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Effect of ageing and chemical form on the bioavailability and toxicity of Pb to the survival and reproduction of the soil invertebrate *Enchytraeus crypticus*

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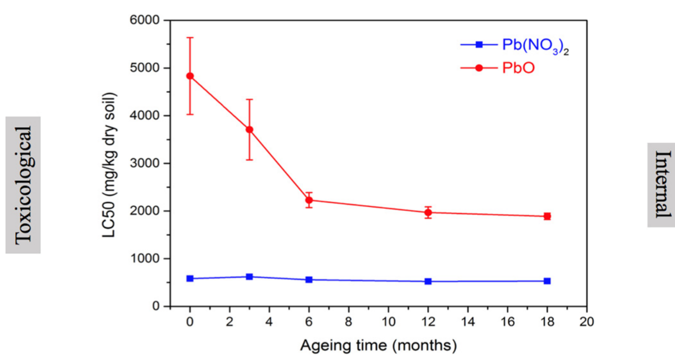
HIGHLIGHTS

- Soil ageing did affect Pb availability, uptake and toxicity to *Enchytraeus crypticus.*
- The effect of ageing on Pb bioavailability did depend on its chemical form.
- Ageing increased bioavailability in soil of PbO but not of $Pb(NO_3)_2$.
- $CaCl_2$ extractable Pb best explained Pb bioaccumulation and toxicity to *E. crypticus.*

GRAPHICAL ABSTRACT



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ABSTRACT

This study investigated the effect of ageing on the bioavailability and toxicity of lead nitrate ($Pb(NO_3)_2$) and lead oxide (PbO) to *Enchytraeus crypticus* in LUFA 2.2 natural soil. The potworms were exposed after 2 weeks pre-incubation and after ageing the spiked soils for 3, 6, 12 and 18 months. Survival and reproduction after 21 d exposure were related to total, 0.01 M $CaCl_2$-extractable and porewater Pb concentrations in the soil and internal Pb concentrations in the surviving animals. Pb concentration in pore water showed little change during ageing for $Pb(NO_3)_2$ but increased strongly for PbO-spiked soils. During ageing, toxicity of $Pb(NO_3)_2$ did not change with LC50s and EC50s for the effect on enchytraeid survival and reproduction based on total soil Pb concentrations being constant at 523–619 and 89.8–99.4 mg Pb/kg dry soil, respectively. Toxicity of PbO, however, increased with LC50s and EC50s decreasing from 4830 to 1889 mg Pb/kg dry soil and from 151 to 97.5 mg Pb/kg dry soil, respectively. When related to internal Pb concentrations LC50s did not differ for both Pb forms at different ageing periods and were 73.4–78.7 mg Pb/kg dry body wt. Survival was better explained from internal Pb concentrations in the worms than from total or available Pb concentrations in the soil. Reproduction toxicity (EC50s) and Pb uptake in the worms however, were better explained from 0.01 M $CaCl_2$-extractable Pb concentrations in the soil. The latter finding could provide a scientific basis for the ecological risk assessment of contaminated soils and the derivation of soil quality standards based on extractable concentrations.

1. Introduction

Pb pollution is regarded as one of the most serious forms of metal contamination, induced by anthropogenic activities such as agriculture,

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industrialization, urbanization and mining, threatening ecological and human health (Alloway, 2013). In soil, Pb may pose a risk to soil invertebrates (e.g. adverse effects on survival, growth and reproduction), like enchytraeids (potworms) that are important contributors to ecosystem services (Didden and Römbke, 2001; Lanno et al., 2004).

Standard ecotoxicity tests with soil invertebrates are usually carried out in freshly spiked soils, often using easily dissolved metal forms not being representative of the form of metals that are actually deposited on soils by industrial and agricultural processes (Lock and Janssen, 2001; Spurgeon and Hopkin, 1999). This may lead to overestimation of metal toxicity in historically contaminated field soils due to the lack of sufficient equilibration time in the laboratory. In addition, the contribution of the associated counterions added with the dissolved metal forms may also have adverse effects on soil organisms (Smolders et al., 2009; Oorts et al., 2006). As a consequence, large differences may appear in metal toxicity between laboratory-spiked and field-contaminated soils (Lock et al., 2006; Smolders et al., 2015). For instance, Lock et al. (2006) compared Pb toxicity to *Folsomia candida* in historically polluted soils and in corresponding freshly $Pb(NO_3)_2$-spiked soils. Total Pb concentrations of 2160–3210 mg Pb/kg dry soil reduced *F. candida* reproduction by 50% in the spiked soils, but median effect concentrations (EC50s) in the historically contaminated soils were more than a factor of 2 higher. Thus, incorporation of the impacts of ageing in the environmental risk assessment of metal-polluted soils might contribute to a more realistic assessment of metal toxicity in terrestrial ecosystems, which should be taken into account in the laboratory tests (Lock and Janssen, 2003b).

Toxicity is often related to total metal concentrations in the soil, however, counterions may also have direct or indirect toxic effects on the test organisms (Peredney and Williams, 2000; Schrader et al., 1998). Zhang and Van Gestel (2017) found that $Pb(NO_3)_2$ and $PbCl_2$ had different toxicities to *Enchytraeus crypticus* in natural standard soils. Chloride ions were reported to affect the reproduction of enchytraeids with an $EC50_{reproduction}$ of around 900 mg Cl/kg in OECD artificial soil (Pereira et al., 2015). Thus, to avoid the influence from the accompanying anions, metal oxide forms like PbO might be a good relevant source to be used in toxicity studies. PbO was also one of the major forms in Pb-contaminated field soils (Nedwed and Clifford, 1998).

