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Zhang, Lulu; Van Gestel, Cornelis A.M.

***published in***

Environmental Toxicology and Chemistry  
2017

***DOI (link to publisher)***

[10.1002/etc.3738](https://doi.org/10.1002/etc.3738)

***document version***

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***citation for published version (APA)***

Zhang, L., & Van Gestel, C. A. M. (2017). The toxicity of different lead salts to *Enchytraeus crypticus* in relation to bioavailability in soil. *Environmental Toxicology and Chemistry*, 36(8), 2083-2091.  
<https://doi.org/10.1002/etc.3738>

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THE TOXICITY OF DIFFERENT LEAD SALTS TO *ENCHYTRAEOUS CRYPTICUS* IN RELATION TO BIOAVAILABILITY IN SOIL

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(Submitted 31 August 2016; Returned for Revision 26 October 2016; Accepted 12 January 2017)

**Abstract:** The present study aimed to assess the bioavailability and toxicity of lead nitrate and lead chloride to *Enchytraeus crypticus* in a natural standard soil. Worms were exposed to Pb-spiked soil for 21 d, and survival and reproduction were related to total, 0.01 M CaCl<sub>2</sub>-extractable, and porewater Pb concentrations in the soil and internal concentrations in the surviving animals. The Pb availability for Pb(NO<sub>3</sub>)<sub>2</sub> and PbCl<sub>2</sub> was similar, as confirmed by Langmuir and Freundlich isotherms. The Pb concentrations in surviving worms increased with increasing Pb concentrations in the soil and did not differ for the 2 Pb salts. Lead was toxic to *E. crypticus* at median lethal concentrations (LC50s) of 543 and 779 mg Pb/kg dry soil and median effect concentrations (EC50s) of 189 and 134 mg Pb/kg dry soil, for Pb(NO<sub>3</sub>)<sub>2</sub> and PbCl<sub>2</sub>, respectively. Mortality of *E. crypticus* was related to internal Pb concentrations in the worms rather than to total or available Pb concentrations in the soil, whereas reproduction toxicity was better explained from Pb concentrations in 0.01 M CaCl<sub>2</sub> extracts or porewater of the test soil than from total Pb concentrations in the soil or Pb concentrations in the worms. Overall, the bioavailability and toxicity of Pb(NO<sub>3</sub>)<sub>2</sub> and PbCl<sub>2</sub> to *E. crypticus* in LUFA 2.2 soil did not differ. *Environ Toxicol Chem* 2017;36:2083–2091. © 2017 SETAC

**Keywords:** Lead Salts Toxicity Bioavailability Soil invertebrates

## INTRODUCTION

Metal pollution is a widespread problem, with metals posing a great risk to aquatic and terrestrial ecosystems. Lead pollution has increased in significance, induced by activities such as transportation, industrial practices (e.g., mining), and waste disposal. This has resulted in increased emissions to the surrounding environment, including soil, through different environmental pathways. Because of the risks of Pb pollution and the high costs of remediation and redevelopment, Pb-contaminated soil is attracting great attention and receiving increased interest from national and international regulatory organizations.

The effects of Pb on human health are well documented [1], but the effects within the terrestrial environment have not been fully studied. The evaluation of soil toxicity is complicated by the the complex interactions among pollutants, organisms, and soil particles with different properties [2]. Therefore, few data are available for lead toxicity to soil invertebrates. Earthworm survival has been reported to be very tolerant to lead, whereas reproduction was more sensitive [3]. Lock and Janssen [4] reported a high median lethal concentration (LC50) value (4530 mg Pb/kg dry soil) and a comparatively low median effect concentration (EC50) value (320 mg Pb/kg dry soil) for Pb toxicity to the related potworm species *Enchytraeus albidus*. The reproduction of the species *Enchytraeus crypticus* showed similar sensitivity to lead in field-contaminated soils from a shooting range [5].

Metal bioaccumulation and toxicity to soil biota is only poorly indicated by total soil concentrations [6]. Bioavailability, defined as the amount of chemical that is actually taken up from the environment and is available to cause a biological response,

is considered a crucial indicator of metal toxicity [7]. Availability of metals in soil is often estimated by extraction with weak acid (e.g., 0.1 M HCl or 0.005 M diethylenetriaminepentaacetic acid) or salt solutions (e.g., 0.01 M CaCl<sub>2</sub> or 0.1 M Ca(NO<sub>3</sub>)<sub>2</sub>) instead of direct measurements in biota, thus providing only an initial indication of the potential risk [8]. Moreover, porewater is the significant route of exposure for soil invertebrates [9]. The bioavailability of Pb, therefore, is strongly influenced by its chemical partitioning in soil, in particular by the concentration of free ions present in the porewater. Free Pb<sup>2+</sup> ion activity, as a function of total metal content, is affected by cation exchange processes and associations with inorganic constituents (e.g., CO<sub>3</sub><sup>2-</sup>, HCO<sub>3</sub><sup>-</sup>, and SO<sub>4</sub><sup>2-</sup>), organic ligands (e.g., fulvic acids, humic acids, and amino acids), or particle surfaces (e.g., Fe-oxides, organic matter, clay particles, and biological materials) in the soil [10]. Because of its strong binding with organic or colloidal materials, only a small part of the total Pb concentration in soil may occur or potentially exist as free ions, and therefore be available for uptake by invertebrates. The activity of free Pb<sup>2+</sup> ions in the porewater also depends on the solubility of the Pb compounds in soil and the Pb species in which Pb is present in the soil. These factors play a crucial role in the availability and toxicity of lead to earthworms [3]. Therefore, evaluating the toxicity of different Pb salts on the basis of not only the total Pb concentration, but also available Pb concentrations, may give a better understanding of Pb toxicity to soil invertebrates. Furthermore, the internal Pb concentration in the organisms may eliminate effects from different routes of exposure, and therefore serve as a better indicator of exposure. As a nonessential metal, Pb is difficult to regulate by organisms after uptake. Thus, internal Pb concentrations may be a good estimator for its bioavailable fraction and its potential risk [11].

In ecotoxicological tests, metal toxicity is usually determined by spiking the test substrate with a metal salt. This means that toxicity is not only related to the concentration of the metal

This article includes online-only Supplemental Data.

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Published online 3 February 2017 in Wiley Online Library (wileyonlinelibrary.com).

DOI: 10.1002/etc.3738

but also potentially affected by the metal salt anions (counterion). Chloride ions were shown to be responsible for the reduction of reproduction in enchytraeids [12]. As an indirect effect, nitrate ions, through their contribution to salinity, did influence partitioning and therefore increased the bioavailability of lead and zinc to make significant contributions to the measured toxicity [13]. Lead nitrate ( $\text{Pb}(\text{NO}_3)_2$ ) and lead chloride ( $\text{PbCl}_2$ ) were also reported to have different toxicities to the springtail *Folsomia candida* [14]. Adding Pb salts to the soil increases the salinity of the soil solution and may induce salinity stress, leading to significant overestimation of metal toxicity [13].

