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Ex-situ induction of Metallothionein gene in *Lybiodrilus violaceous* post cadmium and zinc exposure

Aemere Ogunlaja[a,b,c,*], Vikas Sharma[b,d], Johnson Lin[b]

[a] Department of Biological Sciences, Redeemer's University, Ede Osun State, Nigeria
[b] School of Life Sciences, Discipline of Microbiology, University of Kwazulu-Natal, Durban, South Africa
[c] Africa Centre of Excellence for Water and Environmental Research (ACEWATER), Redeemer's University, Ede Osun State, Nigeria
[d] Department of Biotechnology Engineering, Ambala College of Engineering and Applied Research, Devsthali, P.O Sambhalkha, Ambala 133101, Haryana, India

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ABSTRACT

Background: Earthworms are good indicators used in evaluating the health status of the terrestrial ecosystem and metallothionein (Mt) is a biomarker monitored during environmental studies. There is however a dearth of information on metallothioneins in African earthworms. *Libyodrilus* violaceous is a West African earthworm well investigated and reported including its tolerance to metal pollution but the presence of *Mt gene* in *L. violaceous* is not established.

Methods: The presence of Mt. in *L. violaceous* was determined to validate its potential role in metal tolerance. *L. violaceous* sampled from Ije-Ododo soil were exposed to varying concentrations of Cadmium (Cd) and Zinc (Zn) in a potted experimental set-up and the metallothionein (MT) expressions were determined. The presence of *Mt gene* and metallothionein expression were determined using whole transcriptome sequencing and real-time polymerase chain reaction (PCR), respectively after extracting Ribonucleic acid (RNA) from samples on the 1 st, 3rd and 7th days. The genetic relationship of the detected MT protein sequence was compared to existing MT protein sequences and a phylogenetic tree was constructed.

Results: We established the presence of the MT gene and the phylogenetic tree showed that it had a relationship with other earthworms' MTs. Up-regulation of Mt. expression was observed on the 7th day in *L.violaceous* exposed to concentrations of Cd and Zn.

Conclusion: One of the modes *of survival of L. violaceous* to metal pollution is by the up-regulation of metallothioneins. This earthworm metallothionein protein sequence is the first report of species in the Eudriloides family and the first report in African earthworms deposited in the GenBank repository for reference. The use of *L.violaceous* for Cd and Zn remediation in soils could be exploited.

1. Introduction

The earthworm species, *Libyodrilus violaceous*, was first documented by Beddard, 1891. They are purplish, luminescent earthworms occurring most times twined together forming rope-like bundles (Dada et al., 2013; Ogunlaja, 2014; Okoye et al., 2019) and are found in muddy soils abundantly during the wet seasons (Ogunlaja and Morenikeji, 2013). The species is commonly distributed from the middle belt spanning down the southern part of Nigeria and in Cameroon (Dada et al., 2013). The importance of this earthworm species are highlighted in investigations ranging from; physiology (Idowu et al., 2006), ecology (Ogunlaja and Morenikeji, 2013), nutrition (Dedeke et al., 2013) to ecotoxicology (Dedeke et al., 2016; Dedeke et al., 2018).

Earthworms tolerate metals by passive absorption using chloragogen cells (Świątek et al., 2020) or by biotransformation and biosorption using metal-binding protein, metallothionein (Fouché, 2018) which consequently cause bioaccumulation or sequestrate the pollutants. Since metallothionein (MT) first report in the horse renal cortex (Margoshes and Vallee, 1957), they are used in different fields like their use as molecular markers for monitoring in ecotoxicology. They characteristically have one-third of their amino acids as cysteines (Wang et al., 2014; Slavík et al., 2019), regulate metabolism/homeostasis of essential trace metals, detoxify non-essential metals (Siscar et al., 2014) and manage oxidative stress in organisms across phylogenies (Ruttkay-Nedecky et al., 2013). Earthworm MT was first reported in *L. terristris* by Stürzenbaum et al., 1998 and several recent reports are available

Abbreviations: MT, Meatallothionein; Cd, cadmium; Zn, zinc

* Corresponding author at: Department of Biological Sciences, Redeemer's University, Ede Osun State, Nigeria.
E-mail address: ogunlajaa@run.edu.ng (A. Ogunlaja).