Only a fraction of the total metal concentration in the soil is bioavailable to soil organisms, causing toxic effects (Peijnenburg et al., 2007). Many soil extraction and speciation methods have been developed to predict this bioavailable fraction in soil, providing a first indication of the potential metal risk (Conder and Lanno, 2000; Peijnenburg et al., 2007). Among these methods, porewater concentration was suggested to be a good indicator for metal bioavailability, as pore water is assumed to be the dominant route for metal uptake by soil invertebrates (Van Gestel, 2012). Lock et al. (2006) highlighted that the difference in metal toxicity between laboratory spiked and field-contaminated soils could be explained by metal concentrations in pore water. In our earlier studies, we found that 0.01 M $CaCl_2$ extraction may also provide a good indication of Pb bioavailability in soil (Zhang and Van Gestel, 2017).

Due to the observed differences in the toxicity of different forms of Pb, for our tests we chose nitrate and oxide as very soluble and hardly soluble Pb forms, respectively. And we focused on the dynamics of Pb bioavailability from both chemical forms. So, this study was designed to investigate the influence of ageing and metal form on Pb bioavailability and toxicity to *Enchytraeus crypticus*. We aimed at: (1) assessing the sorption of Pb applied to soil as different forms (PbO, $Pb(NO_3)_2$) with increasing ageing time, (2) evaluating the effect of ageing time on the toxicity of both Pb forms to the survival and reproduction of enchytraeids, (3) comparing the toxicity of the two Pb forms (PbO, $Pb(NO_3)_2$), and (4) determining which measurable Pb concentration could be the best expression of lead toxicity.

2. Materials and methods

2.1. Test organism

Enchytraeus crypticus (Enchytraeidae; Oligochaeta; Annelida) has been cultured for >10 years in the laboratory of the Department of Ecological Science, Vrije Universiteit, Amsterdam. *E. crypticus* were cultured in pots with a layer of agar prepared with an aqueous soil extract, kept in a climate room at 16 °C, 75% relative humidity, and in complete darkness. The potworms were fed twice a week with a mixture of oatmeal, dried yeast, yolk powder, and fish oil (Castro-Ferreira et al., 2012). Adult *E. crypticus* of approximately 1 cm with clitellum were used in the experiments.

2.2. Test soils

Obtained from the LUFA Institute (Landwirtschaftliche Untersuchungs- und Forschungsanstalt) at Speyer, Germany, standard LUFA 2.2 soil was moistened to a final soil moisture content of 24% (w/w), which equals 50% of the maximum water-holding capacity (WHC). It has a nominal pH - 0.01 M $CaCl_2$ of 5.49, 3.5% organic matter, 12% clay and a cation exchange capacity (CEC) of 9.10 $cmol_c$/kg. Because of the low solubility of PbO (0.017 g/L at 25 °C), solid $Pb(NO_3)_2$ and PbO (purity >99.99%; Sigma-Aldrich; USA) were spiked as dry powders into the moist soil, to nominal concentrations of 0, 50, 100, 200, 400, 600, 800, 1600 and 3200 mg Pb/kg dry soil and 0, 78, 156, 312, 625, 1250, 2500, 5000 and 10,000 mg Pb/kg dry soil, respectively. The spiked soils were stored in plastic boxes in a climate room at 20 °C. Soil moisture content was frequently checked by weighing the boxes and moisture lost replenished by adding deionized water. Soils were only disturbed when samples were taken, which was done at five ageing periods (0, 3, 6, 12, 18 months). The first sampling took place after allowing the freshly spiked soils to equilibrate for 2 weeks.

2.3. Toxicity tests

Survival and reproduction tests with *E. crypticus* were conducted following a modification of OECD guideline 220 (OECD, 2016), using the soils incubated for 0, 3, 6, 12 and 18 months after spiking. At each ageing time, ten adult worms were transferred to a glass jar (100 mL) filled with 30 g moist soil and 2 mg oatmeal as food for each treatment (Castro-Ferreira et al., 2012). Test jars were covered with perforated aluminum foil and placed in a climate room at 20 °C, 75% relative humidity with a 16–8 h light:dark cycle. Five replicates were used. Moisture content of the soil was kept constant by replenishing the water loss with deionized water. Food was added once a week. After 21 d, surviving adults were determined and worms three depurated worms each replicate were stored at −20 °C for further analysis. Incubation of the worms in ISO solution containing 294 mg/L $CaCl_2{\cdot}2H_2O$, 123.3 mg/L $MgSO_4{\cdot}7H_2O$, 5.8 mg/L KCl and 64.8 mg/L $NaHCO_3$ (Sigma Aldr>99%) (ISO, 2012) was used for gut depuration for 24 h. The juveniles were stained with Bengal rose (Sigma Aldrich, in 1% ethanol) and counted in the pictures taken using Photoshop CS5.0.

2.4. Chemical analysis

At 0, 3, 6, 12 and 18 months after spiking, soils were dried at 40 °C for 48 h. The total Pb fractions were obtained by digestion of 130 mg dry soil in 2 mL mixture of HNO_3 (65%, Sigma-Aldrich, USA) and HCl (37%, Sigma-Aldrich, USA) (4:1 v/v) in a drying oven (Binder FD). Total soil concentrations in the soil digests were measured by flame atomic absorption spectrometry (AAS; AAnalyst 100, Perkin Elmer, Germany). The certified reference material ISE sample 989 (International Soil-Analytical Exchange) was included for quality control of the analysis and the recoveries of Pb in the reference material were 92.8–96.7%. The detection limit for Pb analysis by flame AAS was 0.027 mg/L.

To determine the body Pb concentrations, the frozen worms were freeze-dried and individually weighed using an analytical microbalance (Mettler Toledo GmbH UMT2). A 300 μL mixture of HNO_3 (65%; Mallbaker Ultrex Ultra-Pure) and $HClO_4$ (70%; Mallbaker Ultrex Ultra-Pure) (7:1 v/v) was used for digestion in a block heater (TCS Metallblock Thermostat) with a heating ramp ranging from 85 to 180 °C for 2 h. Internal Pb concentrations were measured individually by graphite furnace AAS (PinAAcle 900Z, Perkin Elmer, Germany). Quality of the analysis was checked by using the certified reference material DOLT 4 (Dogfish liver, LGC Standards). The Pb recoveries were 89.2–97.9%. The detection limit for Pb in this analysis was 0.21 μg/L.