Enchytraeids (class Oligochaeta, family Enchytraeidae), which are widespread in most soil types, are ecologically relevant terrestrial organisms that play an important role in litter decomposition, bioturbation, and nutrient cycling, and are therefore recommended as a standard test species for soil toxicity testing [15–17]. Compared with other species of enchytraeids, *E. crypticus* has been demonstrated to be a suitable model species for the assessment of soil ecotoxicology because of its short test period, good control performance, and wide tolerance range to distinct soil properties (e.g., pH, texture, organic matter content) [17].

The present study investigated the toxicity and bioaccumulation of different Pb salts, considering total, 0.01 M  $\text{CaCl}_2$ -extractable (potentially available), and porewater (actual available) Pb concentrations in soil and internal (bioavailable) Pb concentrations in the test animals, using *E. crypticus* as the test species. Our aims were to evaluate the toxicity of Pb to *E. crypticus* in natural soil, to investigate which measurable Pb concentration could be the best expression of lead toxicity, and to compare the difference in toxicity of 2 salts,  $\text{Pb}(\text{NO}_3)_2$  and  $\text{PbCl}_2$ .

## MATERIALS AND METHODS

### Test organism

*Enchytraeus crypticus* have been cultured for several years at the Vrije Universiteit, Amsterdam. The animals were cultured on agar prepared with aqueous soil extract at 16 °C, with 75% relative humidity, in a dark room. The worms were fed twice per week with a mixture of oatmeal, dried yeast, yolk powder, and fish oil. Adults of approximately 1 cm with white spots in the clitellum region were used for the tests.

### Test substrates

The natural standard soil LUFA 2.2 was obtained from the Landwirtschaftliche Untersuchungs-und Forschungs Anstalt (LUFA) in Speyer, Germany. The soil has a nominal pH (0.01 M  $\text{CaCl}_2$ ) of 5.49, 3.5% organic matter, a 12% clay content, and a cation exchange capacity (CEC) of 9.10 meq/100 g. Either  $\text{Pb}(\text{NO}_3)_2$  (purity >99.99%; Sigma-Aldrich) or  $\text{PbCl}_2$  (purity >98%; Merck-Schuchardt) was spiked as an aqueous solution to the soil to obtain nominal concentrations of 0, 100, 200, 400, 800, 1600, and 3200 mg Pb/kg dry soil. Because the solubility of  $\text{PbCl}_2$  in water is low, the highest concentration of  $\text{PbCl}_2$  was spiked as follows: the soil was dried at 40 °C, then one-half of the solution was added, after which the soil was dried again at 40 °C, and the rest of the solution of  $\text{PbCl}_2$  was added [14]. In each case, soil was moistened to a final soil moisture content of 24% (w/w), which equals 50% of the maximum water-holding capacity. Before use, the soils were stored in sealed containers for 2 wk in a climate room at 20 °C for equilibration. Moisture loss was frequently checked and replenished by adding deionized water.

### Toxicity tests

Survival and reproduction tests with *E. crypticus* were conducted following modification of Organisation for Economic Co-operation and Development (OECD) guideline 220 [15] described by Castro-Ferreira et al. [17]. Ten adult worms were introduced into each glass jar (100 mL) containing 30 g moist test soil, and 2 mg oatmeal was added for food. Test jars were covered with perforated aluminum foil and incubated in a climate room at 20 °C, 75% relative humidity and a 16:8-h light:dark photoperiod cycle. To assess enchytraeid survival and reproduction, we used 5 and 4 replicates, respectively. Food availability and soil moisture content were checked once per week, and the water loss was replenished by adding deionized water. After 21 d of exposure, the soil with animals from each test jar was transferred to a plastic box (250 mL) to collect the surviving adults. The surviving adults were placed into Petri dishes (100 mm × 15 mm) with 20 mL ISO solution containing 294 mg/L  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 123.3 mg/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 5.8 mg/L KCl, and 64.8 mg/L  $\text{NaHCO}_3$  (Sigma-Aldrich, >99%) [18] for 24 h for depuration. Afterward, 3 animals from each replicate Petri dish were stored at –20 °C for further analysis. The jars were rinsed with 10 mL ethanol (VWR Chemicals, 96%) to fixate the remaining animals. The ethanol was then transferred into the plastic box to also fixate the animals in the soil. After approximately 2 min, the jar was rinsed with 100 mL tap water, which was also transferred into the plastic box. After that, 200  $\mu\text{L}$  Bengal rose (Sigma-Aldrich, in 1% ethanol) was added to stain the animals. The plastic boxes were stored at 4 °C overnight. The stained samples were transferred to a white tray (80 cm × 50 cm) for counting under a magnifier (×2.5).

### Chemical analysis

Soil was left for 2 wk after spiking and dried at 40 °C for 48 h. For the determination of total soil Pb concentration, 130 mg dry soil was digested with a 2 mL mixture of  $\text{HNO}_3$  (65%, Sigma-Aldrich) and HCl (37%, Sigma-Aldrich; 4:1 v/v) by heating in tightly closed Teflon containers in an oven at 140 °C for 7 h. For the assessment of  $\text{CaCl}_2$ -extractable Pb concentrations, 5.0 g dry soil was shaken with 25 mL of 0.01 M  $\text{CaCl}_2$  solution at 200 rpm for 2 h. The containers were incubated overnight to allow settling of particles. After pH measurement using a pH meter (Inolab pH7110; WTW), the supernatant was filtered over a 0.45- $\mu\text{m}$  cellulose nitrate membrane filter. For porewater sampling, soil samples were saturated with deionized water to 100% water-holding capacity and equilibrated for 1 wk at room temperature. Then the samples were centrifuged at 2000 rpm and 16 °C for 45 min over a 0.45- $\mu\text{m}$  membrane filter (Whatman) that was placed in between 2 filter papers (Whatman) [19]. The pH of the porewater was measured. The Pb concentrations in soil, 0.01 M  $\text{CaCl}_2$  extracts, and porewater were measured by flame atomic absorption spectrometry (AAAnalyst 100; PerkinElmer). The detection limit for Pb analysis by flame atomic absorption spectrometry was 0.021 mg/L. Certified reference material ISE sample 989 (International Soil-Analytical Exchange) was included for quality control of the analysis; recoveries were 92.6% to 97.3%. The frozen enchytraeids were freeze-dried and individually digested with 300  $\mu\text{L}$  of a 7:1 (v/v) mixture of  $\text{HNO}_3$  (65%; Mallbaker Ultrex Ultra-Pure) and  $\text{HClO}_4$  (70%; Mallbaker Ultrex Ultra-Pure) in a block heater (TCS Metallblock Thermostat) using a heating ramp from 85 to 180 °C for 2 h. The internal Pb concentrations were measured by graphite furnace atomic absorption spectrometry (PinAAcle 900Z; PerkinElmer). The detection limit for Pb in this analysis was 0.3141  $\mu\text{g/L}$ . Quality of

the analysis was checked by including the certified reference material DOLT 4 (Dogfish liver; LGC Standards); recoveries were 90.6% to 98.8%.

