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Effects of Zinc Exposure on Earthworms, *Lumbricus terrestris*, in an Artificial Soil

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Abstract Earthworms have the potential to act as trophic links for pollutants that accumulate in urban soils. However, many pollutants may act as micronutrients at low concentrations and toxins at higher concentration. When pollutants are also micronutrients, bioaccumulations may initially increase trophic transfer as pollutant concentration increase, but at higher levels toxic effects may limit population size and the potential for trophic transfer. We found support for this model among earthworms exposed to a range of soil Zn levels. Worms showed increasing bioaccumulation of Zn with increasing Zn soil concentrations, but at higher Zn levels worm growth rates decreased.

Keywords Trophic transfer · Metals · Earthworms · Micronutrients · Toxic effects

Earthworms are common components of the soil biota in urban ecosystems and may serve as an important link for trace element transfer of soil contaminants into terrestrial and aquatic food webs. Earthworms directly process soil through their digestive system and may accumulate high levels of trace elements when occupying contaminated soil. Some trace elements (e.g., Cu and Zn) may be both required micro-nutrients and a pollutant at higher environmental concentrations (Spurgeon and Hopkins 1999; Peijnenburg et al. 1999; Vijver et al. 2006; Nahmani et al. 2009). Therefore, some trace elements may act to enhance transfer into terrestrial and aquatic food webs by both enhancing body burdens and earthworm populations simultaneously. On the other hand, these same trace elements can be toxic when at high levels (Nahmani et al. 2007 and references therein), effectively reducing earthworm populations and potential transfer of trace elements into food webs. Therefore, for essential elements, the potential role of earthworms as trophic links for pollutants accumulated in urban soils depends on both the effects of pollutant concentrations on bioaccumulation and earthworm population size. However, few studies have simultaneously investigated the effects on bioaccumulation and population size.

Anthropogenically derived Zn is a significant pollutant of urban soils. Sources of Zn in urban environments include wear of tires and other automobile parts that collect on road surfaces (Councell et al. 2004), collectively referred to as road dust. Road dust is transported to storm water receiving systems during runoff events, resulting in increased Zn concentrations in soils of these systems. Earthworms regulate body Zn concentrations (Sturzenbaum et al. 2001; Demuynck et al. 2007). In intestinal (gut) tissue, defined as the intestine tissue posterior of the clitellum, tissue Zn concentrations increase linearly with Zn concentrations in road dust. In the body, defined as the epidermis posterior of the clitellum, Zn tissue concentrations change little with increasing road dust Zn concentrations (Lev et al. 2008). However, roadway dust is known to contain high levels of Zn and Cu along with other inorganic and organic contaminants (Davis et al. 2001; Councell et al. 2004). The purpose of this study was to isolate the effect of Zn concentration on the earthworm *Lumbricus terrestris* and evaluate the potential for Zn storage in the gut and body tissues at concentrations relevant to urban ecosystems.

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Materials and Methods

We focused on the earthworm *L. terrestris* because it is common in storm water retention basin soils in the Baltimore-Washington region. To investigate the influence of Zn concentrations on gut tissue and body tissue Zn concentration and health we used a standard laboratory approach for determining the toxicity of metals to soil organisms (Lokke et al. 2002). Although the time to reach a steady state concentration will vary by species and soil type, Zn is generally thought to equilibrate rapidly in earthworms exposed in OECD soil (Peijnenburg et al. 1999). Therefore, a 14 day range finding test was conducted (0 to 1,000 mg kg^{-1}) to ensure organisms would survive in laboratory conditions and to design a 21 day definitive test (4.7 to 476.3 mg kg^{-1}) that would allow more accurate modeling of earthworm responses at field relevant Zn concentrations. Here we present results for the definitive test.

Adult earthworms were obtained from Carolina Biological Supply Company (Burlington, NC) and were equilibrated in OECD artificial soil (with a composition by weight of 35% < 200 μm quartz sand, 35% > 200 μm quartz sand, 20% kaolinite clay, and 10% Sphagnum peat moss) prior to each experiment (OECD 2000). The OECD soil was amended with $ZnCl_2$ and calcium carbonate powder was added to adjust pH to approximately 6. Target Zn concentrations for the range finding experiment were: 0, 50, 500, and 1,000 mg kg^{-1}. Based on the results of the range finding experiment, target Zn concentrations for the 21 day definitive test were (measured values in parenthesis) 0 (4.7), 20 (22.9), 60 (52.6), 100 (106.6), 150 (130.7), 200 (173), 250 (259.6), 300 (287.8), 350 (372.5), 400 (399.9), and 450 (476.3) mg kg^{-1}. Measured values for the exposure soils were determined on splits collected from each of the bulk amendments prior to the addition of earthworms. Three replicates were used at each amendment level. Three earthworms were weighed and placed into each microcosm containing 300 g of OECD soil. Each microcosm was placed inside an incubator and held at 70% humidity and 16 ± 3°C on a 12 h dark:light cycle. Microcosms were monitored for mortality. Dead worms were removed when discovered.