For determining the exchangeable Pb fraction, 5.0 g dry soil were shaken for 2 h with 25 mL 0.01 M $CaCl_2$ solution at 200 rpm and the suspensions were filtered through a 0.45 μm membrane (Whatman) after settling overnight. Pore water was collected after saturating the soils with deionized water to 100% WHC and equilibration for one week at room temperature. The pore water was sampled by centrifugation at 2000 rpm and 16 °C for 45 min, and filtration through a 0.45 μm membrane filter (Whatman) placed in between two filter papers (Whatman). Soil pH_{CaCl2} and pH_{pw} values were measured in the $CaCl_2$ and porewater extracts, respectively using a pH meter (WTW, Inolab pH 7110). Pb concentrations in the 0.01 M $CaCl_2$ extracts and pore water were determined by flame AAS or graphite furnace AAS depending on the concentration level.

2.5. Data analysis

To describe the sorption of lead to the test soil a Freundlich isotherm was used:

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

where, C_{sorbed} is the total Pb concentration (mg/kg dry soil), C_{ext} the Pb concentration in the 0.01 M $CaCl_2$ extract or porewater (mg/L), K_F the Freundlich sorption constant ($(L/kg)^n$), and n the shape parameter.

The relationship between Pb uptake by the test organisms and soil Pb concentrations could be described by a Langmuir isotherm:

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

where, C is the internal Pb concentration in the animals (mg/kg dry body wt), C_{max} the maximum Pb uptake capacity (mg/kg dry body wt), C_{exp} the $CaCl_2$-extractable or porewater Pb concentration (mg/kg dry soil or mg/L, respectively), and K_L the Langmuir-based uptake constant.

LC50, LC10 and EC50, EC10 values, giving the 50% and 10% effect levels for effects on survival and reproduction, respectively, were calculated using a logistic dose-response model.

All sorption, uptake and toxicity parameters were estimated by regression in SPSS 24.0, using measured Pb concentrations. To compare differences in parameters between ageing times and Pb forms, one- or two-way ANOVAs and generalized likelihood-ratio tests were applied.

3. Results

3.1. Soil analysis

Soil pH_{CaCl2} and pH_{pw} in pore water decreased with increasing total Pb concentration in the soil for $Pb(NO_3)_2$, but increased with increasing total Pb concentration for PbO (Figs. S1-S2). A significant decrease in soil pH (up to 0.5 unit in pH_{CaCl2} and 1 unit in pH_{pw}) with ageing time was observed for PbO-spiked soils (one-way ANOVA, $p < 0.01$), whereas ageing only slightly affected the pH of $Pb(NO_3)_2$-spiked soils.

The background Pb concentration in the LUFA 2.2 soil was 15–17 mg Pb/kg dry soil. Measured total Pb concentrations in the spiked soils ranged from 80% to 121% of the nominal values. Ageing only slightly affected the total Pb concentrations in the soils. In all treatments, available Pb concentrations in the soil (measured as 0.01 M $CaCl_2$-extractable and porewater Pb concentrations) were positively correlated with total Pb concentrations for both Pb forms, and changed little with ageing time for $Pb(NO_3)_2$, but significantly increased with ageing time for PbO (one-way ANOVA, $p < 0.05$) (Figs. S3-S4). The $CaCl_2$-exchangeable and porewater Pb concentrations were higher in the $Pb(NO_3)_2$-spiked soils than in the PbO-spiked soils (Student-*t*-test, $p < 0.01$).

For both Pb forms, the sorption of Pb was well described by Freundlich isotherms (Table 1). The Freundlich adsorption constants (K_{FP}) based on Pb concentrations in pore water decreased with ageing time from 874 and 22,631 $(L/kg)^n$ at $t = 0$ to 567 and 10,935 $(L/kg)^n$ after 18 months for $Pb(NO_3)_2$ and PbO, respectively. The shape parameter n was lower than 1 for $Pb(NO_3)_2$ and higher than 1 for PbO. The K_{FC} based on $CaCl_2$-extractable Pb concentrations decreased with ageing time from 8668 $(L/kg)^n$ to 4811 $(L/kg)^n$ at 18 months for PbO, whereas it stayed constant at 781–845 $(L/kg)^n$ for $Pb(NO_3)_2$.

3.2. Pb bioaccumulation and toxicity

Measured internal Pb concentrations in the surviving worms exposed for 21 days in control soils did not differ significantly between ageing times and ranged from 0.62 to 3.98 mg Pb/kg dry wt (one-way ANOVA, $p = 0.099$). At each ageing time, Pb concentrations in the animals increased in a dose-related manner with total soil, $CaCl_2$-extractable and porewater Pb concentrations, and for both Pb forms these relations were well described by the Langmuir model (Fig. 1). The maximum Pb uptake capacity (C_{max}) increased with ageing time for PbO, but remained constant for $Pb(NO_3)_2$. Based on total Pb concentrations in the soils, Pb uptake differed between the two Pb forms, with a significantly higher Pb bioaccumulation in $Pb(NO_3)_2$-spiked soils ($\chi^2_{df=1} = 48.1$; $p < 0.001$). No significant differences were found between the two lead forms for all treatments when relating Pb accumulation in the enchytraeids to Pb availability in the soil ($\chi^2_{df=1} \leq 1.39$; $p > 0.05$). $CaCl_2$-extractable and porewater Pb concentrations were the best measures for Pb uptake in *E. crypticus*, and overall Langmuir models could well describe body Pb concentrations from all treatments (Fig. 1C-D). The estimated maximum internal Pb concentrations with corresponding 95% confidence interval were 86.9 (84.4–89.7) and 99.0 (94.7–105) mg Pb/kg dry body wt with $R^2 = 0.895$ and 0.845 based on $CaCl_2$-exchangeable and porewater Pb concentrations, respectively.

