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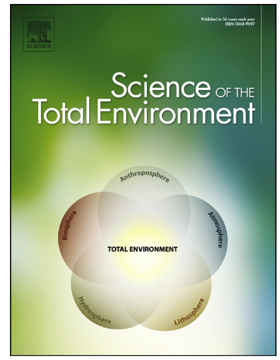

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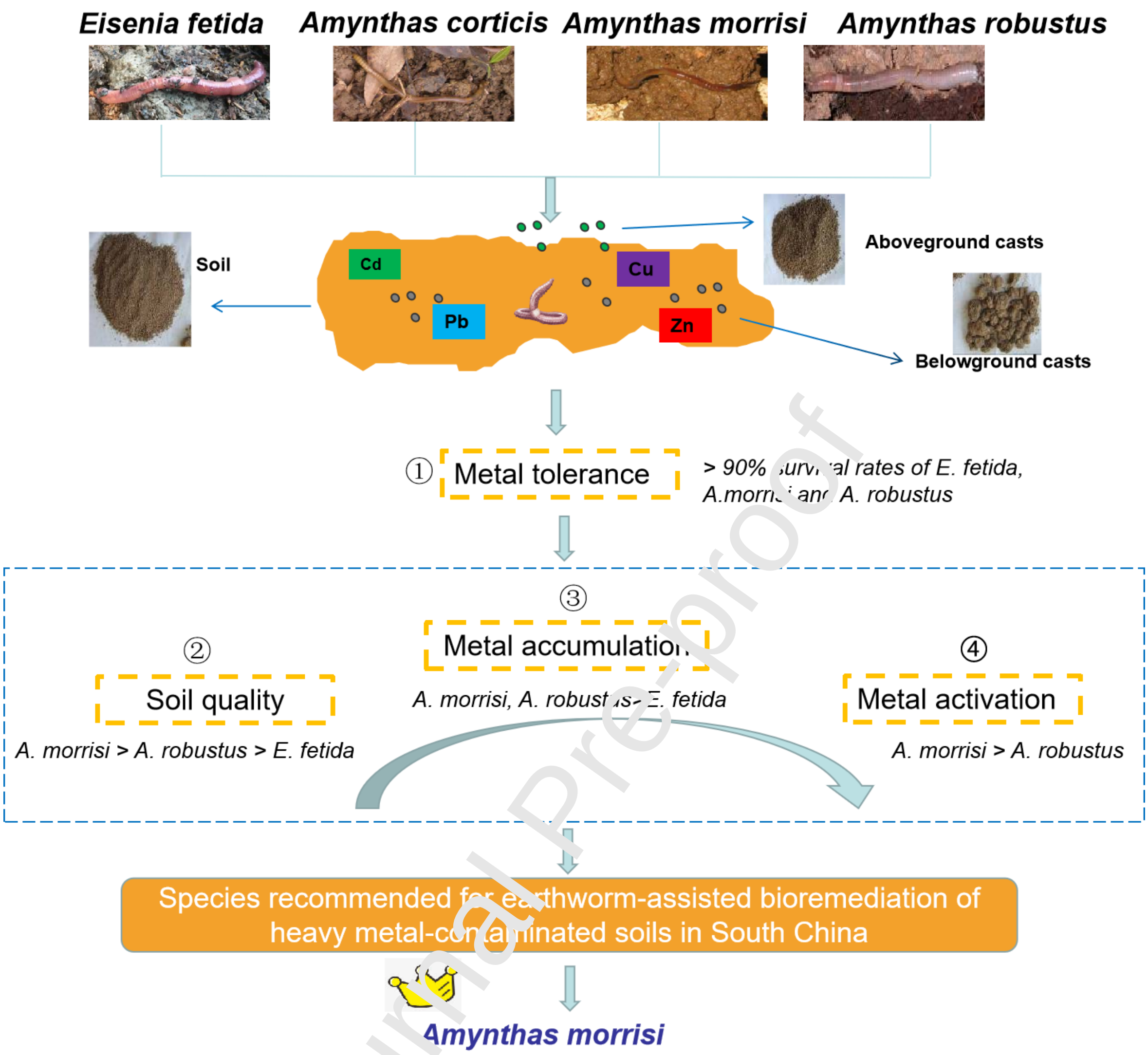
Abstract:

This study was aimed to evaluate the potential of four earthworm species commonly found in South China for the bioremediation of soils contaminated by Cu, Cd, Pb and Zn. Survival rates and metal accumulation of *Eisenia fetida*, *Amynthas morrisi*, *A. robustus* and *A. corticis* and changes in soil physico-chemical properties were investigated in a 60-day incubation experiment with a metal-polluted soil. At the end of the experiment, the survival rates of *E. fetida*, *A. morrisi* and *A. robustus* were significantly higher than that of *A. corticis*. Principal component analysis showed that earthworm activity improved soil quality with the averaging soil quality index being 0.66, 0.64, 0.56, 0.53, and 0.12 for the *A. corticis*, *A. morrisi*, *A. robustus*, *E. fetida*, and control treatments, respectively. The highest total available Cd, Cu, and Pb in casts were found in the treatment with *A. morrisi*, and this species accumulated the smallest amount of metals. Results indicate that *A. morrisi* may be the best candidate for earthworm-assisted bioremediation of metal contaminated soils in South China.

Keywords: Earthworm species; Metals; Accumulation; Availability; Soil quality

GRAPHICAL ABSTRACT



Highlights:

High survival rates of *E. fetida*, *A. morrisi*, and *A. robustus* were found in a soil polluted by multiple metals (Cu, Cd, Pb and Zn).

Native earthworm species showed high metal accumulation and improved soil quality.

The casts of epigeic and epi-endogeic species had higher available metal concentrations than those of endogeic species.

The native species *A. morrisi* is recommended for earthworm-assisted bioremediation of metal-polluted soils in South China.

1. Introduction

Earthworms, known as soil ecosystem engineers, have drawn more attention than ever before in soil quality studies (Lavelle et al., 2016; Guerra *et al*., 2021). They not only influence soil organic matter dynamics, nutrient release, and plant growth (Lavelle and Spain, 2001; Lavelle *et al*., 2020), but also play a vital role in the biogeochemical cycling of trace elements (Sizmur and Richardson, 2020). Due to their important role in numerous ecological functions, earthworms have been used in ecological restoration of soils degraded by intensive tillage or excessive agrochemical inputs such as chemical fertilizers (Bedano *et al*., 2019; Fonte *et al*., 2019; Noguera *et al*., 2010). However, there is limited research on the potential of earthworms in bioremediation of soils contaminated by potentially toxic elements such as metal(loid)s (Zhang *et al*., 2020; Sizmur and Richardson, 2020).

