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Do Earthworms Mobilize Fixed Zinc from Ingested Soil?

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A wide range of organisms inhabit the soil and has to deal with soil-bound metals. The bioavailable fraction of metals may be estimated explicitly using the isotopic dilution technique. In the present paper, we evaluated the isotopic exchange technique for assessing the bioavailability of soil Zn (using ^{65}Zn) to earthworms. To validate the technique, the worms were first exposed to various ^{65}Zn levels, and errors due to soil entrained in the gut were evaluated. This exposure indicated no effect of γ-radiation on growth (wet weight gain) of the organisms and that depuration of the earthworms minimized errors in labile pools determined by isotopic dilution. Our study further showed that the earthworms accessed 55−65% of the total Zn in the soil. The labile pool for the earthworms *Eisenia andrei* was similar to that for the plant *Lactuca sativa,* indicating that earthworms and plants to a large extent access the same fraction of soil Zn. Hence, the isotopic dilution technique has the potential to assess biologically available pool of Zn in soils. As lettuce is not known to significantly mobilize nonlabile metals in soil, this study indicates that Zn uptake by *E. andrei* is predominantly via the exchangeable pools (possibly the soil pore water) rather than dissolution of Zn held within soil particles or within soil organic matter or other food sources.

Introduction

Soils contain and receive a wide range of metals. For each metal, a portion is geogenic, and the rest is derived from anthropogenic inputs such as aerial deposition or from the addition of sewage sludge, fertilizer, or mine waste. These metals are bound in various pools within the soil and vary in their availability to organisms. For example, geogenic metals are often tightly bound to or within soil particles, whereas anthropogenic metals are often bound in more exchangeable and labile pools (*1*, *2*). The sizes of these pools vary, and even a relatively small increase in the total content may cause a large increase in, for example, the exchangeable pool if the metal is added in soluble form. The main determinants for the relative distribution and size of these pools are the physicochemical characteristics of the soil (e.g., soil mineralogy, clay, organic matter, and pH) and possibly also biological factors but also depend on the soil of contamination.

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A wide range of organisms inhabits the soil and has to deal with soil-bound metals. The pathways of metal exposure of these organisms depend on the life-pattern of the organism. For example, earthworms ingest soil particles (*3*, *4*) and many have assumed that they access less labile metal pools as compared to plants, which are believed to take up metals mainly from the soil solution (*5*) and metal pools in equilibrium with the solution. There have been few studies concerning the biological available fraction of soil Zn to invertebrates. These studies generally employed simple chemical extraction procedures that involve mixing of the soil with the extractant, such as water or calcium chloride, after the organisms have been removed and then measuring the Zn concentration in the extraction fluid (*6*−*8*). Such studies usually lack the ability to clearly define the biologically available pool but rather rely on correlation analysis.

The bioavailable fraction of metals may be estimated more explicitly using isotopic dilution principles. The isotopic dilution technique is based on the principle that the added soluble isotope will "equilibrate" with the most labile pool of metal in the soil. In reality, equilibrium is rarely reached during the time course of most experiments, and there is a continuing reaction of isotope with the soil. Measurement of the specific activity of metal in the organism (ratio of radioisotope to unlabeled metal) gives information as to the size of the soil pool detected by the organism. Working with radioactive phosphorus (^{32}P), Larsen coined the term "*L* value" to describe the size of the plant-labile P pool in soils (*9*). Since then, metal availability to plants has been estimated with the use of isotope dilution techniques in several studies (e.g., refs *5*, *10*, and *11*). Using this technique, the ability of rape plants (*Brassica napus* L.) to mobilize P in the rhizosphere was demonstrated (*12*), allowing it to access forms of P not in rapid equilibrium with soil solution. Sheppard et al. (*13*) used isotopes (I, Cs, Mn, Zn, and Cd) to assess uptake and elimination rates of metals in ingested food by earthworms.