The mean survival in the controls was >95%, the mean (±SD; $n = 5$) control juvenile numbers ranged from 640 ± 19 to 855 ± 50 per test jar. The effects of $Pb(NO_3)_2$ and PbO on the survival of *E. crypticus* after 21 d of exposure at different ageing period are shown in Fig. 2. A dose-related decreased survival was found for both Pb forms. The number of juveniles sharply decreased with increasing Pb concentrations in the soil for both Pb forms (Fig. 3). Table 2 summarizes the estimated LC50, LC10 and EC50, and EC10 values for the effects of Pb on enchytraeids survival and reproduction, respectively expressed on the basis of total and 0.01 M $CaCl_2$-extractable Pb concentrations in soil, Pb concentrations in pore water and internal Pb concentrations in the surviving adults at ageing period of 0, 3, 6, 12 and 18 months. Pb toxicity to enchytraeids significantly increased during ageing for PbO with LC50 based on total Pb concentrations decreasing from 4830 mg Pb/kg dry soil at $t = 0$ to 1889 mg Pb/kg dry soil after 18 months ($\chi^2_{df=1} = 149$; $p < 0.001$). For $Pb(NO_3)_2$ the LC50s remained similar at 523–619 mg Pb/kg dry soil. EC50s based on total Pb concentrations declined from 151 mg Pb/kg dry soil at $t = 0$ to 97.5 mg Pb/kg dry soil after 18 months for PbO and were constant at 89.8–99.4 mg Pb/kg dry soil for $Pb(NO_3)_2$. $Pb(NO_3)_2$ was significantly more toxic

Table 1
Parameters describing the sorption of Pb to natural standard LUFA 2.2 soil at different ageing periods after spiking with $Pb(NO_3)_2$ or PbO and related to 0.01 M $CaCl_2$ extractable and porewater Pb concentrations. Shown are the Freundlich sorption constant K_F ($(L/kg)^n$) (K_{FC} and K_{FP} based on $CaCl_2$ extractable and porewater Pb concentration, respectively) and slope parameter n with corresponding 95% confidence intervals.

Treatment	Ageing (months)	Sorption related to $CaCl_2$-extractable Pb concentrations		Sorption related to porewater Pb concentrations	
		K_{FC}	n	K_{FP}	n
$Pb(NO_3)_2$	0	781 (675–887)	0.576 (0.519–0.634)	874 (683–1066)	0.510 (0.428–0.588)
	3	816 (777–860)	0.577 (0.559–0.601)	801 (713–889)	0.470 (0.433–0.507)
	6	845 (813–884)	0.536 (0.522–0.553)	744 (669–819)	0.460 (0.428–0.496)
	12	795 (764–822)	0.543 (0.528–0.556)	704 (651–757)	0.470 (0.443–0.495)
	18	791 (735–847)	0.497 (0.472–0.522)	567 (465–647)	0.510 (0.458–0.593)
PbO	0	8668 (6582–10,752)	1.17 (1.07–1.27)	22,631 (10604–34,660)	1.32 (1.13–1.52)
	3	6087 (5076–7103)	1.07 (1.00–1.14)	21,306 (13447–29,160)	1.67 (1.50–1.84)
	6	5625 (4591–6653)	1.03 (0.95–1.10)	16,188 (10498–21,878)	1.70 (1.53–1.88)
	12	5041 (4045–6035)	1.05 (0.96–1.14)	9292 (5258–13,326)	1.31 (1.11–1.51)
	18	4811 (3678–5895)	1.00 (0.90–1.10)	10,935 (7285–14,586)	1.51 (1.40–1.75)

($\chi^2_{df=1} \geq 28.9$; $p < 0.001$) than PbO in freshly spiked soils (0 months aged soils), with LC50 values of 583 and 4830 mg Pb/kg dry soil and EC50s of 94.6 and 151 mg Pb/kg dry soil for $Pb(NO_3)_2$ and PbO, respectively. Although the LC50 for $Pb(NO_3)_2$ was much lower than that for PbO ($\chi^2_{df=1} = 182$; p < 0.001), EC50s for both Pb forms were similar in the18-months aged soils ($\chi^2_{df=1} = 0.08$; $p > 0.05$).

LC50s based on 0.01 M $CaCl_2$-extractable and porewater Pb concentration ranged from 1.72 to 3.15 mg Pb/kg dry soil and from 0.247 to 0.583 mg Pb/L for both Pb forms (Table 2). The differences in survival and reproduction effects of both Pb forms and ageing periods were well explained by $CaCl_2$-extractable Pb concentrations (Two-way ANOVA, $p \geq 0.478$), giving single overall dose-response curves fitting all data (Figs. 2B, 3B). When expressed as $CaCl_2$-extractable Pb concentration, EC50s were 0.149–0.170 and 0.093–0.101 mg Pb/kg dry soil for $Pb(NO_3)_2$ and $Pb(NO_3)_2$, respectively. EC50s on the basis of porewater Pb concentration were 0.015–0.023 and 0.046–0.050 mg Pb/L, respectively. Internal Pb concentration could well describe enchytraeid mortality, fitting all data for both Pb forms and all ageing periods with a single overall dose-response model ($R^2 = 0.883$) (Fig. 2D) (Two-way ANOVA, $p = 0.813$). LC50s based on internal Pb concentration did not differ between $Pb(NO_3)_2$ and PbO, and were 73.4–78.7 mg Pb/kg dry body wt. Body Pb concentrations did not accurately predict enchytraeid reproduction effects with EC50s ranging from 12.0 to 31.5 mg Pb/kg dry body wt.

4. Discussion

In this study, we investigated the influence of ageing and Pb forms on bioavailability and toxicity of Pb to *E. crypticus* in a natural standard soil. $Pb(NO_3)_2$ was more toxic than PbO in freshly spiked soils, but whereas the toxicity of PbO increased with ageing time, $Pb(NO_3)_2$ toxicity remained constant.