At the end of the experimental period, earthworms were re-weighed, euthanized in ethanol, and preserved in a 4% formaldehyde solution. This approach deviates from the standard toxicity protocols but was necessary to separate the gut tissue from the body before the loss of Zn, which might occur if the organisms were allowed to purge gut contents during a depuration period. To prevent any potential metal loss from preserved tissues, earthworms were dissected within 48 h of preservation. This short-term storage in dilute formalin is not expected to leach Zn from tissues (Gellein et al. 2008 and references therein). Gut contents were rinsed out using deionised water and the gut and the body tissues from the posterior portion of the worm were removed. Samples were dried in a Teflon beaker for 24 h at 50°C. Dried samples were digested in a sealed Teflon vial at 150°C for 12 h using trace metal grade 7 N HNO_3 followed by 30% H_2O_2. Digested samples were analyzed for Zn using a ThermoElemental PQExCell ICP-MS. Each block of digested samples was analyzed with the external standard (NIST SRM 2709), replicates, and method blanks. The Zn concentration of the experimental soils was determined using the same methods with the omission of the 30% H_2O_2 digestion step. Measured Zn values for the standard reference material were within 85–105% recovery, blanks were below the detection limit (1 μg L^{-1}), and replicate samples were typically within 10% of the initial measured value over the data collection period.

For both gut and body tissue concentrations of Zn, we anticipated two potential responses. If body concentrations were regulated tightly, we expected body concentrations to rise to an asymptote as exposure concentrations increased. Alternatively, if regulation was poor, then the relationship between exposure concentration and body concentration will be near linear. For simplicity we represented our asymptotic and linear hypotheses using a second-order polynomial and linear model, respectively. Similarly, we considered both a polynomial and linear response for gut concentrations. We used a generalized linear modeling approach as implemented in the GENMOD procedure of SAS to fit both models and compared models using Akaike Information Criteria (AIC) and model weights (w_i; Burnham and Anderson 2002). Because gut and body burdens of Zn were normally distributed, we included a normally distributed error term in the models. We corrected AIC values for small sample size (AICc) and evaluated the significance of parameters in each model using Wald chi-squared tests. We also calculated 95% Wald confidence intervals for model parameters.

Because we also had two hypotheses concerning relationships between sublethal effects of Zn, we followed the same analysis procedure as described above for body burdens. The simple hypothesis that Zn would have a consistently increasing impact on growth was represented by a linear model and the potential for Zn to be a limiting factor on growth generating a unimodal response (Lev et al. 2008; Kamitani and Kaneko 2007; Lukkari et al. 2004) was represented by a 2nd order polynomial model.

Results and Discussion

At the end of the exposure period in the definitive test, Zn concentration in the gut tissue ranged from 690.2 to

1,901.3 mg kg^{-1} and from 40.5 to 89.3 mg kg^{-1} in the body (Table 1). Concentrations of Zn in gut tissues showed a linear response to increasing Zn exposure (Fig. 1a). Both the linear and second-order polynomial models had similar weights ($w_i = 0.53$ and 0.47, respectively), but the polynomial term was not significant (Table 2) in the polynomial model. In contrast, body tissue Zn concentrations were best modeled by a polynomial equation (Fig. 1b); the relative support of polynomial model ($w_i = 0.78$) was more than three times that of the linear model ($w_i = 0.22$) and all terms in the model were significant (Table 2).