So far, many studies have been conducted on the exposure of earthworms to metals. Some studies focused on the metal tolerance of earthworm species. For instance, Nahmani et al. (2007) investigated the tolerance of *Eisenia fetida* and some European earthworm species (e.g., *Lumbricus rubellus*, *Lumbricus castaneus*, *Lumbricus terrestris*, *Lumbricus friendi*, *Aporrectodea rosea*, *Aporrectodea longa*, *Aporrectodea caliginosa* and *Allolobophora chlorotica*) to metal contaminations. Other studies focused on metal accumulation by earthworms (Dai *et al*., 2004; Nahmani *et al*., 2007; Coelho *et al*., 2018; Xiao *et al*., 2020) or metal mobilization and activation in earthworm-inhabited soil and plant uptake due to passage through the earthworm gut and change in microenvironment (Mondal *et al*., 2019; Ji et al., 2020; Sizmur and Richardson, 2020). However, the key ecological functions of earthworms, such as those associated with C and N cycling, soil structure changes, and microbial activity, have seldom been investigated in these studies (Velasquez et al., 2007; Li et al., 2009a; Dominguez-Haydar et al., 2019).

Therefore, a more integrated approach is needed to understand the influence of earthworms on the quality improvement of metal-contaminated soils. This is especially important in China where 16.1% of arable land is polluted by heavy metals (Meng, 2014). Earthworm-assisted bioremediation technologies have been proposed for metal-contaminated soils due to their low cost, environmentally friendliness, and sustainability (Zhang *et al*., 2018, 2020). Earthworm, as the stimulators of phytoremediation processes, could tolerate rather high amounts of contamination, enhance metal bioavailability and improve plants growth (Zhang *et al*., 2020). However, the tolerance of Asian earthworm species to metals and their influence on metal dynamics and soil quality still remain unknown (Xiao *et al*., 2020). Therefore, our present study was aimed to evaluate the metal tolerance and accumulation of four earthworm species commonly found in China and to assess their influence on soil quality as well.

2. Materials and methods

2.1 Experimental design

Surface soil samples (0–20 cm) were collected from a paddy field (24°30′ N, 113°45′ E) contaminated with Cu, Cd, Pb and Zn in the upstream region of Yang He River near the Dabaoshan opencast mine, Guangdong Province, China. Soils in our study is a granite based soil with low organic C and N contents and acidic pH value but distributed widely in South China (Lu, 2017). Low microbial activity and metal availability in polluted soil were also found in this region (Li *et al*., 2009ab). Samples were homogenized and sieved to 2 mm after air-drying. Soil pH value, contents in total organic C, N and clay-sized particles (<2 μm) and the C:N ratio were 4.18 g kg^{-1} ±0.03, 15.3 ±0.23 g kg^{-1}, 1.47 ±0.05 g kg^{-1}, 33.6% ±1.0 and 10. 4±0.22, respectively. Total Cu, Cd, Pb and Zn concentrations were 394 ±10.2, 0.639± 0.06, 439±8.44 and 405±29.0 mg kg-1, respectively.

Coco coir and cattle dung were purchased from a flower market in Guangzhou, China. Coco coir and cattle dung were air-dried, ground, and sieved to 2 mm. Total organic C, total N, and C/N ratio were 50.3%, 0.66%, and 76.0, respectively, for coco coir and 15.7%, 0.62%, and 25.4, respectively, for cattle dung. Contents of total Cd，Pb，Cu and Zn reached 0.8, 0.0, 16.2 and 123.7 mg kg^{-1} for cattle dung and 0.7, 0.0, 0.0 and 50.7 mg kg^{-1} for coco bran, respectively. These concentrations were lower than the national quality standard for organic fertilizer of China (NY525-2012). Coco coir and cattle dung were mixed at 4:1 (w/w) for later use as an organic amendment (pH 6.8).

Four earthworm species commonly found in South China were used in this study. *Eisenia fetida* (Savigny, 1826), the reference species of several international standard toxicity tests, was collected from a local vermicompost operation. *Amynthas corticis* (Kinberg, 1867), *A. morrisi* (Beddard, 1892), and *A. robustus* (Perrier, 1872), native species in Asia, were collected from uncontaminated soils in Guangdong Province, China. Of the four, *E. fetida* and *A. corticis* are epigeic species, whereas *A. morrisi* and *A. robustus* are epi-endogeic and endogeic, respectively. The earthworms were cultured in an uncontaminated soil with organic matter addition under suitable moisture and temperature conditions for one month before use.

The soil and coco coir/cattle dung mixture were mixed thoroughly at 9:1 (w/w) and added to pots (5 cm lower diameter, 11 cm upper diameter, 13.5 cm height). Then, five treatments were set up in triplicates: control, EF, AC, AM, and AR. In control, no earthworm was added, whereas in EF, AC, AM, and AR, approximately 15 g of healthy adult *E. fetida* (averaging 0.3 g $individual^{-1}$), *A. corticis* (averaging 0.4 g $individual^{-1}$), *A. morrisi* (averaging 0.4 g $individual^{-1}$), and *A. robustus* (averaging 1.5 g $individual^{-1}$) with clitella were added, respectively. During the experiment, soil moisture was maintained at field capacity and temperature at 25°C. After 60 days of incubation, earthworms in each pot were collected, counted, weighed, and placed on moist filter papers in Petri dishes to allow for excretion of contaminated soil from their guts at 25^{o}C for 7 days. Then, earthworms were killed in liquid nitrogen and oven-dried at 100^{o}C for 24 h. Aboveground and belowground casts and non ingested soil were manually separated, air-dried, weighed, ground, and sieved to 2 (for determination of pH, enzyme activities, and available metals) and 0.149 mm (for determination of organic C, total N, and total metals) based on the Chinese state environmental protection standards.

2.2 Laboratory analyses

The soil pH was measured using a 1:2.5 solid-to-water ratio. The organic C content was determined by the dichromate digestion method, and total N was determined by Kjeldahl digestion (Sparks, 1996).

Total Cu, Zn, Pb, and Cd concentrations were determined after digestion with HF, HNO_3, and $HClO_4$ (Amacher, 1996). Available Cu, Zn, Pb, and Cd were extracted with diethylene triamine penta acetic acid (DTPA) (Lindsay and Norvell, 1978) and determined by atomic absorption spectrophotometry (AAS).

Soil enzyme activities were considered biological indicators of soil quality (Dick et al., 1996). β-glucosidase and N-acetyl-β-D-glucosaminidase, acid and alkaline phosphatase activities involved in soil organic C, N and P cycles and microbial activities (Mora et al., 2005; Li *et al*., 2009a). 2 grams of soil or earthworm casts were moistened with 0.5 mL distilled water and incubated during 48 h at 25℃, to which 3 mL of water were added to obtain soil solution. Activities of acid phosphatase and alkaline phosphatase were determined at pH 5 (citrate buffer) and 9 (borate buffer), respectively, using *p*-nitrophenyl phosphate as substrate (Mora *et al.*, 2005). Activities of β-glucosidase and N-acetyl-β-D-glucosaminidase were determined at pH 5 (phosphate buffer) using *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-N-acetyl-β-D-glucosaminide as substrate, respectively (Mora *et al.*, 2005). Overall enzyme activity was evaluated with fluorescein diacetate (FDA) hydrolytic activity which was related with total microbial activities (Schnvrer and Rosswall, 1982).

Metal (Zn, Cd, Cu, and Pb) concentrations of earthworms were analyzed as described by Dai *et al.* (2004). Earthworm responses to metal pollution were evaluated with survival rate and the biota-to-soil accumulation factor (BSAF). The survival rate I_1 was calculated as:

$$I_1 = A_{60}/A_0 \times 100\%$$

where A_0 and A_{60} are earthworm number at the beginning (day 0) and end (day 60) of the experiment, respectively.