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(Anderson et al., 2017; Drechsel et al., 2017), including their genes, nucleotide and protein sequences. However, those of indigenous African earthworms are scarce except for a few reports on metallothionein expression in *L. violaceous* emanating from biochemical studies (Dedeke et al., 2016). Although its tolerance to metals and metal bioaccumulation is known (Ogunlaja and Morenikeji, 2013; Dedeke et al., 2016; Dedeke et al., 2018), its mode of survival is not known. This study set out to investigate the presence of the MT gene and establish its protein sequence; we also determined the phylogenetic relationship between this MT gene and similar earthworm MTs. MT gene expression over seven days of exposure to varying concentrations of cadmium and zinc was also carried out.

2. Materials and methods

2.1. Sample collection

The soil and earthworms used in this study were collected from Ije-Ododo main town, 2 Km away from Agaye in Alimosho LGA of Lagos State Nigeria (Lat. 06o 29′N

and Long. 03o 15′ E). The soil and earthworms used for the metal experimental set-up were distributed and prepared/spiked as described by Dada et al., 2013 and Lourenço et al., 2011, respectively, with slight modifications. Conventional identification of the earthworm species was by Prof. Owa, an earthworm taxonomist from the Zoology Department, Osun State University, Nigeria (Ogunlaja and Morenikeji, 2013).

2.2. Metallothionein induction study

The soil collected was air-dried and passed through a 2 mm sieve. The initial concentrations of Cd and Zn in soil were 0.01 ± 0.002 mg/kg and 21.45 ± 0.02 mg/kg, respectively. Zinc Sulphate ($ZnSO_4$) and cadmium chloride ($CaCl_2$) reagents used were analytical grade from Sigma-Aldreich Laboratories, USA. One Kilogram of soil each was spiked with 427 mg/kg, 366 mg/kg and 305 mg/kg of Cd and each soil were distributed into plastic buckets designated $LVCd^1$, $LVCd^2$, $LVCd^3$, respectively. Similarly, one Kilogram of soil each was spiked with 240 mg/kg, 200 mg/kg and 160 mg/kg of Zn and each soil were also distributed into plastic buckets designated $LVZn^1$, $LVZn^2$ and $LVZn^3$. One Kilogram of soil was also placed in a pot without metal spiking and was used as the control; it was labelled LV_{Cont}. The moist state of the soil was maintained to mimic the field status throughout the experimental period with deionized water obtained from the laboratory.

Earthworms were left to acclimatize for two days, and ten earthworms were distributed into each pot of soil under room temperature conditions. Three earthworms per pot were collected on day 1, 3rd, and 7th day. They were left to depurate overnight after which their anterior portions were cut, ensuring them being void of gut content, they were preserved in RNA shield (ZYMO RESEARCH) for future analysis.

2.2.1. RNA extraction and cDNA library preparation for MT gene detection study

GeneJET RNA Purification kit (Invitrogen) was used according to the manufacturer's instruction to extract RNA and the extracts were labelled and kept in −80 °C freezer for future use. To determine the presence of MT gene(s), RNA extract from $LVCd^3$ sampled on the 7th day was sent to Stellenbosch University for cDNA library preparation. Total RNA from this sample was initially analyzed for RNA integrity scores (RIN) and quantity on the BioAnalyzer 2100 using the RNA 6000 Nano Chip and reagents (Agilent Technologies, Waldbronn, Germany) according to the protocol, G2938-90037 REV.C. The integrity and quantity of the sample were adequate for downstream applications with RIN number 6 and 89 ng/μl, respectively. The whole transcriptome sequencing was done on the Ion S5™ next-generation sequencing platform (ThermoFisher Scientific, Waltham, MA, USA). MT-2 sequence (*Lumbricus rubellus*) was used as a probe to detect the MT gene.

