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Effect of the transit through the gut of earthworm (*Eisenia fetida*) on fractionation of Cu and Zn in pig manure

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ABSTRACT

To investigate the effect of the transit through the gut of earthworm (*Eisenia fetida*) on the fractionation of Cu and Zn in pig manure, earthworms were reared with pig manure in the greenhouse. Both the pig manure and the earthworm casts were subjected to a five-step sequential extraction of Cu and Zn. The content of Cu bound to organic matter in pig manure increased from 60% to 75% after transit through the gut of earthworm, whereas that of Zn decreased from 50% to 25%. It demonstrated that Cu had a strong affinity towards organic matter. The share of Cu and Zn in the exchangeable fraction was reduced by the transit through the gut of earthworm. Based on these changes, Cu was more bioavailable, whereas Zn was less bioavailable. The factors affecting metal fractionation, like pH, organic matter (OM) and total phosphorous (TP) contents, and total metal concentration, were also affected significantly by the transit through the gut of earthworm. Stepwise multiple regression analysis revealed that the fractionation of Cu in the earthworm casts was influenced by OM, TP and the amount of Cu in the earthworm casts. The total Zn concentration in the earthworm casts was the primary factor that explained most of the variation in Zn fractionation. The present study demonstrated that the digestive activity in the gut of *E. fetida* played an important role in the fraction redistribution of Cu and Zn in pig manure.

1. Introduction

The swine industry has developed rapidly in recent two decades. Consequently, the amount of pig manure increases dramatically and its component varies a lot compared with that before. Pig manure is moderately to heavily polluted with heavy metals [1,2]. This is caused by feed additives overuse (largely exceeds physiological requirement levels for swine disease control and weight improvement). In pig farming, the amount of Cu eliminated through the animal feces corresponds to 72–80% of the amount ingested, whereas for Zn, it could be as high as 92–96% [3,4]. These metals are present in the pig manure in various chemical forms and bound to different pig manure fractions [5].

In China the most common agricultural organic waste is livestock manure, especially the pig manure, which is usually processed in farm to rear earthworms. Earthworms constitute a major component in soil functioning, and they play an important role in chemical elements transformations [6]. Additionally, earthworms satisfy all the conditions, especially metal pollution, of a good biological indicator. Previous research has shown that earthworms actively alter soil physical and chemical properties responsible for heavy metal fractionation [7–11]. Indeed it has been reported that, after treatment with earthworms, the distribution of heavy metals in soil fractions was changed significantly [9,10,12]. Earthworms ingest both mineral and organic fragments and, through comminution and mixing during digestion process, the material is subjected to a biochemical and physical modification [13]. Earthworm casts offer micro-environmental conditions very different from those occurring in the surrounding environment [12,14]. The estimated annual rates of earthworm cast production range from 5 to more than 250 tons ha^{-1} [15].

Earthworm cast may be a valuable source of N, P, essential trace elements, and organic matter that improve soil physical and chemical properties and plant nutrient status. However, besides the beneficial effects, potential risks associated with high metal content in earthworm casts (e.g. Pb, Zn, Cd, Cu) have received increasing attention [9,12,16,17]. Generally, most of them [12,16,17] only paid attention to the total metal concentrations in earthworm casts, and neglected the fractionation of metal in earthworm casts. Knowledge of the total metal concentrations of the earthworm casts does not suffice to assess their environmental hazard because the metals can be present in a variety of forms. The forms mainly include the exchangeable, bound to carbonates, bound to Fe and Mn oxides, bound to organic matter and residual fraction [18–20]. It is essential to know both total content and the chemical forms of each species present in the earthworm casts, because

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Table 1
Selected physicochemical properties of four pig manure samples.

	pH	MC[a] (%)	OM[b] (g kg^{-1})	TP[c] (g kg^{-1})	Total Cu (mg kg^{-1})	Total Zn (mg kg^{-1})
S1[d]	7.53 ± 0.03[e]	75.58 ± 0.08	222.72 ± 3.97	41.59 ± 0.12	932.82 ± 3.83	1383.42 ± 5.27
S2	7.41 ± 0.03	77.68 ± 0.31	178.60 ± 2.70	44.92 ± 0.16	819.20 ± 23.79	2520.61 ± 119.04
S3	7.43 ± 0.04	78.73 ± 0.15	202.77 ± 6.14	47.11 ± 0.16	445.11 ± 6.20	1791.97 ± 20.35
S4	7.51 ± 0.02	77.90 ± 0.27	193.04 ± 3.96	40.17 ± 0.28	1410.95 ± 34.92	2822.47 ± 115.27