BSAF was calculated as:

$$BSAF = M_{worm}/M_{soil}$$

where M_{worm} and M_{soil} are metal concentrations of earthworm and soil, respectively.

Cast production was the sum of aboveground casts and belowground casts.

2.3 Statistical analyses

Data were analyzed using one-way analysis of variance (ANOVA) in SAS 8.0 (SAS Institute Inc., Cary, NC, USA). Duncan's test was used to analyze the differences between treatments and the significance level was set at $P < 0.05$. Multivariate analyses, including principal component analysis (PCA) and co-inertia analysis (CIA), were performed using the ADE-4 package in R (R Development Core Team, 2009; Thioulouse et al., 1997). A Monte Carlo permutation test was performed to check the significance of the co-inertia using the ADE-4 package as well. Quality indices of aboveground casts, belowground casts, and non ingested soil were calculated using the methodology proposed by Velasquez *et al*. (2007). The pH, organic C and total N contents, cast production, β-glucosidase and N-acetyl-β-D-glucosaminidase, acid and alkaline phosphatase activities and FDA hydrolytic activity, which are associated with C, N and P cycling, soil structure changes, and microbial activity, were chosen as soil quality variables in this study. For the first three axes of the PCA (F2 and F3 were very close in their contributions to the total variance), the variables with the highest contributions were selected. The values of each variable set, adjusted to a range of 0.1 to 1.0 via homothetic transformation, were multiplied by their respective weight factors and summed, giving soil/casts quality index I as:

$$I = F1 \times (\alpha_1 a + \beta_1 b + \gamma_1 c) + F2 \times (\alpha_2 a + \beta_2 b + \gamma_2 c) + F3 \times (\alpha_3 a + \beta_3 b + \gamma_3 c)$$

where F1, F2 and F3 are the percentages of variance explained by the PCA on axis 1, axis 2 and axis 3, respectively, α, β, and γ represent the contributions of the variables to the formation of their respective axes, and a, b, and c are the values of the selected variables on their corresponding axes (Velasquez *et al*., 2007; Rodriguez *et al*., 2021).

3. Results

3.1 Earthworm survival rates and metal accumulation

The survival rates of *E. fetida*, *A. morrisi*, and *A. robustus* reached 94.8, 115, and 99.7%, respectively, significantly higher than that of *A. corticis* (Fig. 1a). *A. morrisi* reproduced during the experiment, and its quantity exceeded 100%. After 60 days of incubation, the biomasses of *E. fetida*, *A. corticis*, and *A. morrisi* decreased, while that of *A. robustus* increased (Fig. 1b). Moreover, the biomasses of *E. fetida* and *A. morrisi* were significantly higher than that of *A. corticis*.

Metal accumulation in earthworm tissues varied according to the type of metal. Metal concentration in earthworm tissues ranked as Zn >> Pb and Cu >> Cd (Tab. 1). Compared with *E. fetida* and *A. morrisi*, Zn concentration of *A. corticis* was 33.3–92.1% higher ($P < 0.05$). Significantly higher Cu and Cd concentrations were found in the native species (i.e., *A. corticis*, *A. morrisi*, and *A. robustus*) as compared with the non-native species *E. fetida*. In addition, significantly higher Pb concentration was observed in the endogeic species (i.e., *A. robustus*) as compared with the epigeic and epi-endogeic species.

In terms of BSAF, the heavy metals ranked as Cd > Zn > Pb and Cu, and the BSAFs of Zn, Cu, and Pb were less than 1 (Tab. 1). The BSAFs of Cd and Cu of the native species (i.e., *A. corticis*, *A. morrisi*, and *A. robustus)* were markedly higher than those of *E. fetida*, while the BSAF of Pb of endogeic species (i.e., *A. robustus)* was significantly higher than those of the epigeic/epi-endogeic.

3. 2 Earthworm casts production and casts and non ingested soil properties

Earthworm casts production ranked as *A. morrisi* > *E. fetida* > *A. robustus* > *A. corticis* (Fig. 2). *Eisenia fetida* produced the largest amounts of aboveground casts while *A. robustus* produced the smallest amount. Additionally, *A. morrisi* and *A. robustus* produced more belowground casts than *E. fetida* and *A. corticis*.

Compared with the control, pH values of the aboveground casts and non ingested soil in EF, AM and AR increased by 0.33, 0.13, 0.09, and 0.12 units, respectively (Tab. 2). In EF, pH value of the aboveground casts was significantly higher than that of the belowground casts. The highest pH values of aboveground and belowground casts and non ingested soil were found in EF, while the lowest pH values of aboveground casts and non ingested soil were observed in AC and AR respectively ($P<0.05$).

The organic C content of casts was 15.4–31.4% higher than that of the soil in control ($P < 0.05$). Higher organic C contents were always found in the earthworm casts than in the non ingested soil, especially in EF, AC, and AR. Of the four treatments with earthworms, EF showed the lowest organic C content in aboveground casts and in non ingested soil as well ($P < 0.05$).

Compared with the control, the presence of earthworms in the other treatments increased the total N contents of non ingested soil and casts by 4.74–29.4%. In both EF and AC, the total N content of aboveground casts was significantly higher than that of belowground casts. In EF, the total N contents of both the aboveground and belowground casts were significantly higher than that of the non ingested soil. Of the four treatments, EF displayed the lowest total N content of non ingested soil and the highest total N contents of aboveground and belowground casts.

Earthworm inoculation generally decreased the enzyme activities in soil (Tab. 2). β-Glucosidase activity in non ingested soil was significantly higher in AC than in EF and AM. The enzyme activity in aboveground casts was significantly higher in EF than in AC, and the enzyme activity in belowground casts was significantly higher in EF than in AM and AR. In AC, the enzyme activity was significantly lower in aboveground and belowground casts than in non ingested soil. In general, N-acetyl-β-D-glucosaminidase activity ranked as non ingested soil > aboveground casts > belowground casts. However, in EF, the enzyme activity was significantly higher in aboveground casts than in non ingested soil. The enzyme activity in belowground casts was significantly higher in EF than in AM. Alkaline phosphatase activity of the four treatments with earthworms ranked as AR > AM > AC > EF but as AC > AR > AM > EF for belowground casts. In AR, the enzyme activity was significantly lower in belowground casts than in non ingested soil and aboveground casts. Acid phosphatase activity in non ingested soil of the four treatments with earthworms ranked as AC > AR > AM > EF. In AC and AR, the enzyme activity was significantly higher in non ingested soil than in belowground casts. In AC, FDA hydrolytic activity was significantly higher in belowground casts than in non ingested soil and aboveground casts, whereas casts of AR had significantly lower activity than non ingested soil. The activity in non ingested soil and aboveground casts was higher in AR than in the other three treatments with epigeic species. In particular, the differences in FDA hydrolytic activity of non ingested soil were significant between the three treatments with native species.