The mean weight change of worms from each exposure level over the experimental period ranged from −1.9% to −16.1%. The lack of growth for all treatments (with the exception of one earthworm), even the control, was not unexpected since there was no supplemental food source added to maximize exposure to the soil. Both the linear and the polymonial models provided a significant fit for the relationship between growth and Zn exposure concentration (Table 2). However, the polynomial model was much more supported ($w = 0.88$) and accounted for more of the variation ($R^2 = 0.45$) than the linear model ($w = 0.12$;

Table 1 Means and standard errors (in parentheses) of *Lumbricus terrestris* response to Zn exposure in OEDC soils

Zn dose	% Survival	% Weight change	Gut tissue (mg kg^{-1})	Body tissue (mg kg^{-1})
4.7	66.7	−16.1 (0.1)	720.2 (243.2)	55.8 (15.3)
22.9	100	−15.1 (3.1)	690.2 (102)	40.5 (2.2)
52.7	66.7	−17.1 (2.6)	848.9 (136.1)	54.4 (11.8)
106.6	100	−13.6 (5)	1260.7 (255.8)	49.4 (5.9)
130.7	100	−9.4 (2.4)	1133.7 (127.5)	77.5 (9.1)
173	66.7	−9.7 (2.4)	1234.8 (192.1)	74.9 (11.9)
259.6	100	−7.9 (1.9)	1284.3 (123.1)	74.2 (12.8)
275.8	100	−6.8 (2.9)	1487.7 (380)	74.2 (14.3)
287.8	100	−1.9 (1.9)	1154.3 (266.2)	86.7 (9.9)
399.9	100	−5.5 (3.8)	1877.5 (508.5)	89.3 (7.7)
372.5	66.7	−4.8 (0.9)	1037 (209.5)	78.1 (15.4)
476.3	100	−8.7 (4.8)	1910.3 (424.4)	81.4 (23.3)

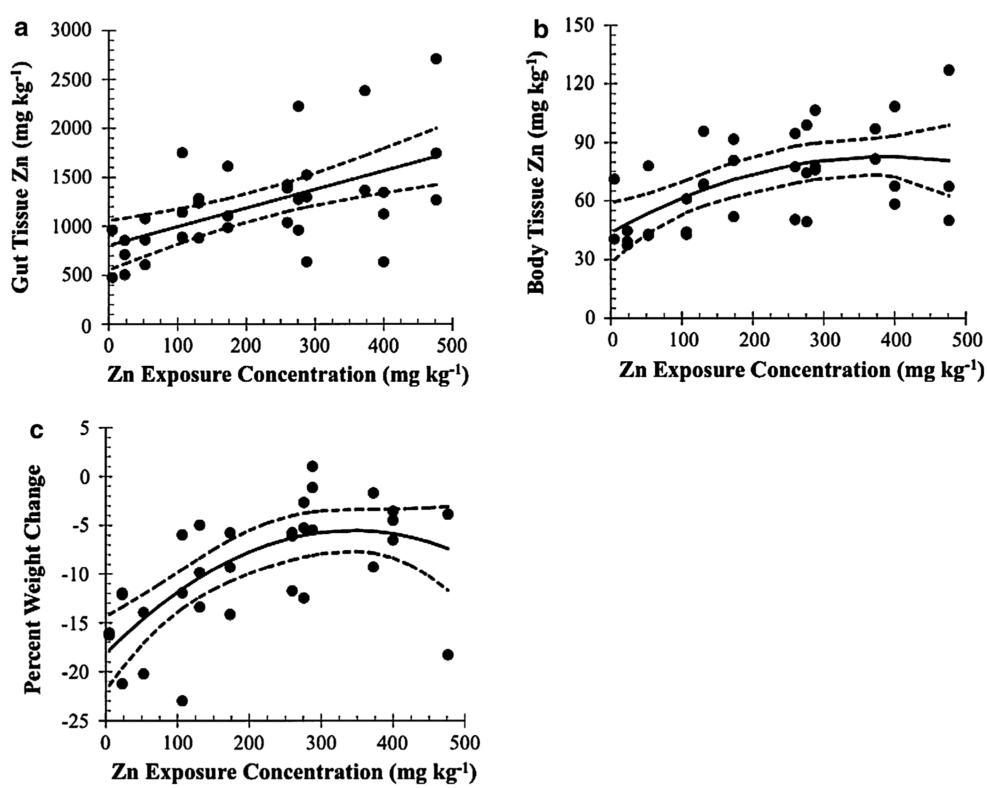

Fig. 1 Relationships between Zn exposure concentration and Zn tissue concentration in *L. terrestris* after the 21 day exposure period are plotted in **a** and **b**. *Solid lines* represent the fit of a linear model (**a**) or a 2nd order polynomial model (**b**) to the data. *Dashed lines* represent 95% confidence envelopes. In (**c**), Zn exposure concentration and percent change in weight of *L. terrestris* after the 21 day exposure period are plotted. The *solid line* represents the fit of a 2nd order polynomial model to describe a uni-modal response. *Dashed lines* represent the 95% confidence envelope