[a] Represents moisture content.
[b] Represents organic matter.
[c] Represents total phosphorous.
[d] Represents four different sites.
[e] The values are represented as means of three replicates ± s.d.

bioavailability and toxicity depend thereon. The bioavailability of heavy metal is highly dependent on the fractionation of heavy metal [21].

The aims of this study are therefore to: (1) investigate the fractionation of Cu and Zn in the earthworm (*Eisenia fetida*) casts from pig manure, (2) investigate the effect of the gut transit on the factors affecting the metal fractionation, like pH, organic matter (OM), total phosphorous (TP) and the amount of Cu and Zn, (3) apply multivariate statistical analysis to investigate the relationship between fractionation of metal and factors.

2. Materials and methods

2.1. Pig manure and earthworms characterization

Fresh pig manure samples were collected from four different sampling sites in a pig-breeding farm located Yuhang country, Zhejiang Province, China. The sites selected were Site 1 (S1), Site 2 (S2), Site 3 (S3) and Site 4 (S4). The pig manure samples were homogenized in each pit and then stored in sealed plastic boxes and kept in refrigerator at 4 °C until use. Pig manure was air-dried, ground, and passed through a 1.0 mm nylon fiber sieve for physical and chemical analysis.

The earthworms used for the experiment were *E. fetida* (confirmation of earthworm identification was carried out by Mr. Jianping Zhang, an expert in earthworm species) from the same farm. The mature earthworms, with fresh weight ranging between 0.45 and 0.55 g, were washed with deionized water, and placed into plastic Petri dish lined with a piece of filter paper moistened with deionized water at a constant temperature of 20 °C. In order to clear their guts of soil, the earthworms were kept on the moist filter paper for 48 h without food, during which the filter paper was changed every 12 h [22].

The moisture content (MC) of the pig manure was determined after drying at 105 °C for 24 h, and the OM content after ashing at 550 °C for 4 h [23]. The TP was colorimetrically measured on mineralized samples with the molybdate acid procedure [23]. The pH was measured with a glass electrode using a 1:5 sample/0.01 M $CaCl_2$ ratio [23]. To determine total Cu and Zn concentrations, samples were digested by tri-acid mixture ($HF/HNO_3/HClO_4$) [23] and were measured by flame atomic absorption spectrometry (FAAS, Thermo Element MKII-M6). Some selected physical and chemical properties of the pig manure are shown in Table 1.

2.2. Experimental procedure

Each of the pig manure sample was divided into three subsamples (500 g pig manure each placed in a 6 l volume of plastic pot which was filled to two-third of its capacity with moistened vermiculite). Twenty individuals of *E. fetida* were inoculated in each pot located in the greenhouse, with pig manure as food source. Deionized water was added to the pig manure to achieve a moisture content of 80 ± 5% during the experimental process. The earthworms were reared for 4 weeks until they egested sufficient earthworm casts to perform the subsequent analysis. Earthworm casts were collected from the pots by plastic forceps. The earthworm casts were air-dried, ground and sieved through a 1.0 mm nylon mesh before analysis.

2.3. Five-step sequential extraction

A five-step sequential extraction procedure recommended by Tessier et al. [18] was used to determine the fractionation of Cu and Zn in both the pig manure and the earthworm casts, into five fractions: exchangeable (F1), bound to carbonates (F2), bound to Fe and Mn oxides (F3), bound to organic matter (F4), and residual (F5). Specific details of the fractionation procedure are given below.