#### Data analysis

The sorption of lead to the test soil was described in 2 ways, by the Freundlich isotherm and the Langmuir isotherm. The Freundlich isotherm reads

$$C_{\text{sorbed}} = K_F \times C_{\text{ext}}^n \quad (1)$$

where  $C_{\text{sorbed}}$  is the total lead concentration (mg/kg dry soil),  $C_{\text{ext}}$  is the lead concentration in the 0.01 M  $\text{CaCl}_2$  extract or in the porewater (mg/L),  $K_F$  is the Freundlich sorption constant ( $[\text{L}/\text{kg}]^n$ ), and  $n$  is the shape parameter.

The Langmuir isotherm reads

$$C = \frac{C_{\text{max}} \times K_L \times C_{\text{exp}}}{1 + K_L \times C_{\text{exp}}} \quad (2)$$

where  $C$  is the total lead concentration (mg/kg dry soil),  $C_{\text{max}}$  is the maximum lead sorption capacity (mg/kg dry soil),  $C_{\text{exp}}$  is the dissolved Pb concentration in the solution (mg/L), and  $K_L$  is the Langmuir sorption constant (L/kg).

The relationship between Pb uptake by the test organisms and soil Pb concentrations also could be described by a Langmuir isotherm, with  $C$  being the internal Pb concentration in the animals (mg/kg dry body wt);  $C_{\text{max}}$  the maximum Pb uptake capacity (mg/kg dry body wt);  $C_{\text{exp}}$  the total soil,  $\text{CaCl}_2$ -extractable, or porewater Pb concentration (mg/kg dry soil, mg/kg dry soil, or mg/L, respectively); and  $K_L$  the Langmuir-based uptake constant.

Bioaccumulation factors (BAFs), defined as the ratio of Pb body concentrations and total Pb concentrations in soil, and bioconcentration factors (BCFs), defined as the ratio of Pb body concentrations and available Pb concentrations in porewater, were also calculated.

A logistic dose-response model was used to describe the relationship between survival or juvenile numbers and exposure concentration  $C_{\text{exp}}$ , as

$$Y(c) = \frac{Y_{\text{max}}}{1 + \left(\frac{x}{100-x}\right) \left(\frac{C_{\text{exp}}}{L/EC_x}\right)^b} \quad (3)$$

where  $Y(c)$  is the survival or reproduction after 21-d exposure,  $Y_{\text{max}}$  is the estimated survival or reproduction in the untreated control,  $C_{\text{exp}}$  is the Pb exposure concentration, expressed as total or  $\text{CaCl}_2$ -extractable concentration in the soil (mg/kg dry soil), concentration in porewater (mg/L), or internal concentration in the surviving animals (mg/kg dry body wt),  $L/EC_x$  is the estimated effect concentration (mg/kg or mg/L) associated with  $x\%$  reduction in survival or reproduction compared with the control, and  $b$  is the slope parameter.

All parameters mentioned above were estimated by nonlinear regression in SPSS 21.0. A generalized likelihood ratio test was used to compare the results obtained for the different lead salts.

## RESULTS

### Soil properties and Pb sorption

The measured total Pb concentrations in soil agreed with nominal values (Supplemental Data, Table S1). The control

LUF 2.2 soil contained 17.9 mg Pb/kg dry soil. All data reported are based on measured concentrations. Soil  $\text{pH}_{\text{CaCl}_2}$  decreased with increasing total soil Pb concentrations, from 5.61 and 5.76 in the controls to 4.86 and 4.95 at the highest Pb concentrations, for  $\text{Pb}(\text{NO}_3)_2$  and  $\text{PbCl}_2$ , respectively (Supplemental Data, Table S2). Overall, no difference in  $\text{pH}_{\text{CaCl}_2}$  was seen between the 2 Pb salts (Student's  $t$  test,  $p > 0.05$ ). Electrical conductivity increased in a dose-related manner with increasing Pb concentration, from  $773 \pm 13 \mu\text{s}/\text{cm}$  and  $768 \pm 8 \mu\text{s}/\text{cm}$  in the controls to  $6790 \pm 56 \mu\text{s}/\text{cm}$  and  $7205 \pm 148 \mu\text{s}/\text{cm}$  at 3200 mg Pb/kg dry soil for  $\text{Pb}(\text{NO}_3)_2$  and  $\text{PbCl}_2$ , respectively, but did not differ between the 2 Pb salts (Student's  $t$  test,  $p > 0.05$ ; Supplemental Data, Table S3).

For both Pb salts, the sorption of lead was well described by the Freundlich isotherms, with  $K_F$  of 807 to 891  $(\text{L}/\text{kg})^n$  and corresponding  $n$  of 0.390 to 0.463, with  $R^2 = 0.941$  to 0.992, with sorption parameters for  $\text{Pb}(\text{NO}_3)_2$  and  $\text{PbCl}_2$  being similar (Table 1). When related to porewater concentrations, the sorption of Pb was slightly better described by the Langmuir isotherm than by the Freundlich isotherm, while for the  $\text{CaCl}_2$ -extractable Pb concentrations, there was no difference in the goodness of fit of the 2 models (Table 1). The Langmuir sorption parameter  $C_{\text{max}}$  was somewhat higher (3283–3385 mg Pb/kg dry soil vs 3016–3063 mg Pb/kg dry soil) and the  $K_L$  somewhat lower (0.347–0.380 L/kg vs 0.582–0.667 L/kg) for the  $\text{CaCl}_2$ -extractable Pb compared with the porewater Pb (Table 1). No significant difference was found between the sorption parameters for  $\text{Pb}(\text{NO}_3)_2$  and  $\text{PbCl}_2$  ( $\chi^2_{\text{df}=1} \leq 0.61$ ; not significant (n.s.)).