In the present paper, we evaluated the isotopic exchange technique for assessing the bioavailability of soil Zn to earthworms in order to determine the relative importance of exchangeable pools and soil pathways of exposure. The specific activity of Zn accumulated by earthworms was compared to that of plants grown in the same soil to determine if earthworms can mobilize soil Zn not available to higher plants. Our hypothesis was that the specific activity of Zn accumulated by earthworms would be lower than that accumulated by plants and that this difference could be used to determine the relative importance of soil exchangeable pools (probably pore water) relative to other pathways of earthworm exposure to soil Zn.

Materials and Methods

Organisms. *Eisenia andrei* was obtained from a local commercial outdoor culture and incubated in a laboratory culture using the same soil as for the experiment. Juvenile *E. andrei* were incubated under conditions identical to the experimental conditions (in the absence of ^{65}Zn) for 3 weeks prior to introduction of earthworms into spiked soils. The juvenile worms ranged in live weight from 10 to 30 mg and were maintained on moist filter paper for 24 h to allow them to evacuate the gut before introduction into treated soils. The worms were randomly assigned to the replicated to ensure no bias distribution.

Lactuca sativa cv. Marksman (lettuce) was used as the test plant for comparison of metal availability between plant and invertebrates.

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Soil. The soil was sampled from a dairy property approximately 50 km south of Adelaide, Australia. For the initial characterization of the soil, pH was determined in a 1:5 soil:water extract (*14*). Total soil carbon content was determined by LECO furnace (*14*) and cation exchange capacity (CEC) by leaching with 1.0 m NH_4Cl (*14*).

Carrier-free zinc (^{65}Zn, activity at day of use 35.5 MBq/mL) isotope was mixed into soils by spraying a solution of ^{65}Zn onto the soil. This was done by spreading out the soil on a tray and using an atomizer spray to distribute the ^{65}Zn, while continuous mixing was performed with a Teflon spatula to ensure a homogeneous distribution of ^{65}Zn in the soil. Soils were then incubated for 20 days at 50% of maximum water holding capacity (MWHC) prior to introduction of the worms into the labeled soil.

Growth Conditions. *Eisenia andrei*. The earthworm experiment was conducted in sealed, but not airtight, plastic containers containing 300 g of dry soil. To assess if γ-radiation from the ^{65}Zn could affect earthworm growth, the earthworms (5 per pot) were exposed to several activities of ^{65}Zn—0, 400, 800, and 1600 KBq/kg of soil on a dry weight basis—with three replicates per exposure. The experiment was continued for 42 days at a constant temperature of 25 °C. No food was added, and water lost from the containers by evaporation was replenished every 7 days.

Lactuca sativa. *Latuca sativa* were grown in soil having ^{65}Zn activities of 400 KBq/kg of soil on a dry weight basis, with three replicated pots per exposure, as described above. Twelve *L. sativa* Iceberg cv. Marksman seeds were planted into each pot containing 180 g of soil prewatered to 50% MWHC. Pots were maintained at constant temperature (25 °C) and placed in a growth room with a 12 h day/night cycle. Ten days after planting, seedlings were thinned to 4 plants per pot. Pots were watered daily to 50% MWHC by weight, and plants were harvested on day 36.

Sample Preparation, Analysis, and Measurements. At the end of the experiment the worms were collected, counted, and weighed after 24 h of depuration on moist filter paper. The depuration time (24 h) was optimized prior to the experiment to ensure full emptying of the gut in order to avoid errors in determination of specific activities of absorbed Zn.

Lettuce plants were cut 1 cm above the soil surface at harvest, and the fresh shoot weight per plant was determined immediately, while the dry weight was determined after drying at 60 °C.

All dried biological material (worms and plants) was digested with concentrated nitric acid (20 mL of acid/100 mg of tissue heated to a maximum temperature of 130 °C), and metals in the digest solutions were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES) (*15*). Soil pH and EC were measured in a 1:5 soil:water extract (*14*). All soils were also digested using aqua regia (1 g of soil, 10 mL of acid), and total metal concentrations in digests were determined with ICP-AES. Activities of ^{65}Zn in plant, earthworm, and soil digests were determined using γ-spectroscopy.