(1) F1. For each sample, 16 ml of 1.0 M $MgCl_2$ was added to 1.0000 g air-dried pig manure or earthworm casts in a 50 ml polypropylene centrifuge tube and shaken for 2 h at room temperature. The extract was separated from the solid residue by centrifugation (4500 rpm) and decantation of the supernatant liquid into a 50 ml volumetric flask which was filled up with deionized water.
(2) F2. 16 ml of 1.0 M NaOAc was added to the residue from step 1 in the centrifuge tube and shaken for 5 h at room temperature. Again, the extraction was performed as described in step 1.
(3) F3. 40 ml of 0.04 M $NH_2OH{\cdot}HCl$ was added to the residue from step 2 in the centrifuge tube and shaken for 4 h at 85 °C in a water bath. Again, the extraction was performed as described in step 1.
(4) F4. 10 ml of 30% (v/v) H_2O_2 was added to the residue from step 3 in the centrifuge tube and shaken for 1 h at 85 °C in a water bath. A second aliquot of 6 ml of 30% H_2O_2 was then added and the mixture was heated at the same temperature for 4 h. After cooling, 10 ml of 3.2 M NH_4OAc in 20% (v/v) HNO_3 were added and shaken for 0.5 h at room temperature. Again, the extraction was performed as described in step 1.
(5) F5. The residue from step 4 was digested by tri-acid mixture ($HF/HNO_3/HClO_4$).

Extracts were conserved with 1% (v/v) HNO_3 and stored at 4 °C prior to analysis. Cu in extracts was determined by FAAS directly, whereas Zn in extracts was determined by FAAS with deuterium background correction.

2.4. Mathematical description of the relationship between fractionation and factors

Since the fractionation of metal was largely dependent on the factors, such as pH, OM, TP and total metal concentration, we developed a mathematical representation (Eq. (1)) to indicate the relationship between fractionation of metal and factors, based on an empirical regression model [24,25].

$$\log\,[\text{metal}_{\text{fraction X}}]_{\text{solid}} = a + b\,\log(C_1) + c\,\log(C_2) + d\,\log(C_3) + e\,\log(C_x) + \cdots \quad (1)$$

where $[\text{metal}_{\text{fraction X}}]_{\text{solid}}$ is the concentration of metal fraction in solid, letters such as a, b, c, d and e are constant, and C_x is a measured characteristic of solid, which may include pH, OM content, TP content, and total metal concentration.

2.5. Statistical analysis

The results were presented as the average of three replicates. Statistical analysis was performed using the software SPSS 11.0 for Windows. A probability level of 0.05 was considered to be statistically significant.

3. Results and discussion

3.1. Effect of gut transit on Cu and Zn fractionation

Similar trends of Cu or Zn fractionation were observed in all four pig manure samples (Fig. 1). The exchangeable Cu content in the earthworm casts was significantly lower ($p < 0.05$) by nearly 8% than that of the pig manure. A similar trend was observed for exchangeable Zn. The content of Cu bound to carbonates decreased from 10% to 3% due to the gut transit, but that of Zn increased from 15% to 30%. The significant increase of Zn bound to carbonates can be a consequence of the co-precipitation as $ZnCO_3$ [26]. Earthworm calciferous glands in the esophagus secrete mucus contain calcium carbonate and endue the earthworm to expel excess calcium and carbonate. So, in further studies, the concentration of Ca in the earthworm casts should also be measured. On the other hand, Zn can precipitate with phosphates [27]. This can be related to the increase of Zn fraction bound to carbonates.

The effect of gut transit significantly increased the content of Cu bound to organic matter, corresponding to about 75% of total Cu in the earthworm casts compared to about 60% in the pig manure. This indicated that Cu was mostly present in the organic matter in both the pig manure and the earthworm casts. This is because Cu is associated strongly with organic matter, and the decomposition of organic matter by earthworm tends to promote the formation of mini-molecule organic acid that has a high capacity for Cu^{2+} complexation [28].

Generally, the fraction of Zn bound to organic matter and the residual Zn fraction predominated, which was congruent with the results in the pig manure. However, the contents of Zn bound to organic matter in four pig manure samples decreased from about 50% to about 25% after transit through the gut of *E. fetida*. In contrast to our results, Udovic and Lestan [9] reported the increase (by a factor of 1.5) of Zn bound to organic matter in casts of *L. rubellus* produced in the remediated soil. The diverse results might be attributed to the different species of earthworms and the different research objects.