Table 2 Parameter estimates, standard errors, and significant for models of Zn effects on *Lumbricus terrestris* growth and bioaccumulation

Response	Model	Parameter	DF	Estimate	Standard Error	*P*
Percent Growth	Linear	Intercept	1	764.5776	125.3387	<0.001
		Dose	1	2.2075	0.4848	<0.001
		Scale	1	396.6293	49.5787	
	Polynomial	Intercept	1	−18.1261	1.9216	<0.001
		Dose	1	0.0733	0.0192	<0.001
		Dose 2	1	−0.0001	<0.0001	0.007
		Scale	1	4.5119	0.5554	
Gut Zn	Linear	Intercept	1	52.0621	6.0001	<0.001
		Dose	1	0.0812	0.0231	<0.001
		Scale	1	19.7018	2.3892	
	Polynomial	Intercept	1	43.746	7.8469	<0.001
		Dose	1	0.2022	0.0802	0.012
		Dose 2	1	−0.0003	0.0002	0.116
		Scale	1	19.0232	2.3069	
Body Zn	Linear	Intercept	1	805.0831	128.0072	<0.001
		Dose	1	1.908	0.492	<0.001
		Scale	1	420.3239	50.9718	
	Polynomial	Intercept	1	763.5302	173.0589	<0.001
		Dose	1	2.5128	1.7685	0.155
		Dose 2	1	−0.0013	0.0037	0.722
		Scale	1	419.5427	50.877	

$R^2 = 0.33$) (Fig. 1c). Only worms at a Zn exposure concentration of $\sim$288 mg kg^{-1} showed positive growth. The magnitude of weight loss among worms decreased through $\sim$288 mg kg^{-1} and then increased again through $\sim$476 mg kg^{-1} (Fig. 1c).

Our results support the idea that over a range of field-relevant Zn concentrations, the potential for *L. terrestris* to act as a trophic link will be a non-linear function of Zn concentration because Zn affects both populations of worms as well as bioaccumulation. As has been reported for other species (Spurgeon and Hopkins 1999; Peijnenburg et al. 1999; Vijver et al. 2006), Zn appears to be acting as a micronutrient for *L. terrestris*. The difference in the models used to describe Zn uptake in the gut tissue (linear) versus the body tissue (polynomial) support the hypothesis that Zn body burdens in *L. terrestris* are regulated. Moreover, the only positive growth (one individual) of worms occurred at intermediate levels of soil Zn concentrations, suggesting that at both low and high Zn concentrations, *L. terrestris* populations might be limited by Zn availability and toxicity, respectively. The observed differences in bioaccumulation between the gut tissue and body tissue in this study suggest that it is necessary to examine the distribution of metals in different earthworm tissue compartments in order to develop more accurate models of bioaccumulation and toxicity in metal contaminated systems. The results from this investigation support the suggestion by Vijver et al. (2006) that exposure response relationships for internal metal compartments will differ from the whole body response and therefore must be carefully evaluated.

Zinc bioaccumulation appears to also be a nonlinear function of Zn soil concentration because of Zn regulation. When a near linear increase in gut Zn concentrations is combined with an asymptotic body increase, a pattern of decreasing bioaccumulation with increasing soil concentration appears as has been reported previously for OECD and contaminated field soils (Neuhauser et al. 1995; Spurgeon and Hopkins 1999, Peijnenburg et al. 1999). Therefore, at least during the early stages of soil contamination, the highest rates of transfer of Zn into terrestrial food webs via earthworms would occur at intermediate levels of contamination when earthworm densities are highest and bioaccumulation is still occurring. However, in the later stages of contamination (or in cases of continued low levels of input) earthworms may adapt to higher concentrations of Zn in the soil (Neuhauser et al. 1995; Spurgeon and Hopkin 1999, Peijnenburg et al. 1999), shifting the tolerance level of worms and increasing the potential for Zn transfer to terrestrial food webs.

Finally, we observed little or no positive growth among the *L. terrestris* included in our experiments. In addition to no amended food source during the exposure period, we believe this is at least partially a result of the low nutrient content of OECD soils. We chose OECD soils because of their low trace element content, which allowed us to isolate the effects of Zn on *L. terrestris*. Others have also observed negative growth rates among species housed in OECD soils (i.e., Peijnenburg et al. 1999; Nahmani et al. 2009). While we feel that our results can be used in a comparative fashion, there is clearly further experimentation and field-work needed to extend our results to the more complicated nature of field soils.

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