Determination of Plant and Earthworm Labile Zn Pools in Soils. The isotopically exchangeable pool of soil Zn as assessed by the worms and plants was determined using the specific activity of Zn in the organisms (eq 1);

$$\text{specific activity} = \frac{^{65}\text{Zn}_{\text{final organism}}}{(\text{Zn}_{\text{final organism}} - \text{Zn}_{\text{start organism}})} \quad (1)$$

The units were $Zn_{\text{final organism}}$ (Bq) and unlabeled Zn (μg). Amounts of Zn in juvenile worms ($Zn_{\text{start organism}}$) were not subtracted from the final Zn content of the adult worms since it, at most, accounted for a 10% error (due to the small size of juveniles as compared to adults). Corrections for seed Zn in the lettuce plants was also unnecessary due to the small amount of Zn in seeds as a proportion of plant uptake. Labile Zn pools in soil, as determined by the two organisms, was calculated as a percentage of total Zn in the soil as follows (eqs 2 and 3):

$$\text{labile Zn pool} = \frac{\text{isotope activity in soil (Bq/g of soil)}}{\text{specific activity in organism (Bq/}\mu\text{g of Zn)}} \quad (2)$$

$$\%\ \text{labile} = \frac{\text{labile pool (}\mu\text{g/g of soil)}}{\text{total metal in soil (}\mu\text{g/g of soil)}} \times 100 \quad (3)$$

Statistics. The data were checked for normality using a Kolmogorov−Smirnov test and for homogeneity of variance by Barlett's test. The exposure regimes were compared using one-way ANOVA with a posthoc Tukey's studentized range (HSD) test (*16*).

TABLE 1. Mean (± SEM) Growth of *Eisenia andrei* Exposed to Various Levels of ^{65}Zn in a Sandy Soil

radiation level (KBq/kg)	growth (g dw/pot)	± SEM
400	0.1704	0.0235
800	0.1928	0.0233
1600	0.1754	0.0122

TABLE 2. Internal (mean ± SEM) Zn Concentration of *Eisenia andrei* and *Lactuca sativa* Exposed to Various Levels of ^{65}Zn in a Sandy Loam Soil

radiation level (KBq/kg)	organism	Zn (μg/g dry weight)	± SEM
0	*E. andrei*	96.8	1.96
400	*E. andrei*	83.5	4.98
800	*E. andrei*	91.0	2.12
1600	*E. andrei*	88.3	2.73
400	*L. sativa*	59.2	8.32

Results

Soils. Soil $pH_{1:5\ \text{soil:water}}$ was 6.8, and cation exchange capacity (CEC (NH_4)) was 15.8 cmol(+)/kg. The soils contained 10.9% clay, 12.4% silt, 54.2% fine sand, 11.5% coarse sand (Sandy loam), 11% organic matter, and 5.5% total carbon. Total Zn concentration was 15 mg/kg.

Method Development. No differences in wet weight gain in earthworms were observed between the various radiation exposure scenarios, indicating no effect of γ-radiation on the organisms ($p \leq 0.05$) (Table 1). The internal Zn concentration was similar for all exposure levels, and the concentrations corresponded with previous observed values for this species (*17*; Table 2). Total Zn uptake by earthworms was 11.2 ± 1.4 μg of Zn/pot, and by lettuce plants was 48.7 ± 8.4 μg of Zn/pot during the exposure period. For one plant replicate, the internal total Zn concentration was 50% higher than the three other replicates, which resulted in an increased relative availability for this plant. Scrutinizing the data, we found that the most likely explanation for this was a contamination of the metal digest tube, and hence an artifact, as this value was more that 5 SD from the mean of the three other values. The replicate has been mentioned as "outlier?" in the text of Figure 1.

