metals (Table 1), mixing for at least 5 minutes to achieve an even distribution of the metals. Thereafter, set-ups were allowed to stabilize for 24 hours. Ten *L. violaceus* were placed on the surface of the soil in each container. The containers were covered with transparent perforated lids to prevent the earthworms from escaping, allow sufficient air, and prevent excessive water loss. To ensure that worms remained in the test medium throughout the duration of the test, testing was done under continuous light at the prevailing room temperature (22 – 31^{o} C).

Table 1: Spiking and final concentrations of soil used for screening *L. violaceus* for burrowing and survival responses to heavy metal contaminated soil

Level of Concentration		Concentration (mg/kg)		
		Zn	Pb	Cd
1	Spiking	200.00	150.00	5.00
	Background	2.94	0.24	0.00
	Final	202.94	150.24	5.00
2	Spiking	400.00	300.00	10.00
	Background	2.94	0.24	0.00
	Final	402.94	300.24	10.00
3	Spiking	800.00	600.00	20.00
	Background	2.94	0.24	0.00
	Final	802.94	600.24	20.00
4	Spiking	1,600.00	1,200.00	40.00
	Background	2.94	0.24	0.00
	Final	1,602.94	1,200.24	40.00
5	Spiking	3,200	2,400	80.00
	Background	2.94	0.24	0.00
	Final	3,202.94	2,400.24	80.00

Assessment of burrowing responses

The time taken for all worms or the last worm to burrow in each metal concentration level was recorded. Each worm was regarded to have completely burrowed if no part of its body was seen on the soil surface. Readings for each container was taken only where all the worms completely burrowed. Inability of one or more worms to completely burrow in a container was taken as an evidence of avoidance for that particular metal concentration (ASTM, 1995); hence, burrowing time was not recorded in such cases.

Assessment of survival responses

Mortality was assessed at 7th and 14th days of the experiment. The soil, including the worms, in each container was poured into a plastic tray and spread out into a thin film in order to count live worms. Worms were regarded as dead if they did not respond to mechanical stimulus of touch at the front end. After the 7-day assessment, earthworms and soil were replaced in the test containers. The pH of the soil medium used was 6.9 ± 0.1 while moisture content was 35 ± 2% (OECD, 1984).

Analyses of data

The data obtained from the study were used to calculate the median lethal concentrations of the heavy metals using probit analysis. Two-tailed Pearson correlation co-efficient was calculated for 7 and 14 - day LC_{50} values. Descriptive statistics were used to describe the burrowing time. All analyses were carried out with the SPSS software, 17.0 version.

RESULTS

Background physico - chemical properties of test soil

The result of the baseline physico-chemical study conducted on the air dried test soil showed that the textural composition of the soil was 69 % sand, 14 % silt, and 14 %, clay while the total organic carbon was 2.78 %. The test soil contained 2.94 mg/kg Zn, 0.24 mg/kg Pb, while Cd was not detected. The Na^+, K^+, Mg^{2+}, and Ca^{2+} contents of the test soil were 5.87, 4.07, 8.17, and 14.71 meg/100g, respectively (Table 2).

Burrowing responses of *L. violaceus* to heavy metals

The burrowing responses of *L. violaceus* to heavy metal contaminated soils as presented in Table 3 indicated that in groups A, B, C and E soils containing Zn, total burrowing was not achieved in all the concentrations. In group A and group C soils, total burrowing occurred between 8 and 35 minutes only in concentrations 1 to 3. In group B soil, total burrowing occurred in concentrations 1 to 4 within 8 and 25 minutes, while in group E soil, total burrowing also occurred in concentrations 1 to 4 howbeit at a longer time interval of between 10 and 40 minutes. In groups D, F and G soils, all the worms in each concentration burrowed within a relatively shorter time of between 5 and 20 minutes. *L. violaceus* in concentration levels 1-3 of Cd contaminated soil (group G) burrowed within 5 minutes. The corresponding burrowing time by *L. violaceus* in Pb contaminated soil was 5 to 7 minutes, and for Zn contaminated soil, it was 10 to 40 minutes.