2.2.2. MT gene sequence alignment and phylogenetic tree construction

The MT sequence obtained was compared to other existing earthworm MT nucleotide sequences in the GenBank database with NCBI nucleotide BLAST software. The best matches were aligned with MAFFT algorithm program (Katoh et al., 2002); the L-INS-i algorithm (maximum likelihood) of the MAFFT alignment program was implemented as available on the webserver (https://mafft.cbrc.jp/alignment/server/) with an ultrafast bootstrap of 1000 replicates (Kalyaanamoorthy et al., 2017). A Phylogenetic tree was generated using the command-line version of the IQ-tree software version 1.5.5 (Nguyen et al., 2014).

2.3. Mt. gene expression

2.3.1. Optimization of RT-qPCR amplification for L. violaceous 18SrRNA and MT- genes

The specific housekeeping gene, 18SrRNA (18P2F and 18P2F) and reference gene (MTsP2F and MTsP2R) primers were designed for the species *L. violaceous*, the primers were authenticated, and conditions for amplification were optimized. The housekeeping gene, 18SrRNA nucleotide sequence from our study (accession number KY114795.1) was used to design the reference gene (18P2F; CGCGGCCGGAAATTTTACTT and 18P2R; ACCGTAATACGAATGCCCCC) while the MT nucleotide sequence (MN161784) was used to design the target gene (MTsP2F; TGTGAATTGCAAGTGCGGTG and MTsP2R; CAACTTCCTTTGGCGCA CTG). 18SrRNA genes are stable markers used in the identification of eukaryotes (Wu et al., 2015). They are used as the reference gene for monitoring gene expressions in earthworms like *Dendrobaena octaedra* (Mustonen et al., 2017). Both DNA amplifications were obtained from a final volume of 20 μl using 2 μl of DNA extract. Each mixture consisted of 10 μl Phusion High-Fidelity PCR Master Mix (ThermoFisher), 4 μl of sterile ddH^2O, and 2 μl (10 mM) of each specific forward and reverse primer. The cycling condition used for 18S rRNA gene PCR amplification was one cycle for 30s at 98 °C; 30 cycles for 10s at 98 °C, 30s at 55 °C, 30s at 72 °C; and one cycle at 72 °C for 5 mins. The cycling condition used for MT gene PCR amplification was one cycle for 30s at 98 °C; 30 cycles for 10s at 98 °C, 30s at 68 °C, 30s at 72 °C; and one cycle at 72 °C for 5 mins. The PCR products were documented with 1.5% agarose using high-resolution gel electrophoresis.

The PCR efficiencies for reference and target qPCR amplification were determined; cDNA amplification of ten-fold dilutions per sample was done with the protocol as above and their efficiencies calculated with the formula; $m = -1\ (1/\log E)$, where m is the slope of the line and E is the efficiency.

2.3.2. RT-qPCR analysis using real-time relative quantification for MT gene expression

Roche RT-PCR Lifecycler machine was used for qPCR amplification, and the relative quantification method of determining RNA concentration was used in this analysis. The specific primers of *L. violaceous* (18P2F/18P2R and MTsP2F/MTsP2R) were used to amplify the reference (18SrRNA) and target (*mt*) genes, respectively. The amplifications were obtained from a final volume of 20 μl; each mixture consisted of 10 μl Luna Universal qPCR Master Mix, 4 μl of sterile ddH_2O, 3 μl of (1/50 diluents) RNA extract and 0.5 μl each of forward and reverse primers.

The qPCR protocol conditions used for 18SrRNA gene and *Mt* gene amplification involved one cycle of 60s at 95 °C for Initial Prenaturation; 2 step cycle of 45 cycles for, 29 s at 95 °C, 30s (+ plate reading) at 60 °C, Prenaturation, 30s at 95 °C, Melt curve of 30s at 95 °C and annealing, 30s at 55 °C and 68 °C (for 18S rRNA gene and *Mt* gene respectively); and final extension of one cycle at 95 °C for 1 s. The PCR products were documented with 1.5% agarose using high-resolution gel electrophoresis.

