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Application of Pb isotopes to track the sources and routes of metal uptake in the earthworm *Eisenia fetida*

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Abstract

The aim of this work is to determine the important routes of metal uptake in earthworms to enable a better understanding of the primary source of metal uptake in the environment. Earthworms can take up chemicals from pore water and soil both by ingestion and through contact with their skin. However, it is unclear which pathway is the most important for metal uptake. An experiment was designed in which both soil chemistry and foods were artificially manipulated, producing different pools of soil lead (Pb) with different isotope compositions at a range of Pb concentrations. Earthworms (*Eiseniafetida*) were exposed to different lead concentrations through the addition of 500 mg/kg lead oxide (Pb_3O_4) to soil and 500 mg/kg lead nitrate to food (manure), with distinctly different isotopic compositions. Earthworms were also exposed to combinations of soil only and soil plus food in order to quantify the proportions of Pb taken up from each component. After acid digestion of the earthworm tissues, the Pb isotope composition of the accumulated lead in the earthworms was measured using a Thermo-fisher, iCAPQ, ICP-MS for $^{208}Pb/^{206}Pb$ and $^{207}Pb/^{206}Pb$ ratios measured relative to NIST SRM 981, allowing us to determine the pathway of lead uptake. Mixing calculations have been used to deconvolute the lead isotope signatures and identify the amount of lead taken up by the earthworms from the different soil pools. Differences in bioaccumulation factors and the relative amounts of lead accumulated from different pools changes as a function of concentration in the different pools. Earthworms were shown to uptake lead from bothsoil and food sources through ingestion route. Our findings suggest that a major pathway of lead uptake in earthworm species is heavily influenced by their ecology.

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Introduction

Earthworms are a primary part of the soil system and represent considerable ratio of soil biomass. They may be beneficial indicators of soil health and quality (Edwards, 2004). Organism tissues can be take up metal via the skin route directly or through the ingestion route (Lanno et al., 2004). This pathway might have a link with feeding activity (Jager, 2004). The activity may be difficult to observe because it is under the surface of soil (Jager et al., 2003b). Most earthworm species feed on soil particles (Doube et al., 1997b). However, determination of the condition is made very complex due to the absence of many examples. Firstly, earthworms can fit to choose a special part of the soil which is more organic than bulk soil (Bolton and Phillipson, 1976). Indeed, food selection is probably depends onspecific species (Piearce, 1978). Secondly, the majority of earthworm groups prefer food sources which are high in quality such as manure or litter. Although the preferred food is present, the difference will still comprise a large amount of soil particles

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(Barley, 1958; Hendriksen, 1991). Number of studies have measured ingestion route as an important route of metal uptake in earthworms (Becquer et al., 2005; Hobbelen et al., 2006; Morgan et al., 2004; Viarengo et al., 2014). On the other hand, route and source of ingestion that uptakemetal is still not fully understood in earthworm species. The epigeic species (*Eisenia fetida*) prefers to habit on soil surface and used commonly in ecotoxicological studies (Brulle et al., 2006; Dymond et al., 1997). Our objectives are to highlight the ingestion route of metal absorption in earthwormsepigeic and determining the main source of this route.