### Lead bioaccumulation

With increasing exposure concentration in soil (up to 800 mg Pb/kg dry soil), the Pb concentrations measured in the surviving worms after 21-d exposure increased from 2.94 and 3.12 mg Pb/kg dry body weight to 83.7 and 75.2 mg Pb/kg dry body weight for  $\text{Pb}(\text{NO}_3)_2$  and  $\text{PbCl}_2$ , respectively (Figure 1). At higher concentrations, mortality was too high to allow for determination of body Pb concentrations. Lead uptake by the enchytraeids was not significantly different between worms exposed in  $\text{Pb}(\text{NO}_3)_2$ - and  $\text{PbCl}_2$ -amended soil. The Pb concentrations in the surviving animals were positively correlated with total soil,  $\text{CaCl}_2$ -extractable, and porewater Pb concentrations, and were well described by the Langmuir model (Figure 1 and Table 2). No significant differences were found between the 2 lead forms ( $\chi^2_{\text{df}=1} \leq 0.64$ ; n.s.). Fitting a Langmuir isotherm to the data that took into account the effect of soil pH did not lead to a better fit. Average BAFs for Pb uptake in *E. crypticus* showed little variation at low exposure concentrations (BAF = 0.16–0.19 and 0.16–0.23  $\text{kg}_{\text{soil}}/\text{kg}_{\text{worm}}$  for  $\text{Pb}(\text{NO}_3)_2$  and  $\text{PbCl}_2$ , respectively). The BAFs were significantly lower (analysis of variance [ANOVA], Bonferroni's test,  $p < 0.05$ ) at 800 mg Pb/kg dry soil, with values of 0.11 and 0.10  $\text{kg}_{\text{soil}}/\text{kg}_{\text{worm}}$  for  $\text{Pb}(\text{NO}_3)_2$  and  $\text{PbCl}_2$ , respectively (Supplemental Data, Figure S1). An inverse relationship between BCFs and porewater Pb concentrations was observed (Supplemental Data, Figure S2), which did not differ significantly between the 2 Pb salts ( $\chi^2_{\text{df}=1} = 3.76$ ; n.s.).

### Lead toxicity

The mean enchytraeid survival in the controls was 100% and 96% in the  $\text{Pb}(\text{NO}_3)_2$ - and  $\text{PbCl}_2$ -amended soil, respectively. In general, the number of surviving adults increased in a dose-related manner with increasing Pb concentrations in the soil, and was well described by a logistic function with  $R^2$  values of 0.992 and 0.965 for  $\text{Pb}(\text{NO}_3)_2$ - and  $\text{PbCl}_2$ -amended soil, respectively (Figure 2). The mean numbers of juveniles produced in the

Table 1. Parameters describing the sorption of lead to LUFA 2.2 soil after spiking with  $\text{Pb}(\text{NO}_3)_2$  or  $\text{PbCl}_2$  salts and related to 0.01 M  $\text{CaCl}_2$ -extractable and porewater Pb concentrations<sup>a</sup>

	Langmuir parameters			Freundlich parameters		
	$C_{\text{max}}$	$K_L$	$R^2$	$K_F$	$n$	$R^2$
Sorption related to porewater concentrations						
$\text{Pb}(\text{NO}_3)_2$	3016 (2744–3288)	0.667 (0.437–0.896)	0.978	826 (675–1102)	0.390 (0.322–0.459)	0.941
$\text{PbCl}_2$	3063 (2769–3353)	0.582 (0.368–0.796)	0.977	807 (652–998)	0.416 (0.341–0.491)	0.938
Sorption related to $\text{CaCl}_2$ -extractable concentrations						
$\text{Pb}(\text{NO}_3)_2$	3283 (3063–3502)	0.380 (0.286–0.474)	0.987	891 (824–964)	0.418 (0.391–0.445)	0.992
$\text{PbCl}_2$	3350 (3175–3524)	0.374 (0.302–0.446)	0.985	853 (771–944)	0.463 (0.425–0.501)	0.986

<sup>a</sup>Shown are the maximum sorption capacity ( $C_{\text{max}}$  [mg/kg dry soil]) and Langmuir sorption constant  $K_L$  (L/kg), with corresponding 95% confidence intervals, and the Freundlich sorption constant  $K_F$  ([L/kg]<sup>1/n</sup>) and corresponding slope parameter  $n$ . Also shown is the goodness of fit ( $R^2$ ).

controls were 703 and 756 for the  $\text{Pb}(\text{NO}_3)_2$ - and  $\text{PbCl}_2$ -spiked soil, respectively. A sharp decrease in reproduction with increasing exposure concentration was found for both Pb salts, which was well described by a logistic function (Figure 2;  $R^2 = 0.996$  and  $R^2 = 0.992$  for  $\text{Pb}(\text{NO}_3)_2$  and  $\text{PbCl}_2$ -amended soil, respectively). The  $\text{Pb}(\text{NO}_3)_2$  was more toxic than  $\text{PbCl}_2$  in terms of survival, with LC50 values of 543 and 879 mg Pb/kg dry soil, respectively, but less toxic in terms of reproduction, with EC50 values of 189 and 134 mg Pb/kg dry soil, respectively (Table 3). Because no internal Pb concentrations could be determined for the 2 highest exposure levels, where mortality was higher than 50%, no reliable LC50 based on body concentrations could be estimated for  $\text{PbCl}_2$  (Figure 2D). The LC50 values for the effect on survival related to body Pb concentrations did not differ significantly for both Pb salts ( $\chi^2_{\text{df}=1} = 3.00$ ; n.s.). The EC50 values for the effect of the 2 Pb salts on enchytraeid reproduction were significantly different when expressed on the basis of total soil concentrations and body concentration in the animals ( $\chi^2_{\text{df}=1} \geq 8.20$ ;  $p < 0.05$ ), but not when expressed on the basis of  $\text{CaCl}_2$ -extractable or porewater concentrations ( $\chi^2_{\text{df}=1} = 2.82$  and 3.48, respectively; n.s.; Figure 3).

## DISCUSSION

### Lead sorption

The LUFA 2.2 natural standard soil had similar Pb sorption isotherms for both Pb salts. The Pb concentrations in  $\text{CaCl}_2$  extracts and porewater were quite low because of the strong sorption of Pb to the test soil. At the  $C_{\text{max}}$  estimated with the Langmuir model, applied to both the  $\text{CaCl}_2$ -extractable and porewater concentrations, approximately 30% of the CEC of the soil was occupied by Pb. The  $K_F$  values relating to Pb concentration in 0.01 M  $\text{CaCl}_2$  extracts or porewater were fairly high compared with values found in other studies [20,21], indicating that most Pb was associated with binding sites on the soil particles. The sorption of Pb leveled off at high concentrations. The leveling off of the sorption at high concentrations is probably explained by the decreasing pH because of the addition of excess Pb ions to the soil, which led to an increased solubility and reduced sorption of Pb to the soil. The Pb concentrations in 0.01 M  $\text{CaCl}_2$  extracts or in porewater did not differ between  $\text{Pb}(\text{NO}_3)_2$ - and  $\text{PbCl}_2$ -spiked soil, which is agreement with the study on the toxicity of these lead salts to the springtail *F. candida* in LUFA 2.2 soil [14].

### Lead bioaccumulation

Pb accumulation in *E. crypticus* increased with increasing exposure concentrations, but leveled off at high soil

concentrations of the 2 Pb salts. As for sorption, this might be partly because of lower soil pH at the higher exposure concentrations. According to the assumption of the biotic ligand model (BLM), a lower pH might protect the animals from metal uptake from the soil solution via competition for binding sites between  $\text{H}^+$  and metal ions [22]. This explains why Pb bioaccumulation also correlated well with extractable and porewater Pb concentrations. Similar results were reported in other studies on Pb toxicity to *E. crypticus* [5,21].