To determine the relative expression ratio, the mathematical model

using the $\mathbf{2}^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008) was applied. Normalization of target gene expression was done to compensate for experimental reliability, which is expected during a run to run and from sample to sample. Ratios were calculated with the formula;

Fold change due to treatment $= \mathbf{2}^{-\Delta\Delta Ct} = \frac{(CT\ gene\ of\ interest - CT\ internal\ control)\ sample\ A}{(CT\ gene\ of\ interest - CT\ internal\ control)\ sample\ B}$. where sample A = Treated and sample B = Untreated.

2.4. Statistical analysis

One – way ANOVA analysis was used to compare the means of the *mt* gene expressions over the days of exposure and statistical significance was at $P < .05$.

3. Results

3.1. Methallothionein gene (Mt) and protein sequence of L. violaceous

Mt gene was detected in the earthworm species *L. violaceous* and its MT-protein sequence; MADVGTCNTKCCGKPSCPREGATCACTNCKCTKAECLPNCDKNCCASAGCGSAKCGNVNCKCGADCKCTGSPECATQCAKGSCAAK is deposited in the DDBJ/EMBL/GenBank with ascension number MN161784. The sequence contains 258 bp ORF which encodes 86 amino acids and having four Cys-X-Cys and two Cys-Cys motifs consistent with other earthworm metallothioneins. The MT- protein sequence aligned with other earthworms' MT- protein sequences and indicates the specific conserved domains (α and β) usually noticed in earthworm MT- protein sequence (Fig. 1).

3.2. Phylogenetic relationship of L. violaceous metallothionein (Mt) protein

All MT sequences used for comparison in the phylogenetic analysis were obtained from the NCBI database. The phylogenetic tree generated (Fig. 2) indicates *L.violaceous* has a common ancestor with all other earthworms' MT. All the earthworm species in this phylogenetic tree are of the sub-order Crassiclitellata, but *L. violaceous* of the Eudrilidae family shares no ancestor with any clustered group. Furthermore, the tree shows two major clusters of earthworm species and *Eisenia fetida* occurring singly. The first is a clade of the Megascoleocidae family; *Amynthas aspergilus, Metaphire carlifornia, Metaphire formosae and Metaphire paiwanna. E. Fetida* branches singly while the second cluster is a clade of *Allobophora chlorotica, Apporectodea caliginosa, Eisenia andrei, Lumbricus castaneous, Lumbricus rubellus* and *Lumbricus terristris.* All species of this cluster are of the Lumbricoidea family except *Pontoscolex corethrurus* of the Rhinodrilidae family formerly named *Lumbricus correthrurus* (Muller, 1856) and later *Pontoscolex arenicola* (Schmarda, 1861); both were grouped in the family Glossoscolecidae.

3.3. Primers authenticity and MT protein expression levels

Amplification of 18SrRNA and *L. violaceous* MT showed there were single bands observed at the expected 164 bp for 18SrRNA and 78 bp for *L. violaceous* MT (Fig. 3) when resolved under gel electrophoresis. The calculated efficiency of the reference gene qPCR amplification was 97.8%, while that of target gene qPCR amplification was 101.3%. The qPCR protocol for both primers needed no further optimization. The gene expression on the initial and third day of exposure indicated that the *mt* gene had a low expression of MT protein observed for all levels.

Of cadmium ($LVCd^1$, $LVCd^2$, and $LVCd^3$). However, there were 2-, 2.5- and 4-fold increase of the *mt*-gene expression for the Cd levels ($LVCd^1$, $LVCd^2$, and $LVCd^3$, respectively) exposed to *L. violaceous* on the seventh day and the expression by the earthworms to all these levels showed a dose-dependent response on the 7th day (Fig. 4). There was a statistical difference between the levels of gene expressions based on the days of exposure ($P = .005$).

The *mt*-gene expressions were all reduced from the first to the third day for all levels of zinc exposed to *L. violaceous*. The highest Zn level ($LVZn^3$) exposed to *L. violaceous* caused 3 fold, 0.3 fold, and 12 fold increase in *mt*-gene expression on the 1 st, 3rd and 7th day, respectively (Fig. 5). There was no statistical difference between gene expressions based on the days of exposure ($P = .3$).