Material and Methods

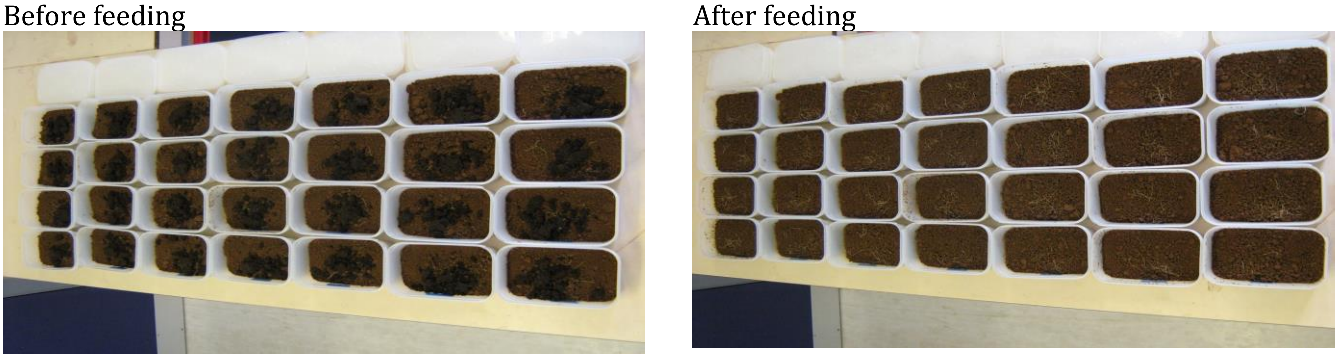
The earthworm species *E. fetida*hasbeen used and adopted as a standard laboratory test organism (OECD, 2009) because this species is easy to culture and propagate in laboratory conditions; we have adopted this species in our experiment. For experimental use, earthworms were provided by Blades Biological Limited (Edenbridge, UK). Worms *E. fetida* were acclimatised in laboratory culture boxes (54 cm × 38 cm × 29 cm), comprising sterilised Kettering loam and Irish peat moss at a ratio of 70:30; (vol:vol) at 20°C for a two-week period. For the experiment, Kettering loam soil was air-dried overnight, sieved to< 2mm, and 500 g of soil was placed into plastic boxes (18 cm × 13 cm × 17 cm). Enough ultra-pure water was added to the soil to provide moisture with content of 60% of the water holding capacity. For soil treatment, Lead oxide (Pb_3O_4) was added as a solid mineral to the soil to give a Pb concentration of 500mg/kg.Lead acetate and lead oxide were added in combination to the soil in one treatment. For food treatment, lead nitrate was dissolved in ultra-pure water and added to horse manure to give a total Pb concentration of 500 and 750 mg/kg (Table 1).

Table 1. The total concentration of lead used in the experiment.

Treatments	Lead acetate in soil (mg/kg)	Lead nitrate in food (mg/kg)	Lead oxide (Pb_3O_4) in soil (mg/kg)
Control	-	-	-
Soil 1	-	-	500
Soil 1 + Soil 2	500	-	500
Manure 1	-	500	-
Soil + Manure 1	-	500	500
Manure 2	-	750	-
Soil + Manure 2	-	750	500

Ten earthworms were added to each container with four replicates of each treatment undertaken and incubated at a temperature of 20°C. During the experiment, earthworms were fed with the horse manure as food source. Approximately 5g of food was added once a week (Figure 1). For each treatment, soil pH was measured at the end of each experiment. Earthworms were exposed to different concentrations of lead oxide and nitrate for 28 days. The following treatments were used in this experiment: (i) control (soil without treatment), (ii) soil treated with 500 ppm lead oxide, (iii) manure treated with 500 ppm lead nitrate in un-spiked soil, (iv) soil+manure 1 treated with 500 ppm lead oxide: 500 ppm lead nitrate, (v) manure treated with 750 ppm lead nitratein un-spiked soiland (vi) soil+manure 2 treated with 500 ppm lead oxide: 750 ppm lead nitrate.(vii)soil1+soil2 with 500ppm Pb- oxide and 500 ppm Pb-acetate.

Before feeding After feeding



S1+S2 S+M2 Manure2 S+M1 Manure1 Soil Control

Figure 1. Laboratory experiment layout showing the effect of different soil and food treatments on earthworms feeding according to the application of different concentrations of lead

At the end of the experiment, the earthworms were collected and placed on Whatman No. 540 filter paper (moistened with ultra-pure water) in a petri dish at 20^{o}C for 48 hours to void them of soil. The filter paper was changed every 12 hours to prevent coprophagy. The earthworms were then placed into clean tubes and kept at –18^{o}C until analysis. Soil pH was routinely measured for each treatment in the experiment.

Prior to analysis, the earthworms were dried on Whatman No. 540 filter paper at 40^{o}C for 24 hours. Then earthworms were weighed and each one cut into small pieces and placed into Kjeldahl digestion tubes. Nitric acid (10ml) was added to each of the tubes, which were then covered with small glass bell covers. The digested samples were transferred into 100 ml volumetric flasks and filtered through Whatman filter paper. The obtained samples were diluted with ultra-pure water for the analysis by ICP-OES. The analysis procedure was calibrated with the use of a certified reference material (China National Analysis Centre, Bushes and leaves, NCS-DC73349). The certified value was 47 mg/kg and the average of the analysed sub-samples was 48.12mg/kg with proportion 102%.

