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Bioavailability pathways underlying zinc-induced avoidance behavior and reproduction toxicity in *Lumbricus rubellus* earthworms

Wei-Chun Ma [a,*], Luc T.C. Bonten [b]

[a] Environmental Sciences Group, Alterra, Wageningen University and Research Centre, P.O. Box 47, 6700 AA Wageningen, The Netherlands
[b] Soil Science Centre, Alterra, Wageningen University and Research Centre, P.O. Box 14 47, 6700 AA Wageningen, The Netherlands

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ABSTRACT

We investigated possible bioavailability pathways underlying zinc-induced avoidance behavior and sublethal reproduction impairment in *Lumbricus rubellus*. Clay-loam (pH 7.3) and sandy soil (three pH values of 4.3–6.0) were amended with zinc sulfate at six soil concentrations of total Zn ranging from 0.1 to 36 mmol/kg dw. Estimated and measured concentrations of free and exchangeable Zn ranged 10^{-4} to 7.1 mmol/l. Avoidance behavior responses were fast and could be directly predicted from the activity of free zinc ions without a modifying pH effect. The repellent effect is thus likely mediated by a direct action of Zn^{2+} ions on epidermal chemosensitive receptors. Body zinc uptake, however, was determined by proton competition with free Zn^{2+} sorption. Excess accumulation of body Zn was a good predictor of reproduction decline, which is indicative of internal zinc poisoning. The results indicated that zinc affects earthworms via both direct and indirect mechanisms of external and internal exposure.

1. Introduction

Bioavailability and bioaccessibility are both essential aspects of ecotoxicological risk modeling and assessment procedures. Distribution of metal speciation forms over soil solid and liquid phases and the fraction of the total bioaccessible amount that may enter into organisms are important factors for risk evaluation of metal-contaminated soils (Peijnenburg et al., 1999; Semple et al., 2004; Nahmani et al., 2007). Earthworms are exposed to trace metals by sorption of the free ionic form across the membrane interface of the epidermis and the intestines (Kiewiet and Ma, 1991; Oste et al., 2001; Saxe et al., 2001; Vijver et al., 2003; Scott-Fordsmand et al., 2004). Apart from acting as a toxicant, trace metals may also have a repellent effect, as has been demonstrated in earthworm avoidance tests with copper and zinc (Ma, 1988; Lukkari et al., 2005). However, it remains unknown whether this avoidance behavior is a sensory-based reaction or more likely a consequence of internal metal poisoning.

The present study focuses on the effect of zinc, one of the most toxic trace metals for earthworms (Lock and Janssen, 2001; Reinecke et al. 1997; Spurgeon and Hopkin, 1996). The objectives were to investigate the significance of chemical speciation forms, in particular of free zinc (Zn^{2+}), in determining the toxic and behavioral effect of zinc. Free Zn was either simulated by weak salt extraction (Gerritse and van Driel, 1984) or estimated using thermodynamic speciation models (Bonten et al., 2008). An acid sandy soil and a calcareous clay were used amended with zinc sulfate. This usually results in greater concentrations of free Zn than are actually found in soils with historical contamination from industrial or agricultural pollution sources (Stephan et al., 2008). However, rather than to mimic the effects of soils with naturally aged zinc contamination, we addressed the basic question to what extent free Zn may account for the specific induction of avoidance behavior and reproduction toxicity in earthworms. Such effects are likely largest under acidic soil conditions when the soluble form of zinc predominates and they can be expected to be least in soils with a high metal sorption capacity and high pH, such as calcareous clays (McBride, 1994). However, some soil properties such as clay content may by themselves influence earthworm reproduction performance (Owojori et al., 2009). Another complicating factor to be addressed is the possible influence of antagonistic zinc–calcium interactions described in animal studies (e.g., Bertolo et al., 2001). Finally, it was also an objective of our study to investigate the question to what extent the repellent and toxic effect of zinc can be predicted from excess

* Corresponding author. Fax: +31 317 41900.
E-mail addresses: wim.ma@wur.nl, wim.ma@yahoo.com (W.-C. Ma), luc.bonten@wur.nl (L.T. Bonten).

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internal body concentrations. Critical internal thresholds have been described for toxicity effects of trace metals in earthworms (van Gestel et al., 1993; Ma, 2005).