One potential source of error using the isotopic exchange technique is the soil Zn content of the earthworm's gut. In the present experiment, the earthworms were depurated for 24 h. The optimal time required for minimizing errors due to the gut soil content was studied in an experiment, and as

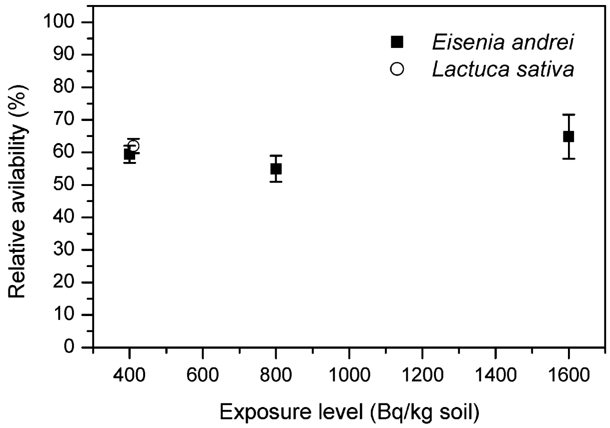

FIGURE 1. Relative availability (mean ± SEM) of exchangeable Zn to earthworm *Eisenia andrei* as compared to the relative availability to the plant *Lactuca sativa*. An assumed "outlier" value for *L. sativa* is not presented in the graph as this was more than 5 SD for the mean of the three other replicates.

TABLE 3. Influence of Soil-Bound Zn in Earthworm Gut on Total Earthworm Zinc Content following Different Depuration Periods

depuration (h)	worm ash (%)	soil ash (%)	max soil in worm[a] (%)	worm Zn (μg/g dry wt)	soil Zn (μg/g dry wt)	Zn from gut soil of total earthworm content (%)
0	18.9	85	16.07	85	14	3.05
1	15.3	85	13.00	85	14	2.40
2	12.6	85	10.71	85	14	1.94
3	13.7	85	11.66	85	14	2.12
4	10.6	85	9.01	85	14	1.60
24	2.4	85	2.04	85	14	0.34

[a] Assuming less than 1% ash in earthworm tissue and 85% (measured) ash in soil.

seen from Table 3, the error when using 24 h of depuration was less than 1%.

Determination of the Available Zn Pool. With the present method the labile (or available) fraction was consistent over all exposures with approximately 55−60% relative availability of the total soil Zn (Figure 1). The pool of soil Zn available to *L. sativa* was identical ($p \leq 0.001$) to that of *E. andrei* (Figure 1). Even if a data "outlier" (see text for Figure 1) is included in the analysis, the pool of soil Zn seen by the two organisms is similar.

Discussion

The present study shows that the isotopic dilution technique is a useful technique to identify the pathways of exposure of earthworms to soil metals. Effects of radiation on earthworm growth were insignificant (Table 1). The growth and total Zn uptake by the worms corresponds with previous studies for this and similar species (*6*, *7*, *15*). The small differences between replicates and the fact that the worms would most likely be in contact with all the soil in the pot during the 42 days of exposure indicates that this is a precise technique. Compared to soil extraction techniques, the isotopic dilution technique is a more biologically relevant assessment of the pool of soil metal available to the organism. However, it should be remembered that the available Zn pool measured using this technique may not necessarily correlate better with earthworm Zn uptake or metal toxicity as it is a measure of the pool of soil Zn *potentially* available to the organism. Not all of this soil Zn pool is taken up by the organisms during the assessment—the plants and earthworms only remove a very small proportion of the labile pool.