4. Discussion

The nucleotide sequence of *L. violaceous* had 24.4% cysteine of its amino acid composition, which is close to 24.5% cysteine of *Lumbricus rubellus* (Stürzenbaum et al., 1998). A comparison with other earthworms MT protein sequences using NCBI BLAST tool showed the protein sequence had closest similarities with the MT-2 sequences of earthworm spp. like *Amynthus aspergillus* (ACD84578.1), *Metaphire californica* (ACD84579.1), *Metaphire formosae* (ACD84580.1), and with *Pontoscolex corethrurus* (ACD84582.1). These earthworms are not of the same family with *L. violaceous*, however, among the 50 earthworm MT nucleotide sequences (including ESTs) and the 19 earthworm MT protein sequences in the NCBI GenBank, MT gene sequence of the genus Eudriloides is absent. The MT sequence for *L. violaceous* is the first report of species from the Eudriloides family found in the GenBank.

M = 50 bp ladder, Lane 1 = 18SrRNA amplicon, Lane 3 = *MT* amplicon.

Tolerance of earthworms to metal pollution could involve the transformation of metal in earthworm guts or is attributed to the action of MT proteins expressed by earthworms under stress. This study has established the presence of the *mt* gene in *L. violaceous* species and is similar to the *mt* genes reported in other studies (Drechsel et al., 2017). Metallothioneins, when induced, serve to detoxify metals by forming clusters with them; this suggests the cellular mechanism of tolerance

Fig. 1. Alignment of *L. violaceous* MT Protein sequence with other earthworm showing four cys-X cys and two cys-cys arrangements marked with a blue box. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

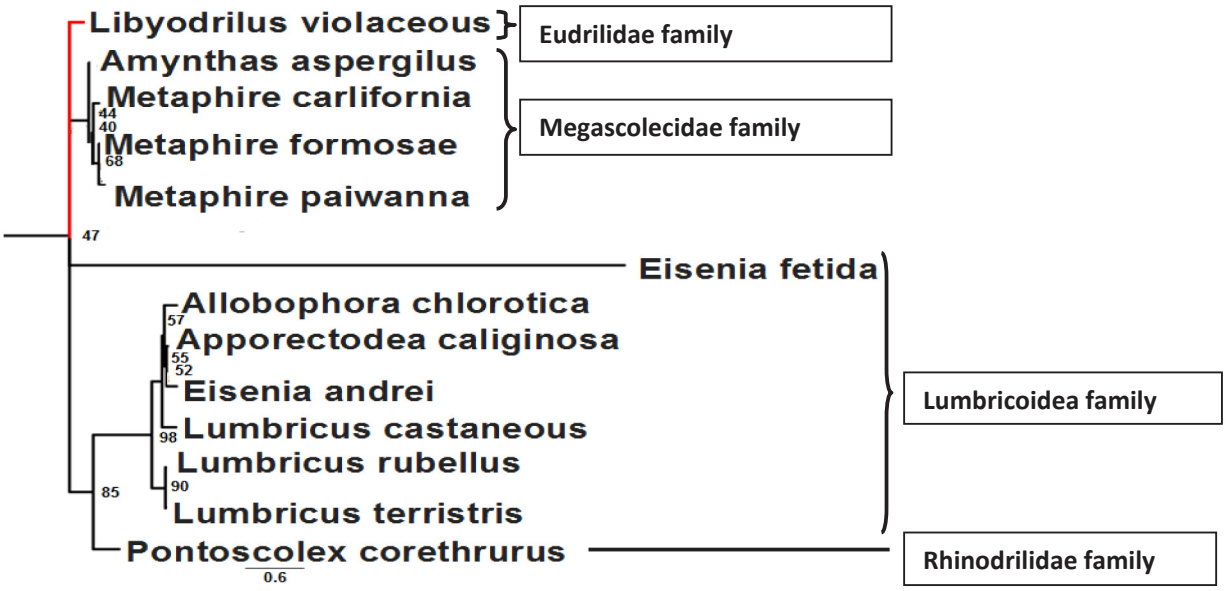

Fig. 2. Phylogenetic tree of MT protein sequence of *L. violaceous* (indicated in red) compared with some earthworm MT protein sequence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