It has been reported that Cu and Zn present in exchangeable and organically bounded fractions is readily available [29]. Clearly, the bioavailability of Cu in the pig manure increased significantly after transit through the gut of earthworm, whereas the Zn was less bioavailable. This showed that the gut transit played a negative role in Cu immobilization, but it played a positive role in Zn immobilization. It was helpful to reduce diffusion of Zn from the earthworm casts to other substrates. Consequently, the Zn in the earthworm casts would be less available for plants as well as other microorganisms. The decrease of availability might be due to precipitation of insoluble salts, such as phosphates, because TP was present at a high content in the earthworm casts.

The contents of Cu and Zn bound to Fe and Mn oxides in the pig manure were not significantly changed after transit through the earthworm gut. Similarly, the amount of residual Cu fraction in the pig manure was not affected by the transit through the gut of earthworm. However, the content of residual Zn fraction was higher in the earthworm casts than in the pig manure. The phenomenon suggests that the possibility of some heavy metals chelating metallophores produced by earthworms, or by microorganisms present in pig manure, which are affected by earthworm activity [10,11,30]. Besides, the effect of the transit through the gut of *E. fetida* on different heavy metals showed diverse results in this study. This might be due to the differences of physiological characteristics of the earthworm digestive system, or the gut enzyme related to the corresponding metal.

3.2. Effect of gut transit on pH

Earthworms change both physical and chemical properties of ingested matter, including the pH, which is an important factor effecting on the fractionation and mobility of heavy metals. Cheng and Wong [8] reported that earthworm activity increased soil pH due to excretion of calcium compounds into environment by calciferous glands. In contrast to their result, the pH value was significantly lower ($p < 0.05$) in the earthworm casts compared with the pig manure (Table 2). The pH values in all pig manure samples transformed from low alkali to neutral of faintly acid after transit through the earthworm gut, decreasing from 0.46 to 0.67 units, which was consistent with the results reported by Udovic and Lestan [9]. Atiyeh et al. [31] also observed slight changes in pH values in cow manure due to the earthworm activity. The decrease of pH value could be attributed to the bioconversion of the organic matter into intermediate species of organic acids, in view of the decrease of organic matter content in the pig manure. Furthermore, it clearly showed that the higher the pH value of pig manure, the lower the pH value of earthworm casts.

In general, a decreasing pH value enhances the content of metal in exchangeable fraction. Chlopecka et al. [32] reported that lowering the pH increased the share of Zn in the exchangeable fraction. This, however, was not observed in our study, in which the decrease of pH did not promote the content of metal in exchangeable fraction. Instead, the amount of Cu and Zn in exchangeable fraction decreased significantly.

3.3. Effect of gut transit on OM

As well as the pH, OM content decreased significantly ($p < 0.05$). The contents of OM in all pig manure samples decreased from 48.80 to 66.21 g kg^{-1} (Table 2), which showed that the digestive activity in the gut of *E. fetida* played an important role in the decomposition of OM. This is because earthworms ingest a considerable amount of OM, both in order to derive nutrients and the need of well balanced metabolism. Liu et al. [33] also reported that the content of OM in sewage sludge treated with earthworm was less than that without earthworm treatment. In contrary to, Zhang and Schrader [34] observed about 1.2–2-fold increase in total C in earthworm casts compared with surrounding soil. The differences of these results may contribute to the different earthworm species and experimental conditions.

OM is considered as one of physicochemical factors affecting the amount of metal available to earthworm [35]. The decrease of OM in the pig manure affected not only the pH, but also the content of Cu bound to organic matter. As shown in Fig. 1, the content of Cu bound to organic matter increased by about 15% due to the gut transit.