4.1. Pb availability

In non-aged soils, the partition coefficients K_{FC} and K_{FP} for $Pb(NO_3)_2$ and PbO differed by a factor of 10 and 25, respectively. Higher porewater Zn concentrations were found in $ZnCl_2$- than in ZnO-spiked

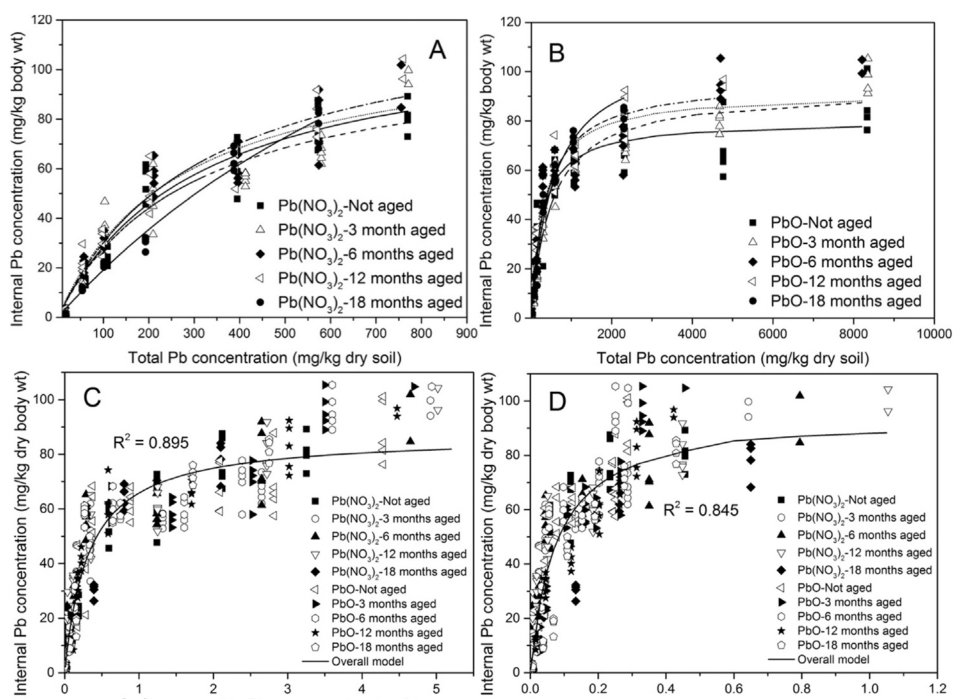

Fig. 1. Lead concentrations in surviving adult *Enchytraeus crypticus* after 3 weeks exposure in natural LUFA 2.2 soil spiked with $Pb(NO_3)_2$ or PbO and aged for different periods, related to total Pb concentration in soil (A and B), 0.01 M $CaCl_2$-extractable Pb concentrations in soil (C) and Pb concentrations in pore water (D). Dots represent measured concentrations, lines show the fit of a Langmuir isotherm (Eq. 2) to the data.

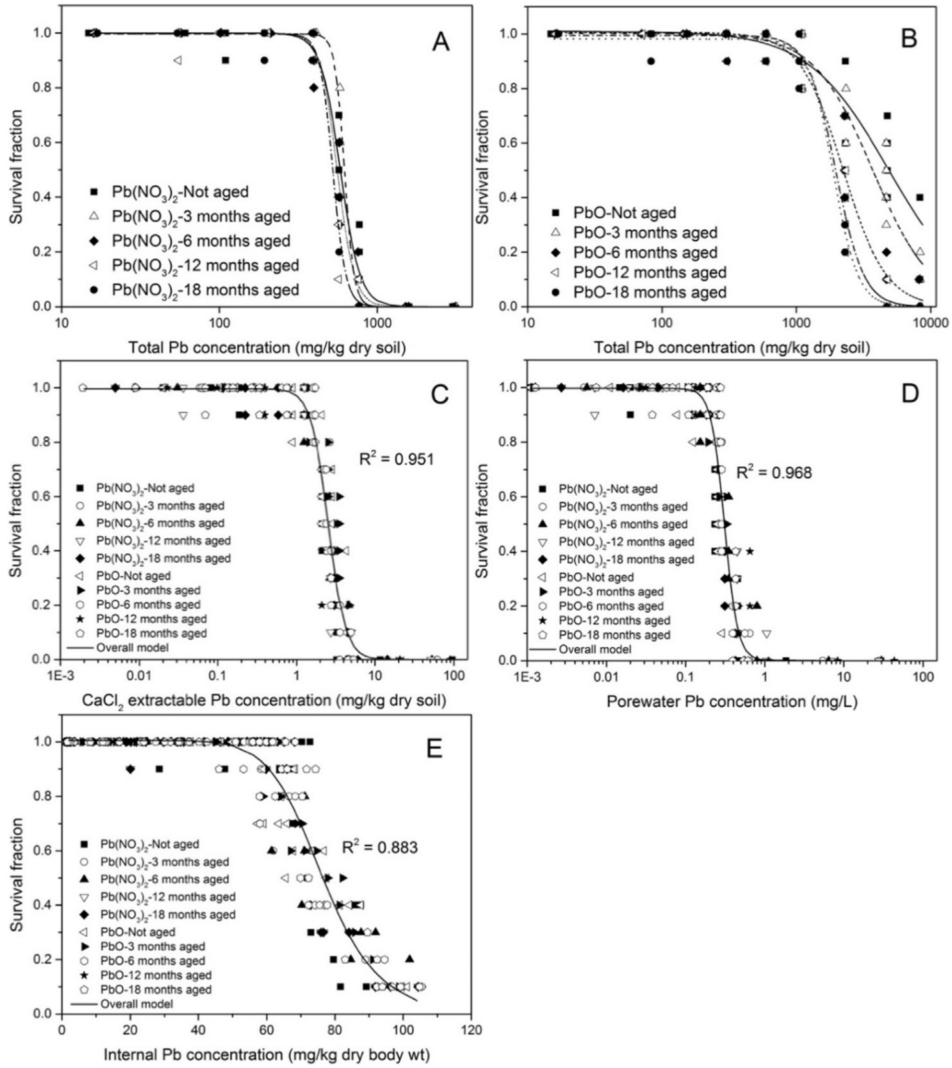

Fig. 2. Effects of $Pb(NO_3)_2$ or PbO on the survival of *Enchytraeus crypticus* after three weeks exposure in natural LUFA 2.2 soil with different ageing periods. Pb concentrations are expressed as total (A and B) and 0.01 M $CaCl_2$ extractable concentrations in soil (C), concentrations in pore water (D), or internal concentrations in surviving adults (E). Lines show the fit of a logistic dose-response curve; in cases where only one curve is shown, the dose-response curves for the different ageing periods and Pb forms did not significantly differ acccording to a likelihood-ratio test.