Luo et al. [5] found that internal Pb concentrations in *E. crypticus* ranged from 10.4 to 776 mg Pb/kg dry body weight, with total Pb concentrations from 15 to 656 mg Pb/kg dry soil in soils from a shooting range. Santorufo et al. [23] reported 17.3 mg to 300 mg Pb/kg dry body wt in *E. crypticus* exposed to urban soils, with total lead concentrations from 18.7 mg to 695 mg Pb/kg dry soil. The concentrations of Pb accumulated in *E. crypticus* in these studies were almost 10-fold higher than those in the present study. Several explanations are possible for the observed difference. First, Pb accumulation measurements could be influenced by the soil that is present in the gut of the animals. The studies mentioned did not allow the enchytraeids to void their gut before analysis, making it likely that the reported body concentrations are influenced by the presence of high Pb concentrations in the soil particles present in the gut. Therefore, the Pb accumulation measured in our study could be more accurate. Second, different soil properties, such as pH, organic matter content, CEC, and texture, could strongly determine the chemical availability of Pb in soil, affecting metal bioavailability to *E. crypticus* [24], and resulting in a different Pb accumulation in the organisms. Third, worms behave in different ways under different conditions, such as temperature and soil moisture content [25]. Finally, metal availability is dependent on properties of the species, with routes of exposure (dermal, oral) [26], habitat, and behavior leading to different accumulation patterns in different species, even in the same soil [27]. Also these factors could also have contributed to differences in body Pb concentrations in our study compared with other studies.

As an indicator of the bioavailability of metals in soil, the BAF may be used. The BAF is assumed to be independent of exposure concentration, which for metals may not always be the case [28]. For Pb accumulation in the springtail *F. candida*, fairly constant BAFs of 0.04 to 0.06 were calculated at total soil concentrations of 406 to 49 200 mg Pb/kg dry soil [29]. In the present study, the BAFs for Pb uptake in *E. crypticus* were more or less constant at low exposure concentrations but showed a slightly decreasing trend at the higher test concentrations of lead nitrate and lead chloride (Supplemental Data, Figure S1), which

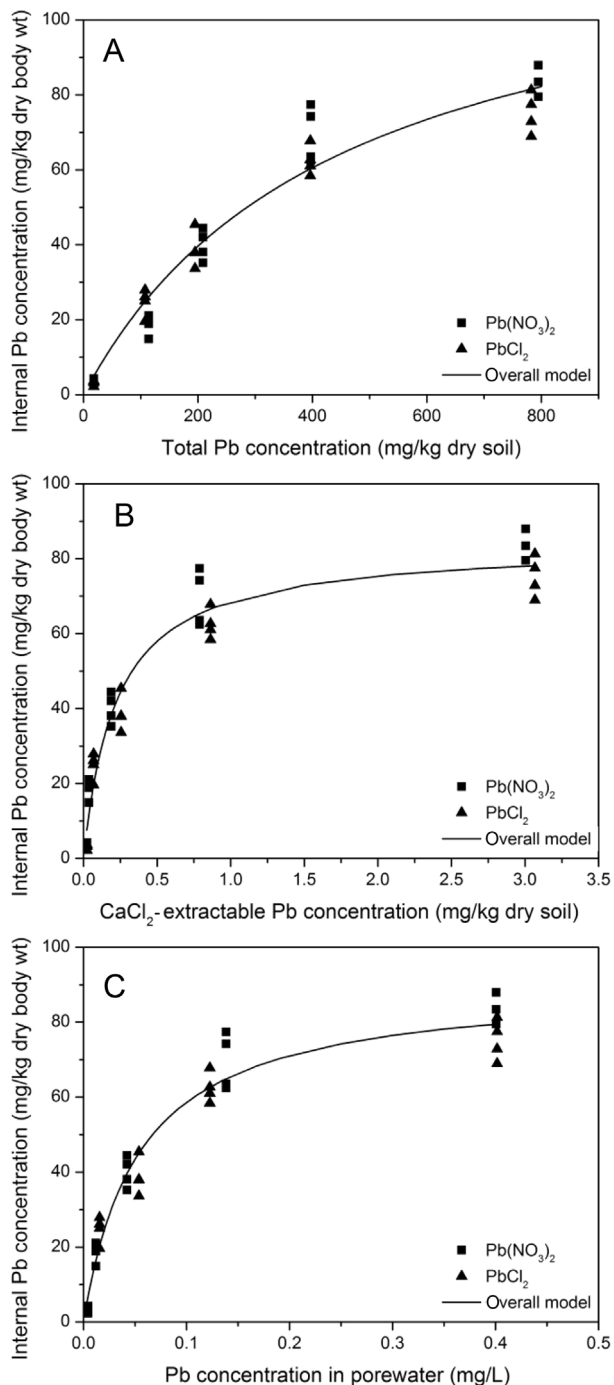


Figure 1. Lead concentrations in surviving adult *Enchytraeus crypticus* after 3-wk exposure to  $\text{Pb}(\text{NO}_3)_2$ - and  $\text{PbCl}_2$ -spiked LUFA 2.2 natural standard soil, related to total (A) and 0.01 M  $\text{CaCl}_2$ -extractable Pb concentrations in soil (B), or Pb concentrations in porewater (C). Dots represent measured concentrations, and lines show the fit of a Langmuir isotherm (Equation 3) to the data. Because there was no significant difference in Pb concentrations between the 2 Pb forms, only one uptake curve is fitted for both Pb forms together.

was in agreement with the findings of Ardestani et al. [30]. The BAFs for *E. crypticus* were much higher than those for *F. candida* reported by Fountain and Hopkin [29], but comparable to those for Pb uptake in *E. andrei* or *F. candida* exposed to field-contaminated soils [31,32]. This might be attributed to species differences, soil properties, and consequent differences in Pb availability in soil. We found an inverse relationship

between BCF and porewater Pb concentrations (Supplemental Data, Figure S2), which was also observed by other authors [28,30]. This indicates that BAF cannot be considered an inherent property of Pb, which undermines its use in risk assessment.