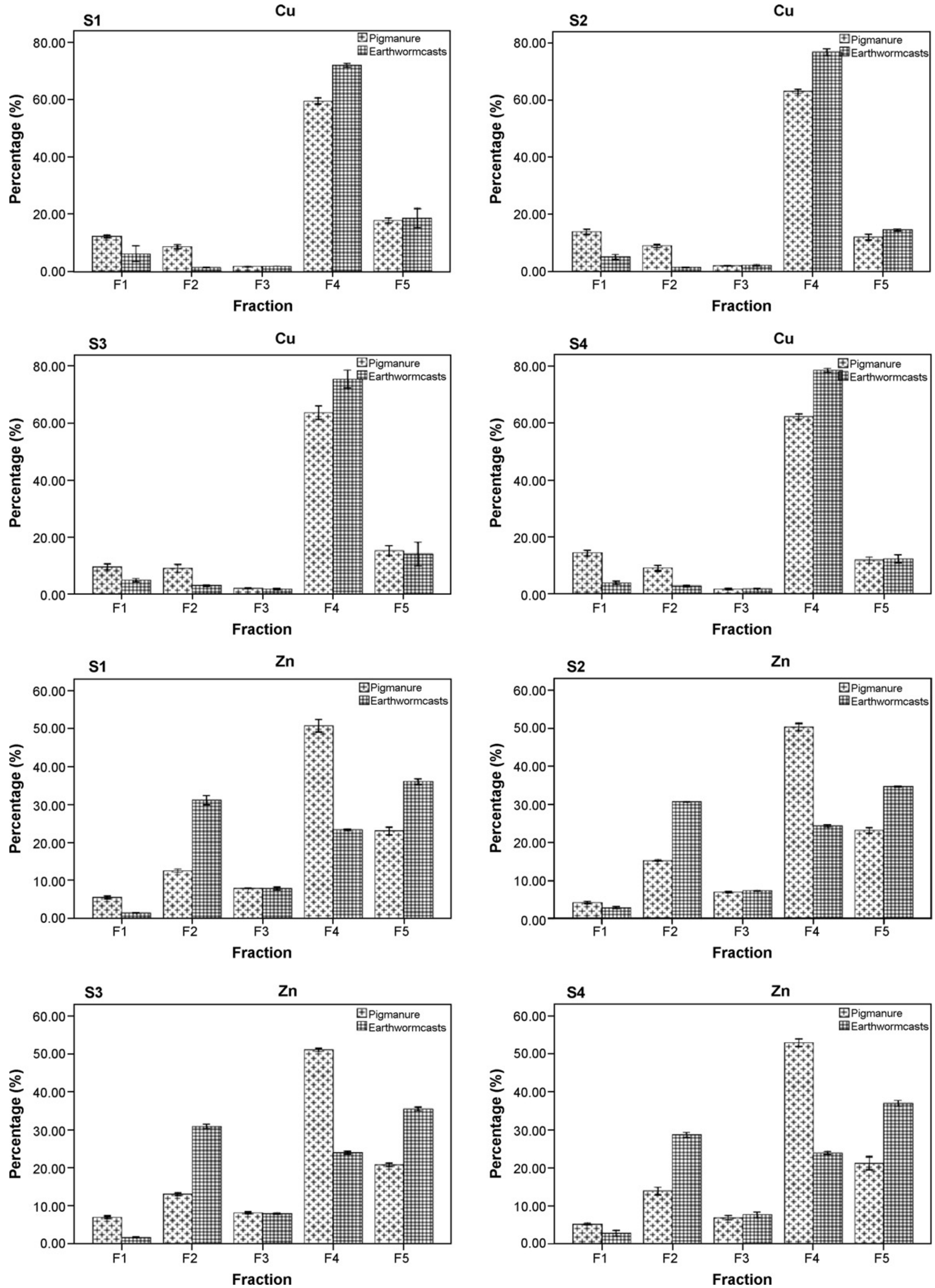

Fig. 1. Changes in fractionation of Cu and Zn (percentage of the metal content determined in all five fractions) in the four pig manure samples after transit through the gut of *Eisenia fetida*. Error bars represent standard deviations ($n = 3$).

3.4. Effect of gut transit on TP

The contents of TP in all pig manure samples were increased from 2.14 to 3.40 g kg^{-1} (Table 2), in good agreement with other reports [36,37]. Buck et al. [13] also reported nutrients, such as P, were generally more concentrated in the earthworm casts than in the parent soil. The remarkable increase of TP indicated that the gut transit played a positive role in the TP. This could be attributed to particle selection operated by earthworms and aggregate instability. Brossard et al. [38] demonstrated that the increase of phosphorous in the earthworm casts could be associated with the selective ingestion of fine soil particles. On the other hand, the increase of TP was most probably due to mineralization of the organic matter [39], which led to relative increase of TP content.