2. Materials and methods

2.1. Experimental soils

KOBG was an acid sandy soil with 1.7% clay, 3.4% organic matter, 0.1% $CaCO_3$, pH-$CaCl_2$ 4.9, and CEC 53 meq/kg. OFLP was an alluvial calcareous clay-loam with 17% clay, 3.5% organic matter, 4.5% $CaCO_3$, pH-$CaCl_2$ 7.3, and CEC 178 meq/kg. Dissolved organic carbon (DOC) in these soils was 18.8 and 25.7 mg/l, respectively. The soils were air-dried and sieved (< 2 mm) before treatment. KOBG was limed with $Ca(OH)_2$ at a concentration of 6 mmol Ca/kg dw and acidified by mixing with sulfur powder at a concentration of 20 mmol S/kg dw. These treatments yielded a mean pH-$CaCl_2$ ($\pm$SE; $N=6$) of 5.6 $\pm$ 0.09 and 4.4 $\pm$ 0.01, respectively. Soils were spiked with analytical grade $ZnSO_4 \cdot 7H_2O$ dissolved in deionized water and applied at nominal dose levels of 0, 2.15, 4.31, 8.62, 17.2, and 34.5 mmol Zn/kg dw. As an additional control, KOBG was amended with analytical grade $CaSO_4 \cdot 2H_2O$ at nominal doses of 17.2 and 34.5 mmol Ca/kg dw, which corresponded to a measured mean Ca concentration ($\pm$SE; $N=6$) of 30.5 $\pm$ 0.61 and 57.5 $\pm$ 1.00 mmol/kg dw, respectively. All treated soils were equilibrated for one month before use in bioassays or chemical analysis.

2.2. Chemical analysis

Total zinc (Zn-total) was determined by destruction of soil samples in aqua-regia, and exchangeable zinc (Zn–$CaCl_2$) by shaking with 0.01 M $CaCl_2$ for 48 h in deionized water at a liquid-to-solid ratio of 4:1. The pH was measured in the suspension. Zinc was measured with inductively coupled plasma-atomic emission spectrophotometry (ICP-AES, Perkin-Elmer Optima 3300DV) after centrifugation and filtering through a 0.45-µm filter. DOC was measured with a total organic carbon (TOC) analyzer (Shimadzu 5500). Concentrations of free zinc (Zn-free) in 0.01 M $CaCl_2$ extracts were calculated using the NICA-Donnan model for sorption to dissolved organic matter (DOM) (Kinniburgh et al., 1999) and for inorganic complexation reactions by using to the MINTEQA2 database (Allison et al., 1991). DOM was assumed to consist of 30% fulvic acids, 30% humic acids, and 40% non-reactive material, similar to Bonten et al. (2008). DOM was assumed to contain 50% organic C. The model accounted for zinc sorption to DOM as well as for inorganic complexation reactions and sorption competition between zinc, calcium, and protons.

Prior to chemical analysis a 48 h depuration period on moist filter paper was applied, which is a proven reliable method for the determination of trace metals in earthworms (Arnold and Hodson, 2007). Composite samples of four or five individuals were made in order to obtain a sufficient amount of biomass for chemical analysis. Only living worms were used for analysis, moribund ones were discarded to avoid possible anomalies due to body metal leakage. Samples were completely digested in concentrated HNO_3 at 100 °C and concentrations of Zn and Ca measured with ICP-AES. Analytical quality assurance was derived from applying ISO/IEC 17025 standards for testing and calibration, using internal reference materials from the International Soil analytical Exchange Program (ISE) (Van Dijk, 2002).

2.3. Bioassay methods

Earthworms were collected from uncontaminated grasslands by digging and samples were sorted for the presence of mature clitellate individuals of *Lumbricus rubellus* (Hoffmeister) with an average fresh body weight of 1.7 g and range 1.1 to 2.2 g. Batch units were made by incorporating five worms in one liter of soil brought to a moisture content of 75% of field capacity in lidded glass containers. The soil was topped with coarsely crushed air-dried leaves of alder (*Alnus glutinosa*) as an ad-libitum food source. Five replicate units were assigned to each combination of soil type, zinc level, and pH following a randomized factorial design. They were incubated for 28 days in continuously illuminated climate chambers set at a constant temperature of 15 °C and 61% relative air humidity.