Table 2: Background physico-chemical properties of air dried test soil

% Sand	% Silt	% Clay	% ToC	% Moisture	pH	Metals (mg/kg)			CEC (meg/100 g)			
						Zn	Pb	Cd	Na^+	K^+	Mg^{2+}	Ca^{2+}
69	14	14	2.78	7.59	6.90	2.94	0.24	ND	5.87	4.07	8.17	14.71

ToC = total organic carbon ND = not detected CEC = cation exchange capacity

Median lethal concentrations (LC_{50}) of heavy metals on *L. violaceus*

The 7-day and 14-day median lethal concentration (LC_{50}) values for different heavy metal mixtures on *L. violaceus* are shown in Table 4. There was a strong positive correlation (r = .99) between the 7-day and 14-day LC_{50} values. The 7-day LC_{50}

values for Zn, Pb and Cd individual metals were 564.24, 1551.55 and 706.66 mg/kg soil respectively. The 7-day and 14-day LC_{50} values for Pb and Cd remained constant while the 14-day LC_{50} value for Zn decreased to 520.06 mg/kg. The LC_{50} of heavy metal mixtures on *L. violaceus*, showed that the pair of Zn and Cd was the most toxic with a 14-day LC_{50} of 626.12 mg/kg soil. The corresponding LC_{50} values for the pairs of Zn and Pb; Pb and Cd; were 1,422.41 mg/kg and 1,454.64 mg/kg soil respectively. The combined action of the three metals (Zn, Pb, Cd) gave a 14-day LC_{50} value of 1,273.47 mg/kg soil.

Table 3: Time (in minutes) taken for all earthworms to burrow in each soil group

Conc.	**Groups**						
	A (Zn, Pb, Cd)	**B (Zn, Pb)**	**C (Zn, Cd)**	**D (Pb, Cd)**	**E (Zn)**	**F (Pb)**	**G (Cd)**
Conc. 1	8 ± 0.50	8 ± 1.50	10 ± 0.50	5 ± 1.00	10 ± 2.00	5 ± 1.00	5 ± 1.00
Conc. 2	8 ± 2.00	9 ± 2.50	14 ± 3.50	5 ± 0.50	14 ± 4.50	5 ± 0.50	5 ± 1.00
Conc. 3	12 ± 4.00	9 ± 2.00	35 ± 6.50	7 ± 2.00	40 ± 8.50	7 ± 3.00	5 ± 3.00
Conc. 4	NB	25 ± 7.00	NB	12 ± 3.50	40 ± 7.00	10 ± 2.00	7 ± 2.50
Conc. 5	NB	NB	NB	20 ± 3.00	NB	16 ± 1.50	7 ± 1.50

Time taken to burrow recorded in minutes after the last earthworm in each of the four replicates had burrowed. NB = No total burrowing (if one or more earthworms did not completely burrow in any of the four replicates). Conc. = Concentration level (mg/kg soil)
Conc. 1= Zn: 202.94; Pb: 150.24; Cd: 5.00; Conc. 2= Zn: 402.94; Pb: 300.24; Cd: 10.00;
Conc. 3= Zn: 802.94; Pb: 600.24; Cd: 20.00; Conc. 4= Zn: 1,602.94; Pb: 1,200.24; Cd: 40.00;
Conc. 5= Zn: 3,202.94; Zn: 2,400.24; Cd: 80.00.

Table 4: Median lethal concentrations (LC_{50}) of soil heavy metals on *L. violaceus*

Period	**Soil group and metal contaminant**	**LC_{50} (mg/kg)**	**95% confidence limit**		**Slope ±S.E**	**D.f.**	**Probit line equation**
			Lower	**Upper**			
7-day	A(Zn,Pb,Cd)	1,273.47	710.50	2,047.70	5.59 ± 0.75	3	Y=5.59+7.41X
	B(Zn,Pb)	1,493.57	248.61	4,873.97	4.37 ± 0.55	3	Y=4.37+7.95X
	C(Zn,Cd)	637.39	-	-	9.02 ± 1.30	3	Y=9.02+6.91X
	D(Pb,Cd)	1,493.68	955.13	2,691.59	7.19 ± 1.09	3	Y=7.19+6.60X
	E(Zn)	564.24	491.42	646.39	4.80 ± 0.63	3	Y=4.80+7.69X
	F(Pb)	1,551.55	-	-	8.26 ± 1.22	3	Y=8.26+6.79X
	G(Cd)	706.66	-	-	2.22 ± 2.81	3	Y=2.22+0.79X
14-day	A(Zn,Pb,Cd)	1,273.47	710.50	2,047.70	5.59 ± 0.75	3	Y=5.59+7.41X
	B(Zn,Pb)	1,422.41	324.88	3,726.83	4.69 ± 0.60	3	Y=4.69+7.80X
	C(Zn,Cd)	626.12	-	-	9.27 ± 1.31	3	Y=9.27+7.09X
	D(Pb,Cd)	1,454.64	-	-	7.95 ± 1.28	3	Y=7.95+6.20X
	E(Zn)	520.06	453.40	594.46	4.96 ± 0.65	3	Y=4.96+7.63X
	F(Pb)	1,551.55	-	-	8.26 ± 1.22	3	Y=8.26+6.79X
	G(Cd)	706.66	-	-	2.22 ± 2.81	3	Y=2.22+0.79X