#### Lead toxicity

Despite the different body Pb concentrations, the LC50 values based on total soil Pb concentrations found in the present study (543 and 779 mg Pb/kg dry soil for  $\text{Pb}(\text{NO}_3)_2$  and  $\text{PbCl}_2$ , respectively) are similar to the findings of Luo et al. [5], who reported an LC50 of 638 mg Pb/kg dry soil for *E. crypticus* exposed to field-contaminated soils from a shooting range. The reproduction of *E. crypticus* was very sensitive to Pb, with EC50 values expressed as total Pb concentration as low as 134 to 189 mg Pb/kg dry soil (Table 3), which was much lower than the EC50 observed by Luo et al. [5]. Although it is hard to directly compare EC50s for standard soil and field soils, these results do show that lead is very toxic for *E. crypticus* reproduction. Lock and Janssen [4] reported that survival of the related potworm species *Enchytraeus albidus* was more tolerant to lead, with a 28-d LC50 based on total Pb of 4530 mg Pb/kg dry soil in an artificial soil, which is approximately 8 times higher than the values found for *E. crypticus* in the present study. However, the 42-d EC50 for effects on the reproduction of *E. albidus* was 320 mg Pb/kg dry soil, which is only double the values found in the present study. Apart from species-specific differences, the difference in soil type could at least partly explain this difference, as soil properties (e.g., pH, CEC, organic matter content) may have influenced metal bioavailability, and metals appear to be more toxic in natural soil than in artificial soil [33]. The earthworm *Eisenia fetida* and the springtail *Paronychiurus kimi* (Lee) also showed low sensitivity to Pb, with 28-d LC50 values of 5395 and 1299 mg Pb/kg dry soil, respectively, and 28-d EC50 values of 993 and 428 mg Pb/kg dry soil, respectively, in OECD artificial soil [34,35].

The availability of Pb (measured as Pb concentration in  $\text{CaCl}_2$  extracts or porewater) was demonstrated to be a better predictor of Pb toxicity (adverse effects on organisms) than total soil Pb concentration, as metal ions might determine toxicity [24,36,37]. Exchangeable metal concentrations in soil have been shown to be better predictors of metal availability than total soil concentrations [33]. In the present study, however, even though  $\text{CaCl}_2$ -extractable and porewater Pb concentrations could describe the mortality of worms well for  $\text{Pb}(\text{NO}_3)_2$  and  $\text{PbCl}_2$  ( $R^2 = 0.966\text{--}0.992$ ), it could not explain results from both salts together (Figure 2B and C). Different from survival, the reproduction of *E. crypticus* was better described by  $\text{CaCl}_2$ -extractable and porewater Pb concentrations when the  $\text{Pb}(\text{NO}_3)_2$  and  $\text{PbCl}_2$  treatments were combined (Figure 3B and C), which is consistent with the finding that Pb concentration in the soil solution was a better measure of Pb toxicity to the reproduction of the springtail *F. candida* than total soil concentration [36]. Luo et al. [5] also concluded that porewater Pb concentration best described *E. crypticus* reproduction in a shooting field.

The LC50s expressed on the basis of Pb concentrations in  $\text{CaCl}_2$  extracts (1.44 and 3.91 mg Pb/kg dry soil for  $\text{Pb}(\text{NO}_3)_2$  and  $\text{PbCl}_2$ , respectively) or porewater (0.224 and 0.524 mg/L, respectively) for *E. crypticus* were lower than those observed in soils from shooting fields (8.5 mg Pb/kg dry soil and 0.643 mg/L, respectively). In that study [5], EC50s expressed on the basis of  $\text{CaCl}_2$ -extractable Pb concentration (1.6 mg Pb/kg dry soil) were much higher than those observed in the present experiment (0.128 and 0.076 mg Pb/kg dry soil for  $\text{Pb}(\text{NO}_3)_2$  and  $\text{PbCl}_2$ ,

Table 2. Parameters describing the uptake of Pb in *Enchytraeus crypticus* after 3-wk exposure in LUFA 2.2 soil spiked with Pb(NO<sub>3</sub>)<sub>2</sub> or PbCl<sub>2</sub> salts and described by a Langmuir model<sup>a</sup>

	Pb(NO <sub>3</sub> ) <sub>2</sub>			PbCl <sub>2</sub>		
	Total Pb (mg/kg dry soil)	CaCl <sub>2</sub> -extractable Pb (mg/kg dry soil)	Porewater Pb (mg/L)	Total Pb (mg/kg dry soil)	CaCl <sub>2</sub> -extractable Pb (mg/kg dry soil)	Porewater Pb (mg/L)
$C_{max}$	150 (111–190)	89.4 (82.0–96.7)	96.7 (88.8–104.6)	112 (97–127)	79.9 (73.5–85.9)	86.3 (79.5–93.1)
$K_L$	0.002 (0.001–0.003)	4.50 (3.17–5.83)	17.3 (13.0–21.7)	0.003 (0.002–0.004)	4.37 (3.01–5.59)	18.7 (14.0–23.3)
$R^2$	0.956	0.969	0.978	0.980	0.964	0.973

<sup>a</sup>Shown are the maximum uptake capacity ( $C_{max}$  [mg/kg dry body wt]), uptake coefficient ( $K_L$  [kg/kg] and [L/kg] for 0.01 M CaCl<sub>2</sub>-extractable Pb and porewater Pb, respectively), with corresponding 95% confidence intervals and the goodness of fit ( $R^2$ ) relating Pb uptake to total and 0.01 M CaCl<sub>2</sub>-extractable Pb concentrations in the soil (in mg Pb/kg dry soil) and Pb concentrations in porewater (in mg/L). See Figure 1 for the model fits.

respectively). However, the EC<sub>50</sub>s expressed as porewater Pb concentrations (0.126 mg/L) were more comparable to the values found in the present study (0.033 and 0.028 mg/L for Pb(NO<sub>3</sub>)<sub>2</sub> and PbCl<sub>2</sub>, respectively) [5]. This difference might result from the higher cation competition in the field soils, which showed a large variation in soil pH. This implies that porewater Pb concentration might be a better predictor of Pb toxicity to the reproduction of *E. crypticus* in soil.

The measured toxicity is not only related to the metal concentration, as the counter-ions could also have contributed to the toxicity of the metal salts [13,14]. In addition to having a direct effect, an indirect effect of the accompanying anion may result from changes in the ionic strength (salinity) on metal speciation after addition of the metal salt. The effect of counter-ions may differ for different metals and test organisms. In our study, the counter-ions (NO<sub>3</sub><sup>-</sup> and Cl<sup>-</sup>) did not seem to affect the

0.01 M CaCl<sub>2</sub>-extractable and porewater Pb concentrations. Also, the test organisms showed similar Pb uptake for the 2 Pb salts. Therefore, the counter-ions (NO<sub>3</sub><sup>-</sup> and Cl<sup>-</sup>) seemed to have similar effects on Pb bioavailability in soil and contributed in a similar way to the observed toxicity of Pb. Bongers et al. [14] found 28-d and 35-d LC<sub>50</sub>s of 980 and 2900 mg Pb/kg dry soil for Pb(NO<sub>3</sub>)<sub>2</sub> and PbCl<sub>2</sub>, respectively, for *F. candida*. In the present study, Pb(NO<sub>3</sub>)<sub>2</sub> was also more toxic to *E. crypticus* than PbCl<sub>2</sub> in terms of survival. Aquatic invertebrates were stated to be sensitive to nitrate, with nitrate toxicity increasing with increasing exposure time [38]. The 120-h LC<sub>50</sub> values for the toxicity of nitrate to the aquatic invertebrates *Echinogammarus echinosetosus*, *Eulimnogammarus toletanus*, and *Hydropsyche exocellata* were 56.2, 73.1, and 230 mg NO<sub>3</sub><sup>-</sup> N/L, respectively [37]. At the soil moisture content of 24% (w/w) used in the present study, the nitrate