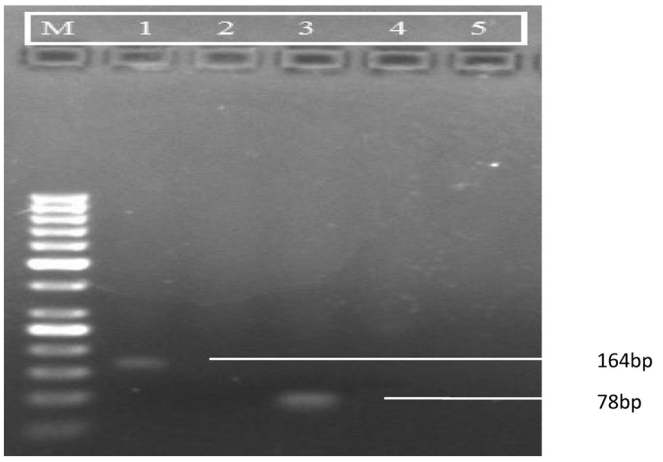

Fig. 3. Gel photo of the expected base pairs for 18SrRNA and *MT* genes of *L. violaceous.*

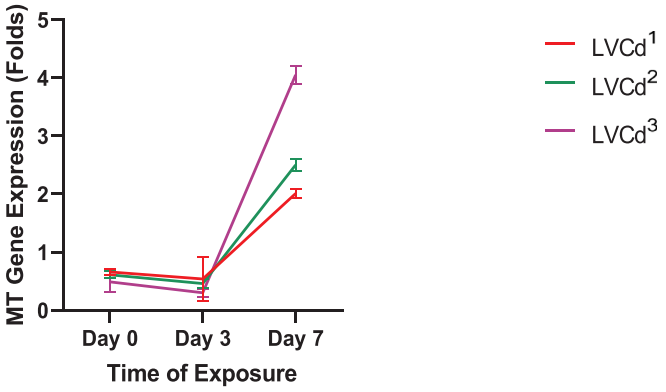

Fig. 4. MT gene expressions with exposure to levels of cadmium.

exhibited by this earthworm species. Zn, Cd or Cu ions are known to form metal-metallothionein clusters (Foster and Robinson, 2011), although the overall metal affinity is species dependent.

The MT expression throughout exposure indicated downregulation

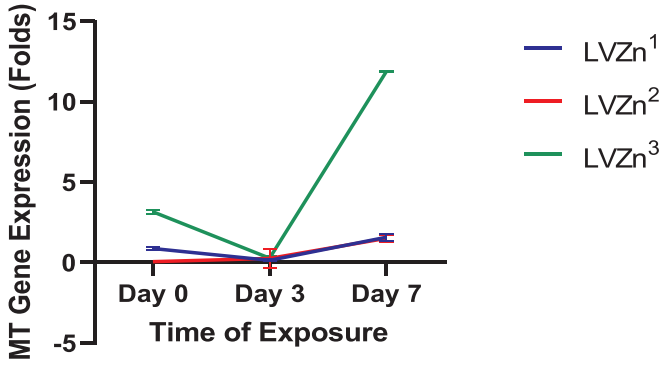

Fig. 5. MT gene expressions with exposure to levels of zinc.

of the gene initially and subsequent up-regulation. The initial down-regulation could owe to the earthworm coping with a sudden exposure to concentrations of Cd and Zn spiked in the soil. Cd caused gene expression levels in a dose-responsive manner on the seventh day. The earthworm, *L. rubellus* exposed to $CdCl_2$, had caused the expression of *wMT-2* in a dose-responsive manner (Owen et al., 2008; Hockner et al., 2015). The temporal exposure of Cd significantly ($P < .005$) caused an increase in MT gene expression with increased days (day 7) while increasing the concentrations of Cd exposed to *L. violaceous* caused no significant difference ($P > .05$) in MT gene expression. The temporal exposure of Zn showed an increase in MT gene expression only on the 7th day for LVZn3. However, this was not statistically different from the other days and compared to the other concentrations.