3. 3 Total and available metals in casts and non ingested soil

Compared with the control soil, total Zn concentration in AC decreased by 10.1% and 5.58% in aboveground and belowground casts, respectively (Tab. 3). In AC, the total Zn concentration of non ingested soil was significantly higher than that of aboveground casts. In AR, the total Zn concentration of non ingested soil was significantly higher than those of casts. The total Zn concentration of belowground casts was significantly higher in AM than in AC. However, there were no significant differences in total Cd concentration between non ingested soil, aboveground casts, and belowground casts whatever the treatments.

In EF, the total Cu and Pb concentrations of belowground casts were significantly higher than those of non ingested soil. In AC and AR, total Pb concentration was significantly higher in non ingested soil than in aboveground and belowground casts. Additionally, total Pb concentration of belowground casts was significantly higher in EF and AM than in AC and AR. Earthworm introduction decreased total soil Pb concentration by 0–19.0% ($P < 0.05$).

In general, casts had higher available metal concentrations than the non ingested soil. The highest metal availability in casts and non ingested soil were found in AC with the epigeic species *A. corticis*, whereas the lowest values were generally observed in AR with the endogeic species *A. robustus* (Tab. 3).

In the three treatments with native species, the available Zn concentrations of casts were significantly higher than that of non ingested soil, which was especially true for AC. Compared with the control, available Zn concentration of aboveground and belowground casts in AC was 13.1% and 6.5%, respectively, higher ($P < 0.05$), and that of aboveground casts was significantly higher than that of belowground casts.

Compared with the control, available Cd concentrations in EF, AC, and AM were 0.4–41.7% higher. In the three treatments with native species (i.e., AC, AM, and AR), available Cd concentration of aboveground casts was significantly higher than that of non ingested soil. Available Cd concentrations of casts were also significantly higher in AC than in the other treatments.

Compared with the control, available Cu concentration of belowground casts in the treatments with epigeic and epi-endogeic species (i.e., EF, AC, and AM) was 1.4–8.7% higher. In the treatments with native species, available Cu concentration was significantly higher in casts than in non ingested soil. The available Cu concentrations of casts were significantly lower in AR than in EF, AC, and AM.

Compared with the control, the available Pb concentrations of casts in EF and AC were 1.3–13.0% higher. It is also worth noticing that in AR with the endogeic species *A. robustus*, the available Pb concentration of non ingested soil was significantly higher than those of casts. In terms of the available Pb concentration of casts, the treatments ranked as AC > EF > AM > AR ($P < 0.05$).

3. 4 Relationship between metal concentration in earthworm tissue and available metal in casts

A co-inertia analysis was performed to explore the relationship between metal concentration in earthworm tissue and available metal in casts (Fig. 3). Metal concentration of earthworm tissue and available metal of casts were significantly different between treatments (P = 0.005). The first axis explained 71.0% of the total variance. Available Cu and Pb in casts were negatively correlated with the axis, while their total concentrations in earthworm tissue were positively correlated with the axis. The second axis explained 18.3% of the total variance. The total concentrations of Zn and Cd in earthworm tissues were positively correlated with the available Zn and Cd in casts. The Pb- and Cu-activating abilities of epigeic species *E. fetida* and *A. corticis* and epi-endogeic species *A. morrisi* were stronger than those of the endogeic species *A. robustus*, but their Pb- and Cu-accumulating abilities were lower. There were significant differences in the Zn- and Cd-accumulating and activating abilities between the three epigeic and epi-endogeic earthworm species (P = 0.005). *Amynthas corticis* had the strongest Zn- and Cd-accumulating and activating abilities, followed by *A. morrisi* and *E. fetida*.

Additionally, total metal accumulation in earthworm tissues and total available metal in earthworm casts and their ratios were calculated (Tab. 4). The highest ratios were found in the treatment with the endogeic species *A. robustus* (P < 0.05). The ratio of Cd was larger than 1.0 for AC, AM, and AR and was considerably larger than those of Zn, Cu, and Pb.

3. 5 Effects of earthworm on soil quality

PCA analysis showed that axis 1 (38.1% variance explained) was mainly related to organic C, cast production, and activities of β-glucosidase, N-acetyl-β-D-glucosaminidase, acid phosphatase and alkaline phosphatase (Fig. 4a, b, c). Axis 2 (17.5% variance explained) was mainly related to total N and FDA hydrolytic activity. Axis 3 (14.0% variance explained) was mainly related to pH and alkaline phosphatase. Organic C and aggregate production were negatively correlated with enzyme activities (Fig. 4a, b, c), and total N were negatively correlated with the overall enzymatic activity (i.e., FDA hydrolytic activity). The differences in non ingested soil and casts properties between the four treatments of EF, AC, AM, and AR were significant ($P = 0.001$). In terms of soil quality index, control < non ingested soil < casts (Fig. 4d). The soil quality indices of non ingested soil ranked as: AM (0.41) > AC (0.40) > EF (0.34) > AR (0.30); those of aboveground casts were in the order of: AC (0.90) > AM (0.63) > EF (0.58) > AR (0.51) ($P < 0.05$); and those of belowground casts were observed as: AM (0.88) > AR (0.85) > EF (0.67) and AC (0.67). Compared with the control (0.12), the average soil quality indices of EF (0.53), AM (0.64), AC (0.66), and AR (0.56) were higher.

4. Discussion

There is an urgently need for soil comprehensive remediation in polluted soil. Transfering metals from soil, improving soil quality and increasing plants growth are the three dimensions of soil remediation, which could be achieved by earthworm-assisted bioremediation technologies (Zhang *et al*., 2018, 2020). When using earthworm for soil bioremediation in the field, it is necessary to choose the most suitable earthworm species and then take several factors into consideration, such as earthworm tolerance to metals, metal accumulation in earthworm, available metals in earthworm casts removed into the aboveground of plants in further, and earthworm effects on soil quality.

4.1 Earthworm tolerance to metals

High tolerance to pollutants is considered an essential characteristic of organisms that are to be used in bioremediation technologies (Macaskie and Dean, 1989). High survival rate in contaminated soil is, obviously, the primary requirement for the selction of earthworm species. In our study, the high survival rates of the four earthworm species in the natural heavy metal-contaminated soil demonstrated their high tolerance to Cu, Cd, Pb, and Zn. Compared with *A. corticis*, the > 90% survival rates of *A. robustus* and *A. morrisi* indicated that the two species may be promising in the field (Fig. 1). *Eisenia fetida* is commonly used in international standard toxicity tests and readily available from commercial supplies. It is not a natural soil species and inhabits organic-rich habitats (Nahmani *et al*., 2007). In addition, it is less sensitive to contaminants than other species (Langdon *et al*., 2005). In our 60-day laboratory experiment with 10% organic matter addition to the soil, a survival rate of 94.8% was found for *E. fetida*, which was not much lower than those of *A. morrisi* and *A. robustus*. Therefore, although not a native species, *E. fetida* may also be considered for bioremediation of metal-contaminated soils in South China. In further study, some subcellar or molecular experiments should be needed for explaining the tolerant mechanism of earthworms on the long-term with the aim to use earthworms in remediation processes.