D.f. = Degree of freedom; S.E. = Standard Error

DISCUSSION

The results obtained from this study indicated that Pb was the most tolerated, and Zn the least. The relatively lower median lethal concentration (LC_{50}) values for Zn indicated higher toxicity of Zn to *L. violaceus*. The results agree with those of Spurgeon *et al*. (1994) and Spurgeon and Hopkin (2000) which concluded that reductions in earthworm populations around highly polluted sites are probably due to the effects of Zn and not other heavy metals like Cd, Cu, and Pb. Spurgeon and Hopkin (2000) investigated the evolution of Zn resistance in *Eisenia fetida* and concluded that there is a relatively low resistance to Zn pollution when compared with Cd pollution. Moreover, it was observed in this study that *L. violaceus* manifested relatively stronger delayed burrowing in all cases where Zn was present. Though burrowing time may not be sensitive enough to be used as a single parameter to measure toxicity (Shin *et al*., 2007), the relatively delayed or non-burrowing responses exhibited by *L. violaceus* in soils contaminated with Zn is a further confirmation of Zn toxicity to the species at high concentrations. The higher toxicity associated with Zn contamination on earthworms may be attributed to the continuous build-up of the metal in the earthworms' tissue (Lourenco *et al*., 2012). This assumption stems from the fact that Zn, being an essential element required for a number of metabolic functions such as healthy immune system, production of certain hormones etc, is difficult to eliminate or excrete (Spurgeon and Hopkin, 2000; Hershfinkel *et al*., 2007; Lourenco *et al*., 2012). Typical of essential metals that have been reported to have bio-importance, Zn intake into the body must be within a limit, as excesses will result in toxicity (Duruibe *et al*., 2007). The relatively higher sensitivity shown to Zn polluted soil suggests that *L. violaceus* should be a good biomonitoring candidate for Zn pollution in its natural habitat, wetland soil.

The relative tolerance shown to Pb in this study as indicated by its higher LC_{50} value also conforms to earlier observations in other studies. Shin *et al*. (2007) compiled a list of LC_{50} values derived from published data for As, Cu, Pb, and Zn on some earthworms and observed a generally low toxicity associated with Pb. However, when the LC_{50} value for Pb derived from this study is compared with those of other species compiled by Shin *et al*. (2007), *L. violaceus* can be considered as only moderately tolerant to Pb. For instance, while the LC_{50} value for Pb in this study was 1,551.55 mg/kg soil, the LC_{50} of other earthworm species such as *Pheretima* spp., *Pheretima guellelmi*, and *Eisenia fetida* ranged between 1,382 mg/kg and 5,941 mg/kg soil. Moreover, Spurgeon *et al*. (1994) also discovered less toxicity of Pb to *Eisenia fetida* relative to Zn and Cu. The relative higher tolerance to Pb observed in *L. violaceus* in this study, and in some other earthworm species by other studies, may be a result of genetic evolution in earthworms to cope with soil Pb pollution or contamination. Since *Pheretima* spp., *Pheretima guellelmi*, and *Eisenia fetida* have been regarded as indicator organisms for soil toxicity (Shin *et al*., 2007; Hirano and Tamae, 2011; Lourenco *et al*., 2012), *L. violaceus* should also be an ideal biomonitoring candidate for heavy metals in wetland soil ecosystem.

The relatively higher LC_{50} value of the binary mixture of Zn and Pb compared to the mixture of Zn and Cd; and the mixture of Zn, Pb, and Cd in this study, suggests a degree of antagonistic relationship between Zn and Pb. Lead probably inhibited the adverse effects of Zn on *L. violaceus* to a certain extent. Though the toxic potential of Pb and Zn are different, the control of intracellular levels of Pb and Zn are not fully understood (Maity *et al*., 2008). *L. violaceus* showed no obvious negative response to Cd except when it was in combination with other metals. This was probably due to the low concentrations of the metal used in this study.

CONCLUSION

The findings of this study have shown that *L. violaceus* is a good candidate to monitor the heavy metal state of wetland soil. Unlike the epigeic and anecic earthworms which may be less exposed to pollutants including heavy metals due to their behavioural, life cycle, and feeding characteristics, *L. violaceus* is an endogeic species which lives in and feeds on mid-layer soils where metals and other pollutants might have accumulated over a long period of time. Using *L. violaceus* to monitor heavy metal pollution therefore presents a convenient, reliable and affordable approach to improving the health of tropical wetland soils.

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