soils (Lock and Janssen, 2003a; Romero-Freire et al., 2017), whereas for $Pb(NO_3)_2$ and $PbCl_2$ similar Pb sorption was reported in several studies (Bongers et al., 2004; Zhang and Van Gestel, 2017). Thus, metal form influences sorption to soils, resulting in different metal availability between soils. This difference mainly depends on metal solubility, which is a factor of 35,000 higher for $Pb(NO_3)_2$ than for PbO. At higher concentrations of $Pb(NO_3)_2$, Pb sorption decreased due to the limited number of effective binding sites on the soil and the competition of H^+ ions, which increased with increasing soil Pb concentration. As a consequence, the sorption isotherms deviated sharply from linearity with shape parameter n being lower than 1. For PbO, sorption did however, increase with increasing concentration, probably as a consequence of the dose-related increase of soil pH, explaining for the n values being higher than 1. Such dose-related increases of soil pH have also been found for other metal oxides, like ZnO (Waalewijn-Kool et al., 2013; Romero-Freire et al., 2017).

With its higher solubility, the $Pb(NO_3)_2$ spiked into the soil as a powder rapidly dissolved and Pb availability (measured as $CaCl_2$-extractable and porewater Pb concentrations) reached equilibrium within 2 weeks, with constant K_{FC} values over the entire ageing period of 18 months. Liang et al. (2014) reported that soluble Pb concentrations significantly decreased to steady levels at different ageing times (2, 4, 16 and 32 weeks) in different soils spiked with $Pb(NO_3)_2$ at 150 and 1500 mg Pb/kg dry soil. Probably due to its large ionic radius, the availability of Pb in soil changed little with ageing time (Lock and Janssen, 2003b). However, ageing effects on Pb availability were observed for PbO. Solid PbO slowly dissolved, leading to increased Pb availability in the LUFA 2.2 soil and decreased K_{FP} values during ageing. The leveling off with time of K_{FP} indicates that PbO dissolved slowly but that the dissolution rate was slowed down, possibly due to the formation of a crust of lead carbonate within 2 months ageing, protecting the PbO particles efficiently against further weathering (Birkefeld et al., 2007). In general, it may be concluded that the ageing effect on Pb availability was metal form dependent.

4.2. Pb bioaccumulation

After 21 days of exposure, Pb uptake in the enchytraeids was positively correlated with total Pb concentration in soil for both Pb forms and at the different ageing times. Zhang and Van Gestel (2017) observed similar Pb availability and Pb uptake in *E. crypticus* exposed to