5. Conclusion

This study successfully detected the presence of the *mt gene* in *L. violaceous* and its protein sequence, thus contributing to the scarce sequences of African indigenous earthworms found in DDBJ/EMBL/GenBank. It is the first report on the metallothionein gene of any species from the Eudrilidae family. Cd and Zn exposed to this earthworm species could induce the expression of metallothionein, suggesting the possible mechanism of tolerance they utilize in Cd and Zn contaminated soils. We also indicated the tolerance of *L. violaceous* to Cd and Zn; hence its use in bioremediation of Cd and Zn polluted soils could be

exploited.

Declarations of competing interest

The authors have no personal or financial commitment to any organization that may inappropriately affect the preparation and publishing of this article.

CRediT authorship contribution statement

Aemere Ogunlaja:Funding acquisition, Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing.**Vikas Sharma**:Conceptualization, Methodology, Investigation.**Johnson Lin**:Supervision, Writing - review & editing.

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References

Anderson, C., Cunha, L., Sechi, P., Kille, P., Spurgeon, D., 2017. Genetic variation in populations of the earthworm, *Lumbricus rubellus*, across contaminated mine sites. BMC Genet. 18 (1), 97.

Beddard, F.E., 1891. Memoirs: on the structure of an earthworm allied to Nemertodrilus, Mich., with observations on the post-embryonic development of certain organs. J. Cell Sci. 2 (128), 539–585. https://jcs.biologists.org/content/joces/s2-32/128/539.full.pdf.

Dada, E.O., Njoku, K.L., Osuntoki, A.A., Akinola, M.O., 2013. Evaluation of the responses of a wetland, tropical earthworm to heavy metal contaminated soil. Analysis 1 (2), 47–52. https://doi.org/10.11648/j.ijema.20130102.12.

Dedeke, G.A., Owa, S.O., Olurin, K.B., Akinfe, A.O., Awotedu, O.O., 2013. Partial replacement of fish meal by earthworm meal (*Libyodrilus violaceus*) in diets for African catfish, *Clarias gariepinus*. International Journal of Fisheries and Aquaculture 5 (9), 229–233. https://doi.org/10.5897/IJFA2013. 0354.

Dedeke, G.A., Owagboriaye, F.O., Adebambo, A.O., Ademolu, K.O., 2016. Earthworm metallothionein production as biomarker of heavy metal pollution in abattoir soil. Appl. Soil Ecol. 104, 42–47.

Dedeke, G.A., Iwuchukwu, P.O., Aladesida, A.A., Afolabi, T.A., Ayanda, I.O., 2018. Impact of heavy metal bioaccumulation on antioxidant activities and DNA profile in two earthworm species and freshwater prawn from Ogun River. Sci. Total Environ. 624, 576–585.

Drechsel, V., Schauer, K., Šrut, M., Höckner, M., 2017. Regulatory plasticity of earthworm *wMT-2* gene expression. Int. J. Mol. Sci. 18 (6), 1113.

Foster, A.W., Robinson, N.J., 2011. Promiscuity and preferences of metallothioneins: the cell rules. BMC Biol. 9, 3–10.

Fouché, J., 2018. The Effect of Cadmium on Earthworms *(Eisenia andrei)* and Their Intestinal Bacteria. Doctoral dissertation. North-West University.

Hockner, M., Dallinger, R., Stürzenbaum, S.R., 2015. Metallothionein gene activation in the earthworm (*Lumbricus rubellus*). Biochem. Biophys. Res. Commun. 460 (3), 537–542.

Idowu, A.B., Edema, M.O., Adeyi, A.O., 2006. Distribution of bacteria and fungi in the earthworm *Libyodrillus violaceous* (Annelida: Oligochaeta), a native earthworm from Nigeria. Rev. Biol. Trop. 54 (1), 49–58.

Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K., von Haeseler, A., Jermiin, L.S., 2017. Model finder: fast model selection for accurate phylogenetic estimates. Nat. Methods 14 (6), 587.