4.2 Earthworm effects on soil quality

Effects of earthworm on soil quality are also crucial when using earthworm in soil bioremediation. Soil health and sustainability should be maintained (Lavelle *et al*., 2016). Soil quality indices reflect soil physical, chemical, and biological processes (Velasquez *et al*., 2007; Dominguez-Hayda *et al*., 2019). In this study, soil quality indices were significantly different between treatments with different earthworm species (Fig. 4c). The higher average soil quality indices in AR (0.56) and AM (0.64) as compared with EF (0.53) may be mainly due to the effects of *A. robustus* and *A. morrisi* on soil aggregates, organic C, and microbial activities. Previous studies have shown that soil-dwelling species living in deeper soil layers produce more stable aggregates than epigeic species (Blouin *et al.*, 2013; Lavelle *et al*., 2020). Similar results were also found in this study that the endogeic species *A. robustus* and the epi-endogeic species *A. morrisi* produced more casts, which are rich in microaggregates, than the epigeic species *A. corticis* (Fig. 2). The high C contents of aboveground and belowground casts (Tab. 2 and Fig. 4) were related to earthworms' feeding habits that they selectively feed on litter or organic-rich soil (Lavelle and Spain, 2001; van Groenigen *et al.*, 2019; Wu *et al.*, 2020; Xiao *et al.*, 2020). Taking their effects on soil quality into account, *A. robustus* and *A. morrisi* were better candidate species than *E. fetida* for bioremediation of metal-contaminated soils.

4.3 Metal accumulation in earthworm and available metal in casts and their relationship

Metal accumulation in earthworm and available metal in casts are key to earthworm environmental application. Metal accumulation in earthworm tissue was significantly influenced by earthworm species (Tabs. 1 and 4). The higher metal concentrations in the tissues of the three native species *A. corticis*, *A. morrisi*, and *A. robustus* as compared with *E. fetida* may be because they ingest larger amounts of soil but excrete smaller amounts of metal (Dai *et al*., 2004; Suthar *et al*., 2008). Total metal accumulation and the ratio of metal accumulation in body to available metal in casts of *A. robustus* were higher than those of the other species (Tab. 4), possibly due to the higher biomass of *A. robustus* (Fig. 1) (Xiao *et al*., 2020). The higher metal availability in earthworm casts than in non ingested soil, which indicates metal activation in earthworm casts, is attributed to the effects of intestinal microbes according to our previous studies (Dai *et al*., 2015; Zhang *et al*., 2016). Metal availability in casts was closely associated with earthworm species (Tab.

3, Fig. 3). The metal-activating effect of epigeic/epi-endogeic species was stronger than that of endogeic species (Tab. 3). Epigeic and epi-endogeic species have stronger organic C-decomposing ability than endogeic species (Lavelle and Spain, 2001) due to some special microbes in their guts which participate in carbohydrate metabolism regulation (Liu *et al*., 2018), resulting in higher availability of elements, including metals. In addition, total available Cd was higher in the casts of *A. morrisi* than in the casts of the other species ($P < 0.05$, Tab. 4), demonstrating that *A. morrisi* is a good Cd activator for Cd bioremediation.

Many studies have focused on metal accumulation (Nahmani *et al*., 2007; Suthar *et al*., 2008; Xiao *et al*., 2020) or metal activation by earthworms (Sizmur and Richardson, 2020, and the references in it), but few studies have looked at their relationship. In this study, the ratio values of metal accumulation in earthworm tissue to total available metal in earthworm casts indicated that a large proportion of the metals ingested by earthworms returned to the soil in available forms instead of entered earthworm tissue except Cd (Tab. 4). Therefore, both stabilizing the Zn, Cu, and Pb in earthworm casts and phytoextraction are plausible remediation methods. As Cd is highly mobile (Nannoni *et al.*, 2011) and easily combines with metallothionein (MT) and non-metallothionein proteins (ie. MIP) in earthworm tissue (Morgan *et al*., 1989; Hussain *et al.*, 2021), Cd accumulation in earthworm should not be overlooked. Metal BSAFs ranked as Cd > Zn > Cu and Pb (Tab. 1, $P < 0.05$), which was consistent with the result of Dai *et al*. (2004). The Cd accumulated in earthworm tissues would re-enter the soil when earthworm dies, posing environmental risk (Zhang *et al*., 2016). Therefore, it is important to reduce Cd accumulation in earthworm tissue. Lanno *et al*. (2004) pointed out that after ingested, Cd is transported to sites of toxic action and persists in earthworm body for a long time. The co-inertia analysis showed that Cd availability in casts was positively related with Cd accumulation in epigeic/epi-endogeic species (Fig. 3). Available Cd in earthworm-soil system is influenced by microbe strains, plant roots, and soil properties (Dai *et al*., 2015; Kaur *et al*., 2019). Employing phytoextraction technology may decrease Cd in soil and earthworm tissue and remediate Cd pollution in soil.

Above all, the highest total available Cd, Cu, and Pb concentrations in *A. morrisi* casts demonstrates that *A. morrisi* is the best option for future application of the three native earthworm species. *A. morrisi*-assisted phytoextraction may be a suitable remediation method for the Cd/Cu/Pb multi-polluted soils in South China. However, it is worth mentioning that only one type of soil was used in our experiment, which lasted only 60 days. Therefore, more studies are required to confirm the results of this study in other environments and over long-term observations.

5. Conclusion

When choosing earthworm species for metal-contaminated soil remediation, special attention should be paid to the tolerance of earthworm to metals, metal accumulation in earthworm tissue, metal availability in earthworm casts, and the effect of earthworm on soil quality. *Eisenia fetida*, *A. morrisi*, and *A. robustus* showed higher survival rates than *A. corticis* in the heavy metal-contaminated soil. Compared with the epigeic species *E. fetida*, the native epi-endogeic species *A. morrisi* and endogeic species *A. robustus* accumulated more metals and had a more beneficial effect on soil quality. In addition, *A. morrisi* had a strong ability in activating Cd, Cu, and Pb in casts. Based on the relationship between metal accumulation in earthworm tissue and metal availability in casts, *A. morrisi* is the most promising of the four for application in heavy metal-contaminated soils, and *A. morrisi*-assisted phytoextraction technology may be exploited for the Cd/Cu/Pb multi-polluted soils in South China.

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CRediT authorship contribution statement

Menghao Zhang: Writing-original draft, Methodology, Data curation. Pascal Jouquet: Methodology, Writing-review&editing, Jun Dai: Writing - review &Editing; Ling Xiao: Writing - review &Editing; Yan Liu: Data curation, Investigation, Kexue Liu: Data curation, Investigation, Mikael Motelica-Heino: Investigation, Patrick Lavelle: Investigation, Hesen Zhong: Investigation, Chi Zhang: Conceptualization, Methodology, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Funding acquisition, Project administration.

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Figure Captions

Fig. 1 Survival rate and biomass of the four earthworm species *Eisenia fetida*, *Amynthas corticis*, *A. morrisi*, and *A. robustus* after 60-day incubation in the Zn/Cu/Cd/Pb multi-polluted soil.

Different lower letters indicate significant differences ($P < 0.05$) between different earthworm species

Fig. 2 Proportions of aboveground and belowground casts and non ingested soil in the four treatments with earthworm species of *Eisenia fetida* (EF), *Amynthas corticis* (AC), *A. morrisi* (AM), and *A. robustus* (AR) added.