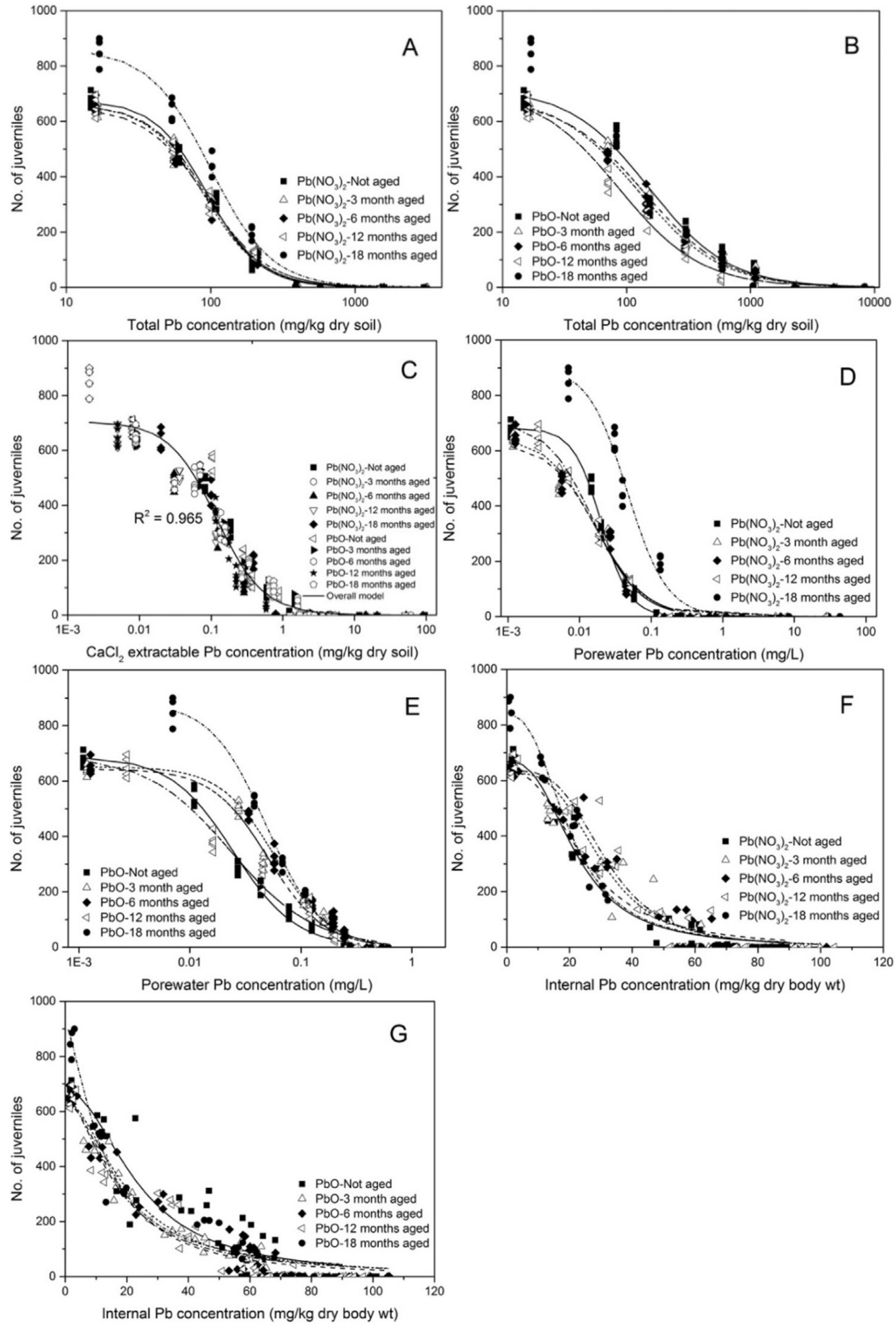

Fig. 3. Effects of $Pb(NO_3)_2$ or PbO on the reproduction of *Enchytraeus crypticus* after three weeks exposure in natural LUFA 2.2 soil with different ageing periods. Pb concentrations are expressed as total (A and B) and 0.01 M $CaCl_2$ extractable concentrations in soil (C), concentrations in pore water (D and E) or internal concentrations in the surviving adults (F and G). Lines show the fit of a logistic dose-response curve; in cases where only one curve is shown, the dose-response curves for the different ageing periods and Pb forms did not significantly differ acccording to a likelihood-ratio test.

$Pb(NO_3)_2$- and $PbCl_2$-spiked LUFA 2.2 soil. Earthworms exposed to Pb-acetate accumulated significantly more Pb than those exposed to Pb $(CO_3)_2$ (Darling and Thomas, 2005). Davies et al. (2003) reported that internal Pb concentrations in earthworms decreased in the order: Pb $(NO_3)_2$ > $PbCO_3$ > PbS following exposure in OECD artificial soil. These findings agree with this study, where the enchytraeids showed higher Pb accumulation in $Pb(NO_3)_2$-spiked soils compared to PbO, indicating that the metal form may have great impacts on the metal uptake by soil organisms. This is probably due to the fact that the highly soluble Pb forms (e.g. $Pb(NO_3)_2$, Pb-acetate and $PbCl_2$) release more Pb into the soil solution than the low soluble forms (e.g. PbO, $PbCO_3$ and PbS).

The Pb concentration in the soil solution or pore water represents the bioavailable pool for Pb accumulation in the enchytraeids. Furthermore, pore water is the major route for metal uptake in soil invertebrates (Van Gestel, 2012). Upon ageing, Pb uptake in the surviving

Table 2
LC50, LC10, EC50 and EC10 values (with 95% confidence intervals) for the effects of lead on the survival and reproduction of *Enchytraeus crypticus* exposed for 21 days in natural standard LUFA 2.2 soil at different ageing periods after spiking with $Pb(NO_3)_2$ or PbO. Effect concentrations are expressed on the basis of total and 0.01 M $CaCl_2$ extractable Pb concentrations in soil, Pb concentrations in pore water and Pb concentrations measured in surviving animals.

	Ageing (months)	$Pb(NO_3)_2$				PbO			
		Total Pb (mg/kg dry soil)	$CaCl_2$ Extractable Pb (mg/kg dry soil)	Porewater Pb (mg/L)	Internal Pb (mg/kg body wt)	Total Pb (mg/kg dry soil)	$CaCl_2$ Extractable Pb (mg/kg dry soil)	Porewater Pb (mg/L)	Internal Pb (mg/kg body wt)
LC50	0	583 (566–600)	2.18 (2.08–2.27)	0.247 (0.231–0.262)	76.2 (67.8–86.8)	4830 (4024–5636)	3.02 (2.74–3.30)	0.262 (0.256–0.268)	78.0 (75.0–81.0)
	3	619 (609–631)	3.06 (2.94–3.18)	0.346 (0.329–0.364)	76.4 (73.4–79.4)	3707 (3356–4057)	3.15 (3.00–3.30)	0.312 (0.302–0.321)	77.7 (76.2–79.2)
	6	557 (540–574)	2.49 (2.34–2.65)	0.328 (0.304–0.353)	77.1 (73.5–80.8)	2229 (2072–2385)	2.36 (2.27–2.45)	0.286 (0.280–0.293)	74.4 (72.0–76.9)
	12	523 (509–538)	2.28 (2.14–2.40)	0.366 (0.336–0.381)	73.4 (72.2–74.7)	1969 (1851–2088)	2.66 (2.54–2.78)	0.302 (0.297–0.305)	78.4 (76.2–80.5)
	18	530 (513–547)	1.72 (1.59–1.86)	0.583 (0.495–0.672)	76.8 (74.9–78.8)	1889 (1753–2025)	2.45 (2.34–2.55)	0.391 (0.373–0.410)	78.7 (77.2–80.3)
LC10	0	426 (393–458)	1.38 (1.23–1.54)	0.128 (0.109–0.148)	64.0 (28.7–63.2)	1067 (571–1565)	1.20 (0.865–1.54)	0.210 (0.196–0.225)	58.1 (53.5–62.7)
	3	531 (518–546)	2.19 (2.07–2.32)	0.244 (0.208–0.241)	60.3 (57,1-63,6)	1220 (937–1502)	1.79 (1.57–2.02)	0.202 (0.187–0.218)	60.0 (58.2–63.0)
	6	424 (387–461)	1.42 (1.17–1.68)	0.165 (0.131–0.199)	59.0 (53.7–64.4)	996 (832–1159)	1.52 (1.37–1.64)	0.232 (0.220–0.245)	60.5 (56.7–64.2)
	12	425 (393–457)	1.49 (1.25–1.71)	0.226 (0.176–0.244)	66.3 (63.5–69.1)	1168 (985–1351)	1.83 (1.62–2.04)	0.269 (0.258–0.279)	65.6 (62.3–69.0)
	18	431 (391–471)	1.03 (0.799–1.27)	0.438 (0.201–0.675)	68.2 (64.5–72.0)	1191 (970–1413)	1.87 (1.66–2.08)	0.317 (0.277–0.358)	71.9 (69.1–74.8)
EC50	0	94.6 (89.5–99.6)	0.149 (0.137–0.169)	0.020 (0.019–0.021)	22.2 (20.5–23.9)	151 (128–174)	0.170 (0.154–0.187)	0.025 (0.023–0.027)	23.7 (18.5–28.8)
	3	89.8 (85.5–94.9)	0.125 (0.115–0.134)	0.016 (0.012–0.019)	24.7 (20.5–28.8)	128 (116–143)	0.135 (0.122–0.148)	0.048 (0.044–0.051)	18.1 (15.7–20.4)
	6	95.7 (90.8–101)	0.090 (0.075–0.106)	0.019 (0.016–0.023)	30.0 (27.2–32.7)	101 (88.5–112)	0.098 (0.087–0.109)	0.050 (0.046–0.054)	19.8 (16.0–23.7)
	12	94.1 (88.5–99.2)	0.103 (0.092–0.113)	0.015 (0.013–0.017)	31.5 (28.4–34.5)	86.1 (72.1–102)	0.138 (0.131–0.145)	0.023 (0.017–0.029)	16.9 (12.6–21.1)
	18	99.4 (91.0–108)	0.093 (0.069–0.117)	0.046 (0.040–0.053)	20.1 (18.2–22.0)	97.5 (83.9–111)	0.101 (0.091–0.112)	0.048 (0.044–0.052)	12.0 (8.9–15.1)
EC10	0	37.3 (32.8–42.2)	0.033 (0.027–0.040)	0.007 (0.006–0.008)	9.7 (8.1–11.3)	29.5 (18.9–39.8)	0.054 (0.042–0.067)	0.006 (0.005–0.007)	8.1 (4.5–11.6)
	3	32.3 (28.6–36.7)	0.029 (0.024–0.035)	0.003 (0.001–0.004)	10.2 (6.7–13.8)	23.6 (18.3–29.7)	0.026 (0.020–0.032)	0.013 (0.011–0.015)	5.6 (4.1–7.2)
	6	35.1 (30.7–39.1)	0.014 (0.009–0.021)	0.004 (0.002–0.006)	15.6 (12.6–18.6)	18.5 (13.5–23.3)	0.017 (0.013–0.032)	0.014 (0.012–0.017)	5.9 (3.5–8.3)
	12	34.8 (30.2–39.2)	0.018 (0.013–0.022)	0.003 (0.002–0.004)	17.0 (13.4–20.6)	15.8 (9.9–22.2)	0.053 (0.045–0.062)	0.003 (0.002–0.005)	4.6 (2.1–7.0)
	18	33.9 (27.1–40.9)	0.011 (0.005–0.017)	0.011 (0.008–0.015)	8.5 (6.6–10.4)	18.8 (12.7–24.9)	0.015 (0.011–0.019)	0.014 (0.011–0.016)	2.7 (1.18–4.23)