Measuring lead isotopes

The lead nitrate and lead acetate were selected because they could be easily dissolved in water for mixture with food and soil. These lead compounds have different isotopic values (Table 2) which made them suitable for a combined experiment. The Pb isotope values were measured on a Thermo-fisher iCAPQ ICP-MS in isotope ratio mode. The sample solutions were diluted until they were in the range of the instrument (typically 4-5 μg/L) and measured ten times each and averaged. The samples were run in a standard-sample-standard bracket configuration in order to correct for any instrumental drift during the run using NIST SRM 981 (Common Lead Isotope Standard), which was found to be less than 1% for both ratios.

Table 2. The value of lead isotopes used in the experiment.

Source of lead	$^{208}Pb/^{206}Pb$	$^{207}Pb/^{206}Pb$	Pb concentration (mg/kg)
Pb nitrate (manure)	2.107 +/- 0.004	0.864 +/- 0.005	500/750
Pb acetate (soil)	2.096+/- 0.004	0.865 +/- 0.005	500
Pboxide (soil)	2.316 +/- 0.006	1.130 +/- 0.006	500

Statistical analysis

Statistical software Minitab (version 16th) was used to analysis all experimental data. Equal variance and normality data were measured to test significant differences in changes in body weight during the experiment period. The one-way ANOVA test was used to identify the significant differences of metal uptake between treatments.

Results and Discussion

Accumulation and soil pH

Earthworms were accumulated lead in their tissue at all treatments (Figure 2). There were significant differences ($P < 0.001$) between treatments in accumulation rate of Pb. Manure treatment (8.36 mg/kg) was higher Pb concentration than soil treatment (5.55 mg/kg). The highest absorption of Pb was observed in Soil + Manure treatment (500:750ppm) (17.68 mg/kg) comparing to control. Values of Pb accumulation were obtained in Manure (750ppm) (10.51 mg/kg), Soil + Manure (500:500ppm) (12.26 mg/kg) and Soil1 + Soil2 (500:500 ppm) (14.77 mg/kg). Food sources (1.76%) had a higher proportion of Pb uptake than soil sources (1.11%) through the ingestion route (Figure 3).

The value of pH was changed in treatments from the beginning and the end of experiment period. After 28days, soil pH was reduced in all treatments (Figure 4).

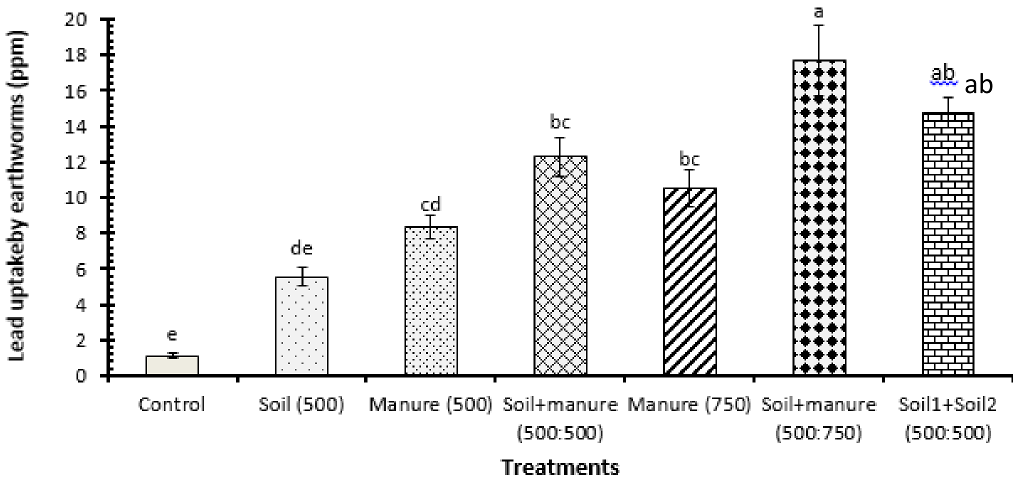


Figure 2. Mean metal accumulation in *E. fetida*tissues using different concentrations of Pb after 28 days of incubation. Bars with different letters show treatments that are significantly different ($p < 0.001$) from one to another, ($n = 4$).