Fig. 3 Co-inertia analysis between metal concentration in earthworm tissue and available metal in earthworm casts.

(a) Projection of metal concentration in earthworm tissue.

(b) Projection of available metal in earthworm casts.

(c) Score plot of treatments with different earthworm species addition.

Fig. 4 Principal component analysis of soil properties in treatments with earthworm addition in factorial F1, F2 and F3 planes.

(a) Correlation circles of soil and cast properties in all treatments in factorial F1 and F2 planes

(b) Correlation circles of soil and cast properties in all treatments in factorial F2 and F3 planes

(c) Projection of experimental points according to treatments.

(d) Soil quality indices of treatments with different earthworm species addition. (Different lower letters indicate significant differences ($P < 0.05$) between aboveground casts, belowground casts, and non ingested soil for a same treatment, and different capital letters indicate significant differences ($P < 0.05$) in soil quality index of aboveground casts/belowground casts/non ingested soil between different treatments.)

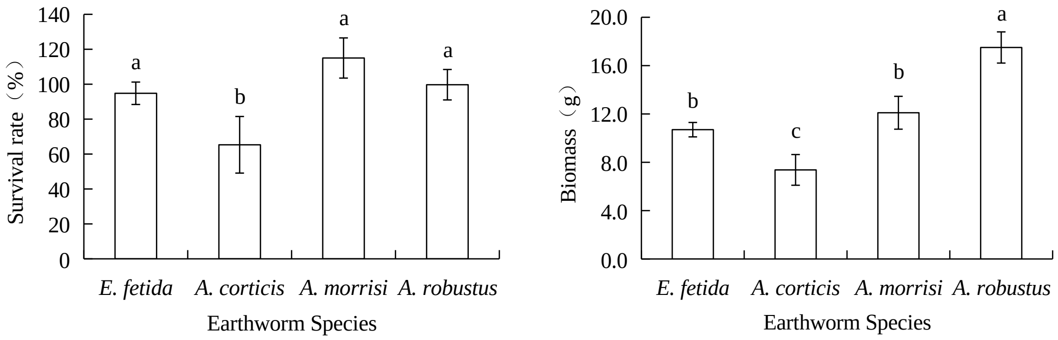


Fig. 1 Survival rate (a) and biomass (b) of *Eisenia fetida*, *Amynthas corticis*, *A. morrisi*, and *A. robustus* after 60-day incubation in the Zn/Cu/Cd/Pb multi-polluted soil. Different letters indicate significant differences ($P < 0.05$) between different earthworm species.

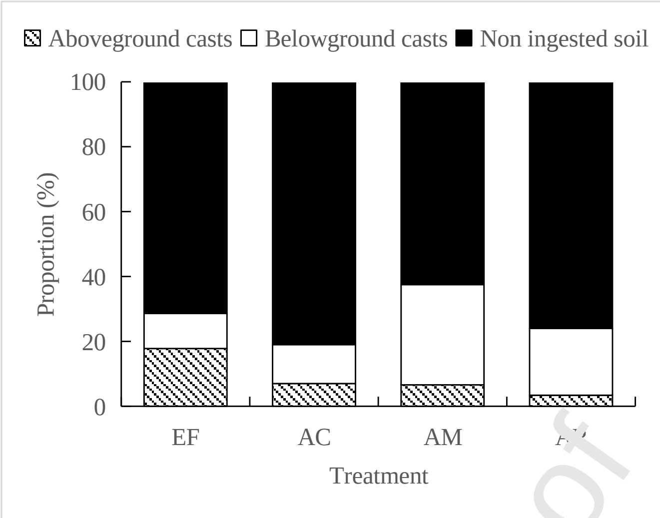
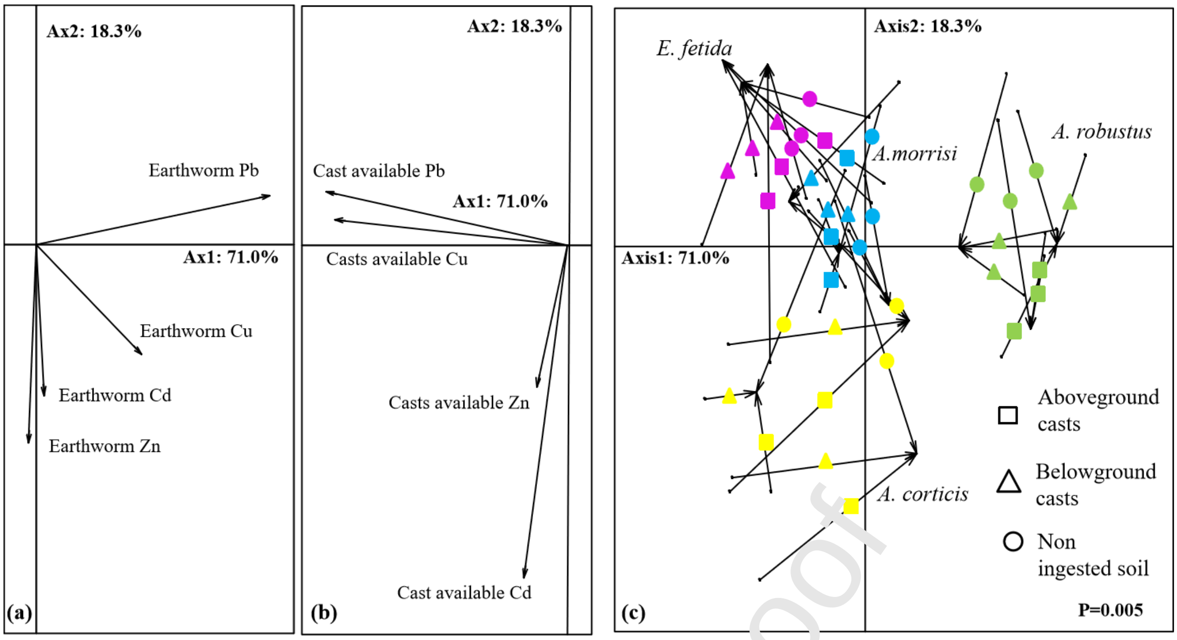


Fig. 2 Proportions of aboveground and belowground casts and non ingested soil in the four treatments with earthworm species of *Eisenia fetida* (EF), *Amynthas corticis* (AC), *A. morrisi* (AM), and *A. robustus* (AR) added.