Bioavailability of metals in soil to invertebrates has been estimated in various ways, for example, based on the ratio between the external total and the internal total and the ratio between the internal total and a chemical extractable fraction of the external (e.g., H_2O- and $CaCl_2$-extractable) (*6*−*8*). These previous studies suggested that the primary route of uptake for Zn in earthworms is through the pore water. However, these were correlative approaches and merely indicated that Zn uptake was related to the most labile metal fractions in soil. Furthermore, these studies were performed in contaminated soils, where the extractable pool of Zn is large. Recently, Saxe et al. (*18*) proposed a model for predicting metal uptake by earthworms, with the assumption that metal entry to the organism was predominantly through soil solution, either dermally or through the gut wall. No direct evidence was presented to verify this assumption. Isotopic dilution provides a definitive answer regarding the pathways of Zn exposure to earthworms. We chose a soil with low available Zn so that mobilization of Zn from soil minerals and organic matter in the soil by the earthworms would be maximized. The isotopic dilution technique offers a dynamic approach to the metal availability by integrating microhabitat conditions created by the organism in the soil. Isotopic data are needed from a wider range of soils and for other soil invertebrates.If these also indicate that exposure is predominantly via soil pore water and associated solid-phase pools, then models such as those of Saxe et al. (*18*) may be widely applicable.

Data from our study has shown that the earthworms had access to 55−65% of the total Zn in the soil. The labile pool for the earthworms *E. andrei* was similar to, or a little less than, the same pool for the plant *L. sativa*, indicating that earthworms and plants to a large extent access the same fraction of the soil Zn pool in the soil tested here. If, as previously suggested, plants access Zn from the soil solution and readily exchangeable pools, this is likely also to be the case for the worms. This also supports the previous speculation that earthworms mainly take up Zn via the soil solution (*7*, *18*, *19*). While specific activities in plants and earthworms were similar, it could be argued that the lettuce plants also mobilized soil Zn to the same extent as the earthworms. This is most unlikely, as lettuce is not a hyperaccumulator of Zn. In previous isotope dilution studies examining a wide range of plants, it has been found that all plants appear to access the same pool of Zn in soils, which is in equilibrium with the soil solution (*5*, *10*).

The similarity in availability is surprising, as we believed that earthworms would access a larger fraction of the more strongly bound metal since they ingest soil particles. Indeed, for snails (*Helix aspersa* Müller), Scheifler et al. (*20*) concluded that approximately 18% of the Cd in these organisms was derived from the nonlabile Cd pool. This could well indicate that various soil invertebrates do access different metal pools, although keeping in mind that two different metals and very different experimental techniques were used. The conclusions from Scheifler et al. (*20*) were interesting in that organisms were exposed for only a short duration of their lifetime; the soil was highly polluted and hence would have had high amounts of exchangeable Cd; and the animals were fed an unlabeled food source, which introduced additional error into determination of specific activities of soil-derived Cd.

It should be noted in our experiments that although the labile pool is similar for the two organisms we used, the earthworms accumulated a higher internal Zn concentration than the plants during a similar exposure interval, although total Zn uptake was greater for the plants. For plants, Tiller et al. (*10*) and Hamon et al. (*5*) showed that species accessing the same soil metal pool could accumulate very different internal metals levels, which is in agreement with the observations from the present experiment. Hence, it is apparently not primarily the metal pool accessed that determines the Zn uptake for an organism, rather it is species-related factors. In the present study additional food sources, such as cow dung or litter, were not introduced, unlike the study of Scheifler et al. (*20*). Such surface-deposited food could be speculated as an important Zn source for the earthworm, but to introduce unlabeled food in the experiments would have compounded errors in determining the specific activity of soil-derived Zn. Furthermore, Sheppard et al. (*13*) studied the uptake in *Lumbricus terrestris* of ^{65}Zn from the food and found this source to constitute a small fraction of the total uptake. In addition, given the fact that Spurgeon and Hopkin (*6*) observed a very rapid Zn uptake from the soil and the fact that worms in the present experiment contained the assumed physiological required levels (approximately 80 μg of Zn/g of dry tissue), we would contend that food is less likely to be an important contributor to the earthworm Zn budget.

In conclusion, isotopic dilution techniques have the potential to assess the biologically available pool of Zn in soils for soil invertebrates. The pools from which Zn is taken up by *E. andrei* correspond to pools from which lettuce takes up Zn.

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