3.5. Heavy metal in earthworm casts

Earthworms are known to inhabit and survive in site where is heavily contaminated with heavy metal [40–42], and the heavy metal would be enriched in the earthworm casts. The total Cu and Zn concentrations in the earthworm casts were higher ($p < 0.05$) than those in the pig manure (Table 2), although the concentrations observed for Zn tended to be higher than Cu, as reported also by Kizilkaya [12]. The increase in Cu and Zn concentrations were by factors of 1.18–3.40 and 1.29–2.46, respectively.

Morgan and Morgan [41] considered that it might be a result of the absorption by organic matter when the heavy metal passed through the earthworm alimentary canal, which led to a significant enrichment of metal in the earthworm casts. However, we speculated that for essential metals, such as Cu and Zn, the mature earthworm had such an ability to regulate metal concentration in its body, and kept a reasonable equilibrium concentration between uptake and excretion of metal for physiological metabolism. So the rate of metal excretion would increase rapidly, surpassing the uptake of metal greatly, if the earthworm was exposed to an environment of high metal pollution level. From this point of view, consequently, the apparently illogical higher concentrations of Cu and Zn in the earthworm casts were observed.

3.6. Relationship between fractionation and factors

In the earthworm casts analyzed, the variability in the distribution of Cu and Zn among different fractions was considerable, and presumably caused by differences in earthworm casts properties that exert an effect on metal fractionation. Stepwise multiple regression analysis was used to show the possible relations and interdependence of Cu and Zn fractionation, and selected earthworm casts properties. The regression equations and statistical parameters are shown in Table 3. All the formulae obtained were statistically significant ($p < 0.05$).

Of the Cu forms, a statistically significant relationship ($p < 0.05$) was found between the Cu bound to carbonates and the earthworm casts TP content. The correlation coefficient was negative - an increasing TP content in the earthworm casts lowered the share of carbonates bound Cu. This further explained why the concentration of Cu bound to carbonates decreased significantly. As described above (Section 3.4, Table 2), the contents of TP in all pig manure samples increased significantly. On the other hand, the decreasing OM content also lowered the share of carbonate bound Cu in the earthworm casts (Table 3). The fraction of Cu bound to organic matter depended and increased with OM content and total Cu concentration in the earthworm casts. Furthermore, the coefficients of the primary and secondary factors had same signs and magnitudes. A significant correlation was also found between the residual Cu fraction and TP and OM contents in the earthworm casts, and the correlation coefficients were positive. Bojeong and Murray [43] reported the quantities of extractable Cu were correlated to the total Cu concentration, regardless of extraction scheme. This was not our case, however, for the total Cu concentration in the earthworm casts was only the secondary factor affecting the fraction of Cu bound to organic matter.

As shown in Table 3, a statistically significant relationship ($p < 0.05$) was found between the fractions of Zn and the total Zn concentration in the earthworm casts. Fraction of Zn bound to organic matter and in the exchangeable fraction decreased with the total Zn concentration in the earthworm casts. In contrast to the before-mentioned two fractions, the residual Zn fraction increased with the total Zn concentration in the earthworm casts.

Table 2
Changes in pH, organic matter, total phosphorous and total metal contents of pig manure after transit through the gut of *Eisenia fetida*.

	pH		OM[a] ($g\,kg^{-1}$)		TP[b] ($g\,kg^{-1}$)		Total Cu ($mg\,kg^{-1}$)		Total Zn ($mg\,kg^{-1}$)	
	Pig manure	Casts	Pig manure	Casts	Pig manure	Casts	Pig manure	Casts	Pig manure	Casts
S1[c]	7.53 ± 0.03[d]	6.86 ± 0.04	222.72 ± 3.97	156.52 ± 4.43	41.59 ± 0.02	45.00 ± 0.02	932.82 ± 3.83	1555.91 ± 17.92	1383.42 ± 5.27	3397.52 ± 47.77
S2	7.41 ± 0.03	6.95 ± 0.02	178.60 ± 2.70	129.80 ± 1.01	44.92 ± 0.16	47.06 ± 0.27	819.20 ± 23.79	1470.20 ± 19.49	2520.61 ± 119.04	3282.25 ± 35.93
S3	7.43 ± 0.04	6.94 ± 0.03	202.77 ± 6.14	148.28 ± 2.41	47.11 ± 0.16	49.64 ± 0.28	445.11 ± 6.20	1378.80 ± 4.04	1791.97 ± 20.35	3037.58 ± 85.02
S4	7.51 ± 0.02	6.93 ± 0.04	193.04 ± 3.96	131.32 ± 3.78	40.17 ± 0.28	43.15 ± 0.24	1410.95 ± 34.92	1658.71 ± 50.17	2822.47 ± 115.27	3644.37 ± 67.72

[a] Represents organic matter.
[b] Represents total phosphorous.
[c] Represents four different sites.
[d] The values are represented as means of three replicates ± s.d.