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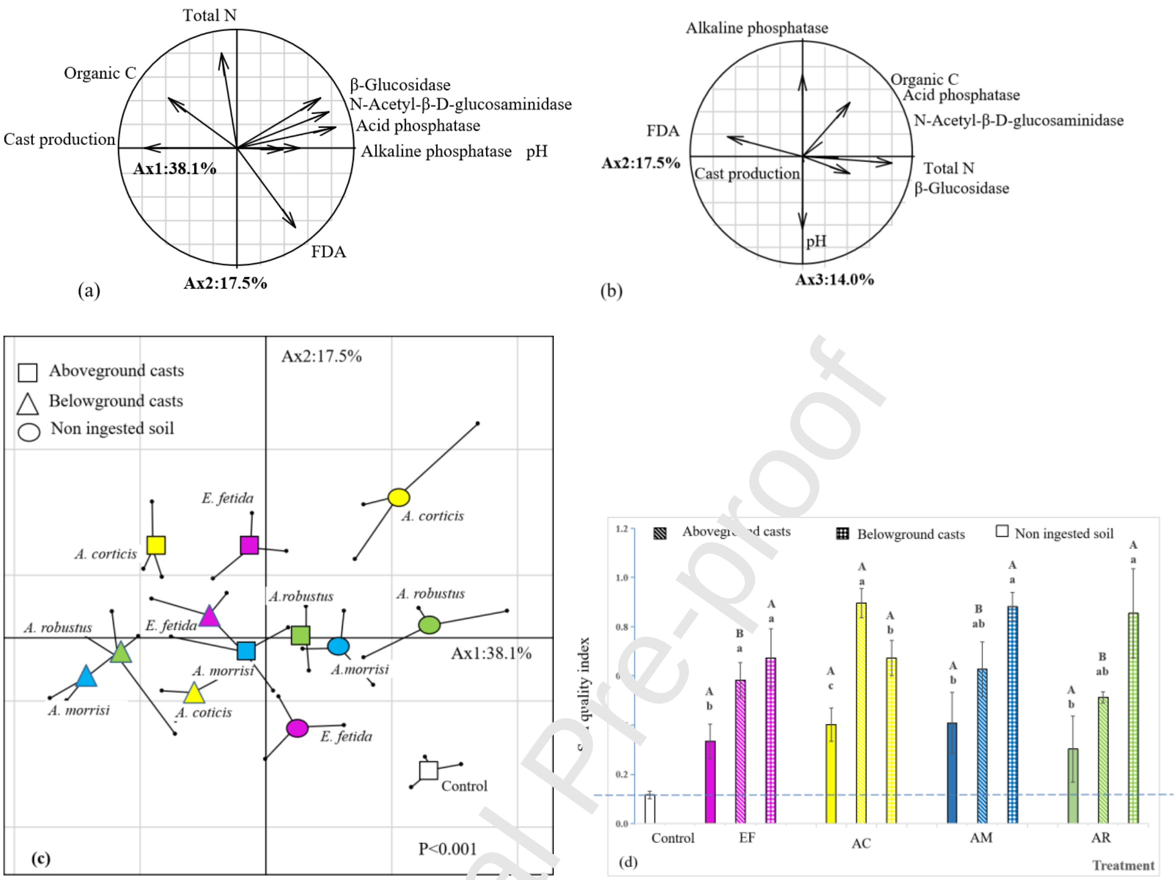

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Table lists:

Tab. 1 Metal concentration and metal biota-to-soil accumulation factor (BSAF) of the four earthworm species (i.e., *Eisenia fetida*, *Amynthas corticis*, *A. morrisi*, and *A. robustus*) after 60-day incubation in the Zn/Cu/Cd/Pb multi-polluted soil (mean ± S.D., n = 3)

Different lower letters indicate significant differences between earthworms ($P < 0.05$).

Tab. 2 Properties of non ingested soil and aboveground and belowground casts in the different treatments (mean ± S.D., n = 3)

Different capital letters indicate significant differences ($P < 0.05$) between non ingested soil, aboveground casts, and belowground casts for a same treatment; different lower case letters indicate significant differences ($P < 0.05$) between treatments for a same component (i.e., non ingested soil, aboveground casts, or belowground casts). The data for non ingested soil and aboveground casts in EF and AM treatments were from Zhang *et al*. (2016).

Tab. 3 Total and available metals in the aboveground and belowground casts and non ingested soil of all treatments (mean ± S.D., n = 3)

Different capital letters indicate significant differences ($P < 0.05$) between non ingested soil, aboveground casts, and belowground casts for a same treatment; different lower case letters indicate significant differences ($P < 0.05$) between treatments for a same component (i.e., non ingested soil, aboveground casts, or below ground casts). The data for non ingested soil and aboveground casts in EF and AM treatments were from Zhang *et al.* (2016).

Tab. 4 Metal accumulation in earthworm tissue, total available metal in earthworm casts and the ratio between them

Different lower case letters indicate significant differences ($P < 0.05$) between treatments.

Tab. 1 Metal concentration and metal biota-to-soil accumulation factor (BSAF) of the four earthworm species (i.e., *Eisenia fetida*, *Amynthas corticis*, *A. morrisi*, and *A. robustus*) after 60-day incubation in the Zn/Cu/Cd/Pb multi-polluted soil (mean ± S.D., n = 3)

Earthworm species	Metal concentration ($mg \cdot kg^{-1}$)				BSAF			
	Zn	Cd	Cu	Pb	Zn	Cd	Cu	Pb
E. fetida	95.8±4.80b	5.03±0.117b	30.9±1.67b	56.8±4.86b	0.308±0.043a	6.76±1.60b	0.084± 0.006b	0.138±0.011b
A. corticis	184±28.3a	15.7±2.62a	72.5±8.74a	62.5±22.7b	0.666±0.099a	24.8± 7.03a	0.194± 0.022a	0.149±0.055b
A. morrisi	138±22.3b	12.0±0.614a	59.9±8.39a	63.6±7.47b	0.508±0.270a	16.6± 5.58ab	0.156± 0.018a	0.150±0.017b
A. robustus	150±9.89ab	12.2±4.31a	72.7±5.35a	97.4±13.7a	0.557±0.059a	24.0± 7.92a	0.196± 0.009a	0.235± 0.034a

Different lower letters indicate significant differences between earthworms (P < 0.05).

Tab. 2 Properties of non ingested soil and aboveground and belowground casts in the different treatments (mean ± S.D., n = 3)

Treatment	pH	Organic C	Total N	β-Glucosidase	N-Acetyl-β-D -glucosaminidase	Alkaline phosphatase	Acid phosphatase	FDA
		mg.kg^{-1}			μg pNP g^{-1} soil h^{-1}			μg fluorescein g^{-1} soil h^{-1}
Control	5.48±0.00	40.8±0.22	2.11±0.14	209±14.1	212±11.9	85.9±34.0	467±50.3	149±19.5
EF								
non ingested soil	5.61±0.06ABa	38.4±1.83Bb	2.30±0.05Cb	166±26.1Abc	114±30.5Ba	31±22.2Ab	350±103Ac	80±8.6Ab
Aboveground cast	5.81±0.12Aa	47.2±1.62Ab	2.73±0.08Aa	213±28.3Aa	186±45.9Aa	25±17.6Ac	324±68.9Aa	61±13.0Aa
Belowground cast	5.45±0.14Ba	48.5±1.59Aa	2.53±0.04Ba	184±49.3Aa	132±8.6ABa	47±33.8Aa	292±47.2Aa	68±19.1Aa
AC								
non ingested soil	5.45±0.08Ab	44.1±2.53Ba	2.59±0.08Aa	293±48.2Aa	201±70.4Aa	72±33.2Aab	615±95.4Aa	22±3.7Bc
Aboveground cast	5.23±0.23Ab	53.6±2.96Aa	2.57±0.25Aa	130±18.3Bb	150±41.9Aa	56±15.6Abc	311±51.1Ba	22±2.7Bb
Belowground cast	5.44±0.16Aa	50.1±1.63Aa	2.21±0.08Ba	135±11.2Bab	98±45.5Aab	73±14.2Aa	338±74.2Ba	69±6.7Aa
AM								
non ingested soil	5.57±0.11Aab	43.6±3.48Aa	2.51±0.19Aab	136±2.6Bc	175±36.2Aa	106±26.7Aab	438±84.9Abc	81±7.6Ab
Aboveground cast	5.31±0.07Ab	48.1±2.28Ab	2.39±0.18Aa	182±24.1Aab	127±56.4ABa	94±37.3Aab	318±61.4Aa	76±18.6Aa
Belowground cast	5.28±0.26Aa	47.1±2.92Aa	2.50±0.09Aa	110±12.9Bb	51±38.8Bb	50±20.2Aa	298±77.8Aa	72±8.7Aa
AR								
non ingested soil	5.43±0.04Ab	44.0±1.90Ba	2.56±0.14Aa	250±73.7Aab	180±56.5Aa	142±42.4Aa	521±54.6Aab	117±19.1Aa
Aboveground cast	5.60±0.03Aa	49.7±0.95Ab	2.40±0.18Aa	164±42.0ABab	154±18.6Aa	117±8.7Aa	409±51.7ABa	79±3.0Ba
Belowground cast	5.31±0.25Aa	50.6±0.66Aa	2.42±0.31Aa	110±36.8Bb	101±36.8Aab	60±8.4Ba	268±98.9Ba	63±5.5Ba