Test endpoints included the induction of avoidance behavior and the sublethal impairment of reproduction. Avoidance responses were assessed by recording the number of individuals appearing on the soil surface after their introduction in soil. The no-choice test situation and the ambient light conditions required the worms to defy their natural negative photo- and geotaxis in order to escape which added to the acuity of the test and which had the added advantage of allowing to measure the rapidity of the behavior response. Reproduction performance was assessed from the number of cocoons collected from soil by stepwise wet-sieving. Only units were considered that retained a complete set of living worms after completion of the test. Results were expressed as a fecundity index, the number of cocoons in units with treated soil relative to the average number in control units with untreated soil. Data were statistically evaluated by analysis of variance and regression analysis, whereby dose–responses were analyzed by fitting the following logistic model:

$$\text{response}\ (\%) = 1 + 10^{(\log \mathrm{EC50} - \log C)n}$$

where C is the concentration, n a slope parameter, and EC_{50} the median effective concentration at which the response is 50% of the maximum possible response.

3. Results

3.1. Soil chemistry

Fig. 1a shows the estimated concentration of free Zn together with the measured concentration of exchangeable Zn (Zn–$CaCl_2$). Concentrations were about two orders of magnitude lower in OFLP as compared with those in KOBG, which indicated the large difference in the availability of zinc between the two soil types. Fig. 1b shows that almost all zinc present in the $CaCl_2$ extract of KOBG was in the free ionic form, with less than 8% bound to dissolved organic carbon (DOC) or inorganic ligands, mainly chloride. As was estimated by the NICA-Donnan model, the percentage of free zinc ions in OFLP was considerably smaller with up to 68% of zinc in the $CaCl_2$ extract bound to DOC due to the high pH of this soil. Complexation by inorganic ligands in OFLP was always below 6% and consequently never played an important role in solution chemistry.

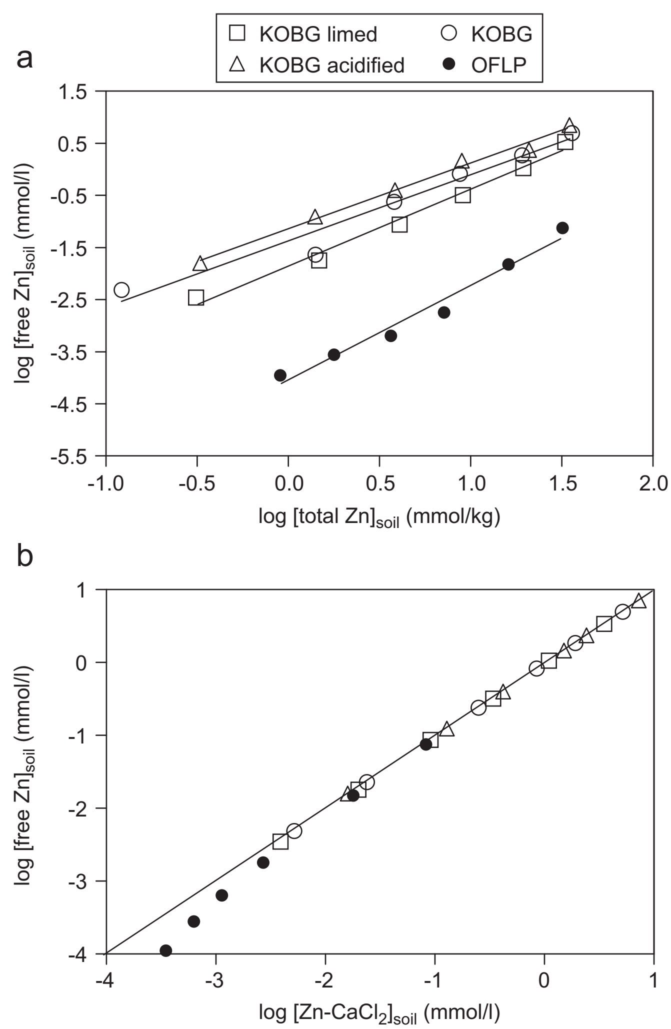

Fig. 1. Interrelationships between concentrations of free Zn, exchangeable Zn (Zn–$CaCl_2$), and total Zn in the various test soils.