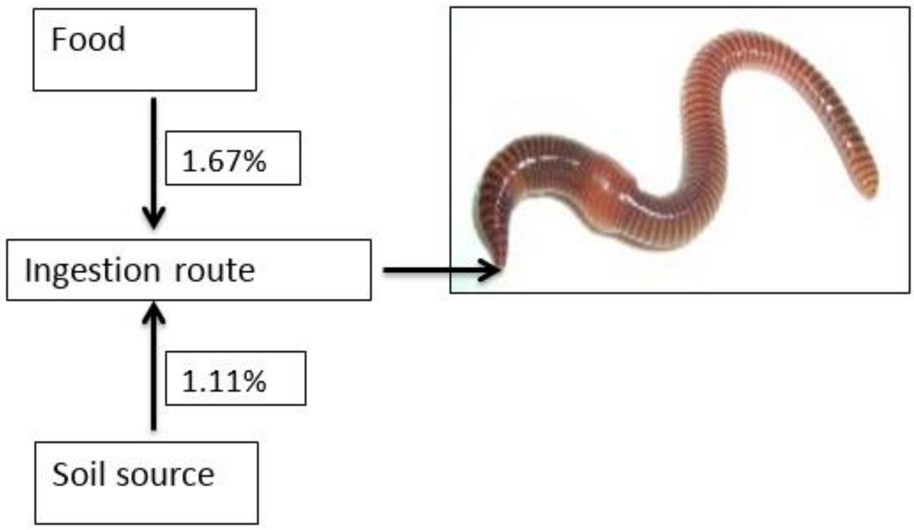


Figure 3. Proportion of Pb accumulation from the ingestion route in E. fetida tissues.

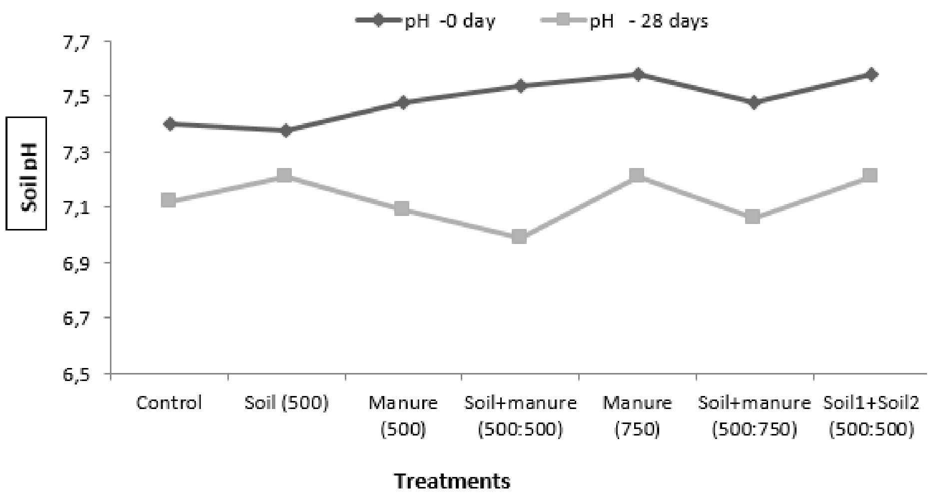


Figure 4. Mean of soil pH as an effect of applying *E. fetida*earthworms to soil-lead treatments at the start and end of the experiment.

Weight change

Body weight of earthworms was recorded from day 0 to day 28 of the experiment (Figure 5). Earthworms weight were increased in all treatments. A significant difference of body weight ($P < 0.05$) was showed between treatments during the experiment period. The higher body weight of earthworms was found in Soil1 + Soil2 treatment (0.49g) and Soil + Manure (500:500 ppm) (0.48g). The body weight was lowest at the end of the experiment for the Soil treatment (0.39g).