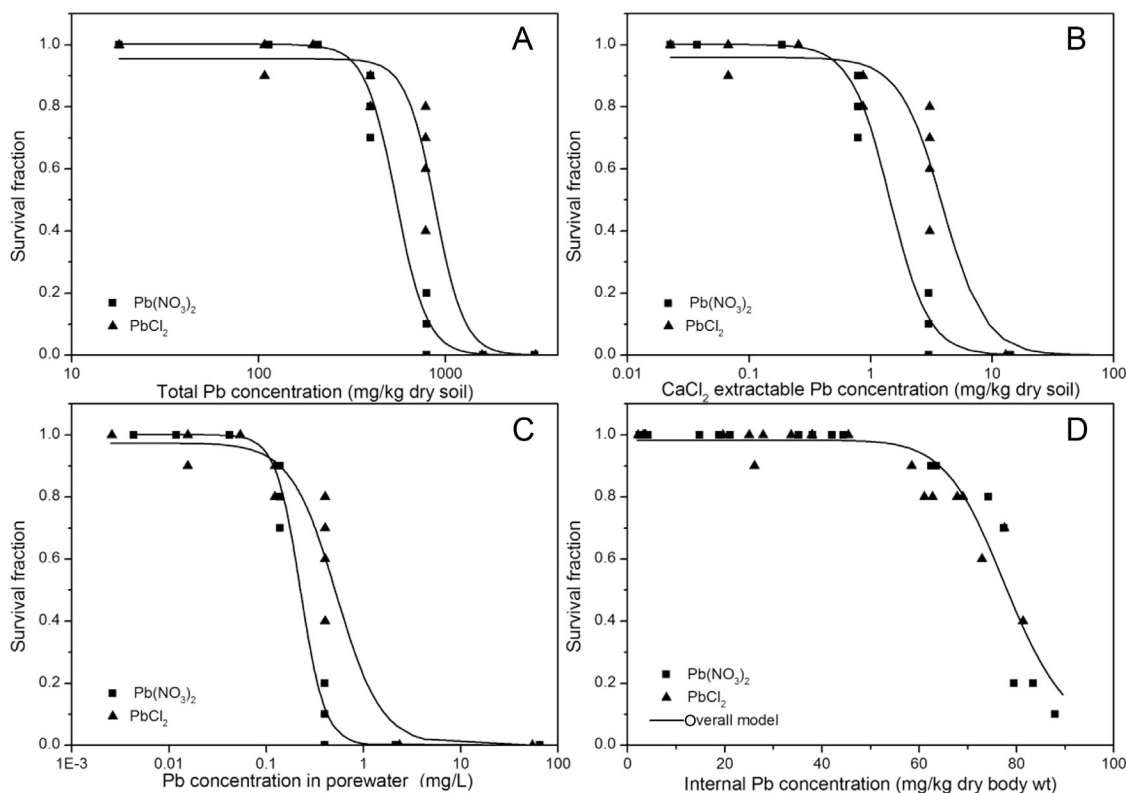


Figure 2. Effects of Pb(NO<sub>3</sub>)<sub>2</sub> and PbCl<sub>2</sub> on the survival of *Enchytraeus crypticus* after 3-wk exposure in LUFA 2.2 natural standard soil. Pb concentrations are expressed as total (A) and 0.01 M CaCl<sub>2</sub> extractable concentrations in soil (B), concentrations in porewater (C), or internal concentrations in surviving adults (D). Lines show the fit of a logistic dose–response curve; in cases where only one curve is shown, the dose–response curves for the 2 Pb forms did not significantly differ according to a generalized likelihood ratio test.

Table 3. LC50, LC10, EC50 and EC10 values (with corresponding 95% confidence intervals) for the effects of lead on the survival and reproduction of *Enchytraeus crypticus* exposed for 21 d to 2 different Pb salts in LUFA 2.2 soil natural standard soil<sup>a</sup>

	Pb(NO <sub>3</sub> ) <sub>2</sub>				PbCl <sub>2</sub>			
	Total Pb (mg/kg dry soil)	CaCl <sub>2</sub> -extractable Pb (mg/kg dry soil)	Porewater Pb (mg/L)	Internal Pb (mg/kg body wt)	Total Pb (mg/kg dry soil)	CaCl <sub>2</sub> -extractable Pb (mg/kg)	Porewater Pb (mg/L)	Internal Pb (mg/kg body wt)
LC50	543* (517–569)	1.44* (1.31–1.58)	0.224* (0.208–0.240)	78.2 (77.2–79.2)	879* (806–955)	3.91* (3.25–4.60)	0.524* (0.429–0.616)	81.1 (77.1–85.1)
LC10	358* (332–384)	0.65* (0.55–0.74)	0.118* (0.105–0.131)	72.8 (70.4–75.3)	599* (494–705)	1.52* (0.96–2.08)	0.161* (0.097–0.223)	58.5 (53.0–64.1)
EC50	189* (181–196)	0.128 (0.088–0.167)	0.033 (0.028–0.037)	34.8* (31.4–38.3)	134* (126–141)	0.076 (0.068–0.094)	0.028 (0.026–0.030)	29.1* (27.2–31.1)
EC10	96.6* (87.8–106)	0.023 (0.006–0.040)	0.008 (0.006–0.011)	17.7* (13.7–21.8)	64.6* (53.6–69.6)	0.018 (0.013–0.023)	0.009 (0.007–0.010)	16.4* (13.7–19.0)

<sup>a</sup>Effect concentrations are expressed on the basis of total and 0.01 M CaCl<sub>2</sub>-extractable Pb concentrations in soil, Pb concentrations in porewater, and Pb concentrations measured in surviving animals.

\* Significantly different between the 2 salts.

LC50 = median lethal concentration; LC10 = 10% lethal concentration; EC50 = median effect concentration; EC10 = 10% effect concentration.

concentration at 100 mg Pb/kg soil in the porewater was estimated to be 54.1 mg NO<sub>3</sub><sup>-</sup> N/L (assuming no losses as a result of biogeochemical transformations or uptake of NO<sub>3</sub><sup>-</sup>), and thus was similar to the lowest LC50 reported. Counter-ion (NO<sub>3</sub><sup>-</sup>) concentrations, therefore, might have had some negative effects on *E. crypticus* during the 21-d exposure period, but the effects of nitrate cannot be judged properly at this time. Pereira et al. [12] reported that *E. crypticus* was sensitive to sodium chloride, with an EC50-reproduction of approximately 900 mg Cl/kg in OECD artificial soil. In the present study, such a high chloride concentration was only reached at 3200 mg Pb/kg dry soil (corresponding to ~1100 mg Cl/kg dry soil). Therefore, it is

unlikely that Cl<sup>-</sup> has contributed to effects on reproduction, as they occurred at much lower Pb concentrations.