Table 3
Correlation between fractionation of metal and earthworm casts characteristics according to: $\log[\text{metal}_{\text{fractionX}}]_{\text{solid}} = a + b\log(C_1) + c\log(C_2) + d\log(C_3) + e\log(C_x) + \cdots$.

Metal	Fraction	Regression equation obtained	Statistics			
			n	R^2_{adj}	F	p
Cu	F2[a]	$(-13.52 \pm 2.02) \cdot \log \text{TP}^{b} + (8.91 \pm 1.30) \cdot \log \text{OM}^{c} + (4.84 \pm 4.67)$	12	0.93	54.04	0.000
	F4	$(0.82 \pm 0.14) \cdot \log \text{OM} + (0.55 \pm 0.18) \cdot \log \text{To}^{d} + (-0.50 \pm 0.60)$	12	0.88	25.56	0.002
	F5	$(5.71 \pm 1.05) \cdot \log \text{TP} + (3.63 \pm 0.67) \cdot \log \text{OM} + (0.43 \pm 2.42)$	12	0.90	34.51	0.001
Zn	F1	$(-10.45 \pm 1.87) \cdot \log \text{To}^{e} + (38.50 \pm 6.58)$	12	0.81	31.32	0.001
	F4	$(-4.77 \pm 1.58) \cdot \log \text{To} + (-13.83 \pm 5.56)$	12	0.54	9.16	0.023
	F5	$(12.87 \pm 3.62) \cdot \log \text{To} + (48.17 \pm 12.78)$	12	0.62	12.60	0.012

[a] Represents fraction of metal in earthworm casts.
[b] Represents total phosphorous content of earthworm casts.
[c] Represents total organic matter content of earthworm casts.
[d] Represents total Cu concentration of earthworm casts.
[e] Represents total Zn concentration of earthworm casts.

This was congruent with the results reported by Leatan et al. [44], although they studied the relationship between Zn fractionation and soil properties. Clearly, we can modify the fraction redistribution of Zn in the earthworm casts by regulating the total Zn concentration, in order to reduce the risks of available Zn in the earthworm casts. Chlopecka et al. [32] reported that the fraction Zn associated with soil organic matter was not affected, while Zn bound to carbonates increased with the total Zn concentration. All of their results were quite different from our results. The diverse results might be ascribed to the different research objects that were soil samples and earthworm casts respectively.

In addition to OM and TP contents, and total metal concentration, the pH did not statistically significantly influence the fractionation of Cu and Zn (Table 3). Torri and Lavado [45] reported Zn bound to inorganic fraction depended on soil pH rather than other factors. They found a positive and significant correlation between the exchangeable Zn and soil pH. Our results indicate, however, that the fractionation of Cu and Zn in the earthworm casts was not affected by pH.

4. Conclusion

The results of our study clearly show that the changes in fractionation of Cu and Zn in pig manure are significant after transit through the gut of earthworms (*E. fetida*). The earthworms can redistribute some heavy metals (e.g. Zn) ingested towards less mobile and bioavailable forms. This is beneficial to reduce diffusion of Zn from earthworm casts to other substrates. Furthermore, in order to modify the fractionation of metal in the earthworm casts, we can regulate the physical and chemical characteristics of the earthworm casts, such as OM and TP. The selected physical and chemical properties in pig manure affecting metal fractionation were altered significantly by the transit through the earthworm gut. Similar results reported by Atiyeh et al. [31], although they studied the effect of earthworm (*Eisenia andre*) activity on the biochemical properties of cow manure.

However, further researches are needed to ascertain whether other earthworm species have the similar effect on the fraction redistribution of heavy metals. More importantly, further works are also needed to investigate the mechanism for those significant changes, in order to reduce the environmental risks of heavy metals in the earthworm casts.

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