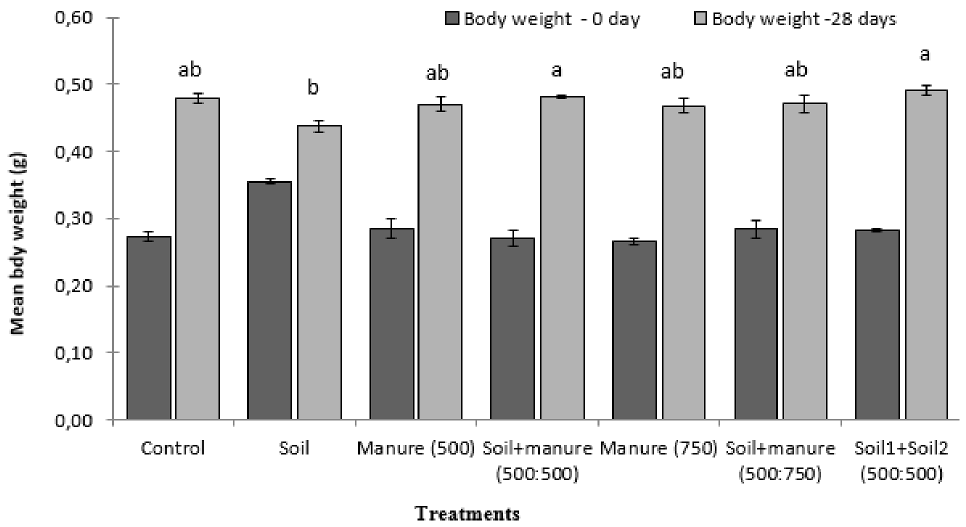


Figure 5. Mean body weight of earthworms*E. fetida*at day 0 and day 28 after exposure to lead concentrations. Error bars are standard errors, (n = 4). Bars with different letters show treatments that are significantly different($P < 0.05$) from one to another.

Lead isotope ratios

The rate of Pb isotopes of ($^{207}Pb/^{206}Pb$) and ($^{208}Pb/^{206}Pb$) was observed in all treatments. As shown in Figure 6, the Pb isotope value ($^{208}Pb/^{206}Pb$) of the manure treatment was different than the soil treatment. Comparing pools with treatments, it was found that the Pb isotope values ($^{208}Pb/^{206}Pb$) in combined treatments Soil + Manure (500:500ppm) and (500:750ppm) as well as Soil1 + Soil2 (500:500ppm) resulted in being situated in the middle between the soil and manure treatments. For ($^{208}Pb/^{206}Pb$), the correlation relationship of Pb uptake was observed. It was found that the highest Pb accumulation with the Pb isotope was shown in the Soil + Manure (500:750ppm) treatment.

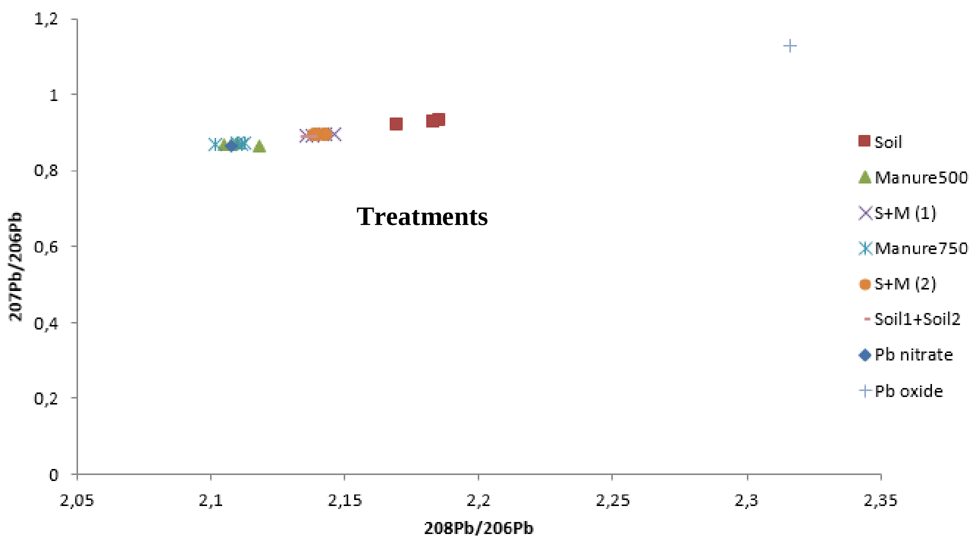


Figure 6. Lead isotopic ratios ($^{207}Pb/^{206}Pb$ and $^{208}Pb/^{206}Pb$) showing the isotopic compositions of different Pb sources in earthworm tissue.