The body Pb concentration may be a good descriptor of metal toxicity to organisms. Previous studies have also demonstrated that reproduction toxicity is often better related to internal metal concentration than to total soil concentrations [24,39,40]. Because of metal detoxification mechanisms, only a portion of the total body concentration is responsible for toxic effects in terrestrial invertebrates [39]. Thus, it is likely that when the internal Pb concentration exceeds a physiological limit (the capacity of Pb detoxification mechanisms), the mortality of *E. crypticus* increases, while metal is slowly sequestered. In the present study,

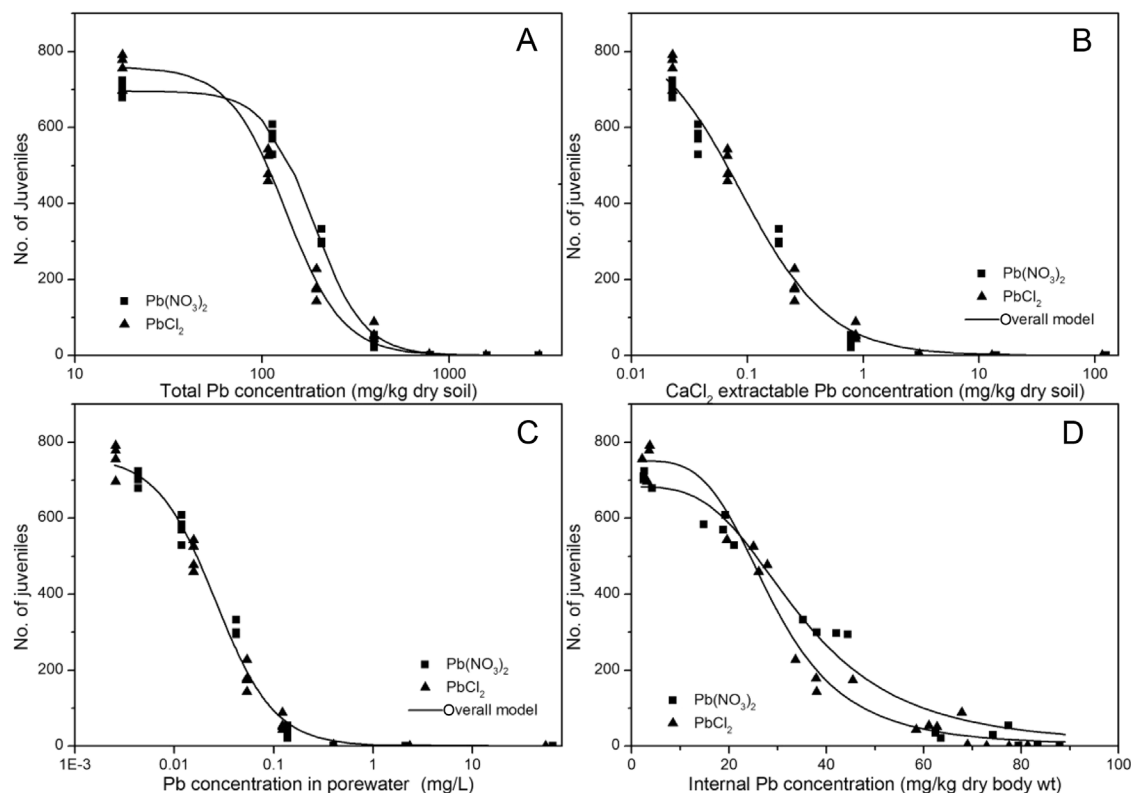


Figure 3. Effects of Pb(NO<sub>3</sub>)<sub>2</sub> and PbCl<sub>2</sub> on the reproduction of *Enchytraeus crypticus* after 3-wk exposure in LUFA 2.2 natural standard soil. Lead concentrations are expressed as total (A) and 0.01 M CaCl<sub>2</sub> extractable concentrations in soil (B), concentrations in porewater (C), or internal concentrations in the surviving adults (D). Lines show the fit of a logistic dose–response curve; in cases where only one curve is shown, the dose–response curves did not significantly differ according to a generalized likelihood ratio test.



mortality occurred when internal Pb concentration exceeded approximately 60 mg Pb/kg dry body wt, which is in agreement with the results observed by Davies et al. [41]. Survival correlated well with Pb body concentrations in *E. crypticus* for both nitrate and chloride salts ( $R^2 = 0.899$ ; Figure 2D). In the literature, limited data could be found on the internal LC50 or EC50 values for the toxicity of Pb to *E. crypticus*. The LC50 and EC50 values based on internal Pb concentrations were much lower for *E. crypticus* in the present study than the values for the earthworm *E. andrei* of 852 and 484 mg Pb/kg dry body wt, respectively, reported by Luo et al. [40]. The inability of *E. crypticus* to regulate the metal and the subsequently high accumulation were found to cause detrimental effects on its physiological performance (e.g., reproduction) [23]. Compared with the control, low reproduction was already observed at fairly low body Pb concentrations in *E. crypticus*, while whereas effects on survival only started at higher concentrations. This indicates that the reproduction of *E. crypticus* was a highly sensitive parameter to estimate effects of Pb in soil.

### CONCLUSIONS

The present study shows that Pb has a high toxicity to *E. crypticus* in freshly spiked natural soil. Compared with most other soil invertebrates, *E. crypticus* was more sensitive to Pb, with low LC50s (543 and 779 mg Pb/kg dry soil for Pb(NO<sub>3</sub>)<sub>2</sub> and PbCl<sub>2</sub>, respectively) and EC50s (189 and 134 mg Pb/kg dry soil for Pb(NO<sub>3</sub>)<sub>2</sub> and PbCl<sub>2</sub>, respectively). Partitioning of Pb between the test soil and soil porewater or 0.01 M CaCl<sub>2</sub> extracts did not differ between the 2 Pb salts. The body Pb concentrations in *E. crypticus* increased with increasing total Pb concentration in soil and did not show a difference between the 2 Pb salts. The mortality of *E. crypticus* was best described by internal Pb concentrations in the animals, whereas reproduction toxicity was more related to Pb concentrations in porewater or 0.01 M CaCl<sub>2</sub> extracts of the test soils. For Pb(NO<sub>3</sub>)<sub>2</sub> and PbCl<sub>2</sub>, only a slight difference in toxicity was observed.

**Supplemental Data**—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3738.

**Acknowledgment**—The PhD scholarship of L. Zhang is supported by the China Scholarship Council (CSC). We thank R.A. Verweij for his assistance in various phases of the work.

**Data availability**—Data, associated metadata, and calculation tools are available from the corresponding author (kees.van.gestel@vu.nl).

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