Katoh, K., Misawa, K., Kuma, K.I., Miyata, T., 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 30 (14), 3059–3066.

Lourenço, J., Pereira, R.O., Silva, A.C., Morgado, J.M., Carvalho, F.P., Oliveira, J.M., Malta, M.P., Paiva, A.A., Mendo, S.A., Gonçalves, F.J., 2011. Genotoxic endpoints in the earthworms sub-lethal assay to evaluate natural soils contaminated by metals and radionuclides. J. Hazard. Mater. 186, 788–795.

Margoshes, M., Vallee, B.L., 1957. A cadmium protein from equine kidney cortex. J. Am. Chem. Soc. 79 (17), 4813–4814.

Mustonen, M., Haimi, J., Kesäniemi, J., Högmander, H., Knott, K.E., 2017. Variation in gene expression within clones of the earthworm *Dendrobaena octaedra*. PLoS One 12 (4), e0174960.

Nguyen, L.T., Schmidt, H.A., von Haeseler, A., Minh, B.Q., 2014. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol. Biol. Evol. 32 (1), 268–274.

Ogunlaja, A., 2014. Earthworm ecology, heavy metals and total petroleum hydrocarbon assessment of an oil spill site in Agaye, Lagos State, Nigeria (Doctoral dissertation). http://ir.library.ui.edu.ng/handle/123456789/193.

Ogunlaja, A., Morenikeji, O.A., 2013. Species diversity and population size of earthworms after oil spillage in a pipeline vandalized area in Lagos State. Nigeria, African Journal of Agricultural Research 8 (8), 671–679. https://doi.org/10.5897/AJAR12.870.

Okoye, C.O., Chiejina, C.O., Ezeorah, C.C., Okeke, J.J., Ezeonyejiaku, C.D., Okonkwo, C.J., 2019. Diversity of earthworms in Nnamdi Azikiwe University, Awka. Annals of Science and Technology 12, 1 Nov. (ahead-of-print).

Owen, J, Hedley, BA, Svendsen, C, Wren, J, Jonker, MJ, Hankard, PK, ... Blaxter, ML, 2008. Transcriptome profiling of developmental and xenobiotic responses in a keystone soil animal, the oligochaete annelid *Lumbricus rubellus*. Bmc Genomics 9 (1), 266–275.

Ruttkay-Nedecky, B., Nejdl, L., Gumulec, J., Zitka, O., Masarik, M., Eckschlager, T., Stiborova, M., Adam, V., Kizek, R., 2013. The role of metallothionein in oxidative stress. Int. J. Mol. Sci. 14 (3), 6044–6066.

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C T method. Nat. Protoc. 3 (6), 1101.

Siscar, R., Koenig, S., Torreblanca, A., Solé, M., 2014. The role of metallothionein and selenium in metal detoxification in the liver of deep-sea fish from the NW Mediterranean Sea. Sci. Total Environ. 466, 898–905.

Slavík, J., Trnková, L., Hubálek, J., 2019. Interaction of selenite with metallothionein studied by Brdička reaction. Monatshefte für Chemie-Chemical Monthly 150 (3), 469–475.

Stürzenbaum, S.R., Kille, P., Morgan, A.J., 1998. The identification, cloning and characterization of earthworm metallothionein. FEBS Lett. 431 (3), 437–442.

Świątek, Z.M., Woźnicka, O., Bednarska, A.J., 2020. Unravelling the ZnO-NPs mechanistic pathway: cellular changes and altered morphology in the gastrointestinal tract of the earthworm *Eisenia andrei*. Ecotoxicol. Environ. Saf. 196, 110532.

Wang, C., Zhang, F., Cao, W., Wang, J., 2014. The identification of metallothionein in rare minnow (*Gobiocypris rarus*) and its expression following heavy metal exposure. Environ. Toxicol. Pharmacol. 37 (3), 1283–1291.

Wu, S., Xiong, J., Yu, Y., 2015. Taxonomic resolutions based on 18S rRNA genes: a case study of subclass copepod. PLoS One 10 (6), e0131498.