The impact of metal bioavailability depends on many factors which include soil properties (Spurgeon and Hopkin, 1999; Van Gestel, 2008; Van Gestel and Mol, 2003). Soil pH change is a better signal of metal bioavailability (Little, 2011). In this work, we showed that the level of pH was decreased in the soil particles by time during the 28 days of experimental period. This reduction in the soil pH is probablybecause of the activity of earthworms. Other scientists found that earthworms activity can decrease the value of pH in the soil (Sizmur et al., 2011). The reduction in pH might be associated with metal in the soil to be more mobile form which may be a guide to uptake by earthworms (Lukkari, 2004). This finding is in agreement with Peijnenburg et al. (1999a) who showed that soil pH had positively correlated with the values of Pb and Cd uptake in the earthworm Eiseniaandrei. Also, Hobbelen et al. (2006) suggested the existence of higher amounts of Pb and Cduptaken by earthworms in low pH soil environment when measured by chemical methods. The reason for increasing body weight of earthworms may be the food provided in the soil surface on weekly interval. Significant differences in weight change in the treatments occurred mostly because of the different types of lead compounds in the soil.

Our results found that the earthworms *E.fetida* take up Pb through the skin and ingestion routes in $Soil_1$ + $Soil_2$ treatment. This probably because there are two different sources of Pb compounds in soil, Pb nitrate was soluble in water and Pb oxide was insoluble.

The findings of this study indicate that the Pb isotope may reflect feeding sources. Earthworms *E.fetida* absorbed Pb from both soil and foodsources. The food source can be one part of Pb uptake in this earthworms group. The possible explanation for some of our results may be that earthworms resort to take up more chemicals from the soil because of earthworms physiology and behaviour (Jager, 2003). *E.fetida* is an epigeic earthworm which can be divided according to feeding habits to detritivores. Detritivores earthworms eat near the soil surface on organic matter (Lee, 1985b; Viljoen and Reinecke, 1992). Another source for the uptake of lead in earthworm tissues is the soil source. It seems possible that the reported results are due to the behaviour of these species. Epigeic earthworms may burrow a hole in soil to shelter from predators (Dunger, 1983). The burrowing technique of earthworms in soil is made by pushing soil particles aside or passing them through the ingestion pathway (Lee and Foster, 1991). Another possible explanation for this is feeding behaviour. Feeding behaviour of earthworms alternates between manure and soil source because they cannot eat continuously on a mix of manure and soil (Jager et al., 2003a). This finding is in agreement with Spurgeon and Hopkin (1999) who showed that Pb and Cd concentration from soil contaminates were increased in *E.fetida* after 21 days. Moreover, lead accumulation in earthworms tissue had a strong correlation with total lead concentration in soil (Spurgeon and Hopkin, 1999).

The most interesting finding was that earthworms *E.fetida* absorb Pb via ingestion route. The possible reason for that can be food type. Horse manure was added to soil surface as a food source. Earthworms highly prefer cattle manure because it is attracting and nourishment type of food source (Barley, 1958; Marhan and Scheu, 2005b; Lowe and Butt, 2005). The feeding activity might be high in these species. Rate of feeding can be dependent on factors such as food quality, palatability, earthworms activity and favourableness of environmental conditions (Curry and Schmidt, 2007). Another reason may be the chemical form which is added to the soil. Lead oxide (Pb_3O_4) is a lead compounds which is insoluble in water (Csuros and Csuros, 2002). Chemicals that have low solubility with water can be taken up through ingestion route (Leppänen and Kukkonen, 1998). The present findings seem to be consistent with other research finding which found that the ingestion route may be the primary route to take up hydrophobic chemicals when earthworms *E.andrei* exposed to (B(a)P) and (TCDD) in soil by the immunohistochemical method (Viarengo et al., 2014). Furthermore, Jager et al. (2003a) reported that the PCB153 chemical(which is less soluble) was accumulated in *E.andrei* by ingestion route. This confirmed by Belfroid et al. (1994a) who showed that ingestion route of *E.andrei* dominate the uptake from food that was contaminated by chemicals (PCB 153) and (OCN).

Conclusion

The purpose of the current study was to determine the importance of ingestion pathway as the main source of metal uptake in *E. fetida* earthworms. This study found that there is a high correlation between ingestion pathway and feeding activity. The evidence from this study suggests that the discovery of the chemical form as the pathway for take up the metal. In conclusion, ingestion pathway could be major pathway to uptake metal in epigeic earthworms from soil and food sources.

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