pNP = p-nitrophenol.

Different capital letters indicate significant differences ($P < 0.05$) between non ingested soil, aboveground casts, and belowground casts for a same treatment; different lower case letters indicate significant differences ($P < 0.05$) between treatments for a same component (i.e., non ingested soil, aboveground casts, or belowground casts). The data for non ingested soil and aboveground casts in EF and AM treatments were from Zhang *et al*. (2016).

Tab. 3 Total and available metals in the aboveground and belowground casts and non ingested soil of all treatments (mean ± S.D., n = 3)

Treatment	Total Zn	Total Cd	Total Cu	Total Pb	Available Zn	Available Cd	Available Cu	Available Pb
				mg.kg^{-1}				
CK	266.69±5.27	0.65±0.24	382.82±2.86	427.64±2.12	19.9±0.645	0.266±0.009	76.3±1.78	84.3±1.86
EF								
non ingested soil	313.21±27.30Aa	0.77±0.19Aa	369.13±8.28Ba	410.65±8.11Ba	17.1±1.32Aa	0.267±0.012Aa	73.6±2.94Aa	79.1±9.69Aa

Aboveground casts	250.14±110.09Aa	0.46±0.22Aa	374.73±2.74ABa	403.62±10.29Ba	18.7±1.69Ab	0.300±0.029Ab	71.4±5.49Aa	85.4±5.37Aa
Belowground casts	406.80±192.68Aab	0.80±0.22Aa	382.08±1.54Aa	427.63±6.10Aa	18.7±1.09Ab	0.275±0.013Ab	81.4±6.47Aa	92.5±7.32Aa
AC								
non ingested soil	275.49±1.52Aa	0.65±0.10Aa	374.73±6.46Aa	421.33±2.58Aa	18.0±0.64Ca	0.272±0.006Ba	73.9±2.06Ba	84.3±1.37Ca
Aboveground casts	239.86±18.74Ba	0.72±0.00Aa	357.62±10.91Aa	394.63±11.11Ba	22.5±0.91Aa	0.377±0.019Aa	77.6±2.57Ba	90.6±2.72Ba
Belowground casts	251.82±19.02ABb	0.62±0.05Aa	373.74±12.11Aa	393.64±7.83Bb	21.2±0.13Ba	0.343±0.025Aa	82.9±1.82Aa	95.3±2.75Aa
AM								
non ingested soil	305.96±107.72Aa	0.77±0.22Aa	384.78±12.06Aa	425.30±11.71Aa	[illegible]	0.272±0.016Ba	71.6±2.34Ba	74.9±1.85Ba
Aboveground casts	376.28±169.45Aa	0.65±0.36Aa	315.45±67.21Aa	346.47±77.04Aa	14.8±1.52Ac	0.316±0.009Ab	74.4±1.36ABa	78.0±4.16Bb
Belowground casts	485.57±127.72Aa	0.83±0.26Aa	384.98±10.61Aa	[illegible]	13.8±0.26ABd	0.292±0.009Bb	77.4±1.43Aa	84.8±1.07Ab
AR								
non ingested soil	269.16±10.82Aa	0.52±0.13Aa	370.26±9.25Aa	413.72±3.23Aa	14.9±0.32Bb	0.241±0.007Bb	55.4±0.86Bb	64.1±2.90Ab
Aboveground casts	234.86±16.79Ba	0.68±0.04Aa	344.63±9.41Aa	380.58±12.15Ba	18.0±0.98Ab	0.297±0.020Ab	55.8±1.48Bb	49.2±2.00Bc
Belowground casts	236.60±10.42Bb	0.70±0.05Aa	[illegible]	391.84±9.12Bb	16.6±0.91Ac	0.265±0.016ABb	63.9±2.46Ab	20.6±1.36Cc

Different capital letters indicate significant differences ($P < 0.05$) between non ingested soil, aboveground casts, and belowground casts for a same treatment; different lower case letters indicate significant differences ($P < 0.05$) between treatments for a same component (i.e., non ingested soil, aboveground casts, or below ground casts). The data for non ingested soil and aboveground casts in EF and AM treatments were from Zhang *et al.* (2016).

Tab. 4 Metal accumulation in earthworm tissue, total available metal in earthworm casts and the ratio between them

Treatment	Metal accumulation in earthworm				Total available metal in earthworm casts				The ratio of metal accumulation in earthworm to total available metal in earthworm casts			
	Zn	Cd	Cu	Pb	Zn	Cd	Cu	Pb	Zn	Cd	Cu	Pb
	mg/pot				mg/pot							
EF	1.03±0.03c	0.054±0.002b	0.33±0.01c	0.61±0.04b	5.33±0.65a	0.083±0.010b	21.42±2.53b	25.10±2.79a	0.1[illegible]±0.[illegible]b	0.66±0.09b	0.016±0.002c	0.024±0.001b
AC	1.36±0.40bc	0.118±0.036b	0.54±0.15b	0.45±0.17b	4.12±0.95a	0.067±0.011b	15.39[illegible]bc	17.[illegible]±4.26b	0.35±0.15b	1.84±0.76b	0.038±0.017bc	0.027±0.013b
AM	1.64±0.12b	0.144±0.010ab	0.72±0.04b	076±0.04b	5.22±0.72a	0.111[illegible]	2[illegible].88±4.55a	31.37±5.10a	0.32±0.06b	1.31±0.14b	0.025±0.005b	0.025±0.004b
AR	2.62±0.34a	0.217±0.092a	1.27±0.11a	1.70±026a	4.[illegible]a	0.064±0.008b	15.05±1.45c	5.90±1.08c	0.65±0.04a	3.31±1.03a	0.085±0.006a	0.291±0.043a

Different lower case letters indicate significant differences (P < 0.05) between treatments.

Conflict of interest

The authors declared that they have no conflicts of interest to this work.

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.