worms remained constant for $Pb(NO_3)_2$ but increased for PbO, which could be explained by the changes in Pb availability in the soils for the different Pb forms mentioned above. Thus, total soil concentration could not well describe Pb concentration in the worms for both Pb forms during ageing, confirming that metal bioaccumulation in soil biota is poorly indicated by total soil concentrations (Bradham et al., 2006; Veltman et al., 2007).

For $Pb(NO_3)_2$, internal Pb concentrations in the enchytraeids increased with increasing porewater Pb concentrations and levelled off at high exposure concentrations (Fig. S5A). For PbO, however, Pb uptake increased linearly with Pb concentrations in the soil pore water (Fig. S5B). There are two possible explanations for the observed difference. First, porewater Pb concentrations were lower for soil spiked with PbO than with $Pb(NO_3)_2$, only showing the part of Langmuir model at relatively low concentrations. Second, according to the biotic ligand model (BLM), the lower pH at high exposure concentrations could be responsible for the (relatively) lower Pb accumulation in the enchytraeids for $Pb(NO_3)_2$ due to increased competition of H^+ ions for the effective binding sites on the surface of the organisms. For PbO, increasing porewater Pb concentrations with increasing pH might have contributed to weaker competition of H^+ at higher concentrations, leading to a linear pattern for Pb uptake in the worms (Ardestani et al., 2015; Thakali et al., 2006). However, the difference in internal Pb concentrations in *E. crypticus* between the two Pb forms and between the different ageing periods could be minimized when relating to Pb availability in the test soils expressed as 0.01 M $CaCl_2$-extractable or porewater concentrations. This is consistent with Zhang and Van Gestel (2017), who found that the body Pb concentrations could be well predicted as a function of Pb availability in soils when enchytraeids were exposed to $Pb(NO_3)_2$- and $PbCl_2$-spiked soils.

4.3. Pb toxicity

The dose-response curves and toxicity values (LC50 and EC50) based on total Pb concentrations differed considerably between the two Pb forms. The toxicity of solid $Pb(NO_3)_2$ with LC50s and EC50s based on total soil Pb concentrations of 523–619 and 89.8–99.4 mg Pb/kg dry soil, respectively was similar to the results of Zhang and Van Gestel (2017), who reported LC50s and EC50s of 543–779 and 134–189 mg Pb/kg dry soil, respectively for *E. crypticus* exposed to LUFA 2.2 natural soil spiked with solutions of $Pb(NO_3)_2$ and $PbCl_2$. This suggests similar toxicity of $Pb(NO_3)_2$ mixed in with the soil as a powder or as an aqueous solution, which is consistent with Davies et al. (2003). Luo et al. (2014) found a comparable LC50 of 638 mg Pb/kg dry soil but with a higher

EC50 of 645 mg Pb/kg dry soil for *E. crypticus* exposed to field-contaminated soils from a shooting range. The difference could partly be explained by the differences in soil properties (e.g. soil pH, CEC, OM content) (Bradham et al., 2006). With LC50s of 1889–4830 mg Pb/kg dry soil, in this study PbO was less toxic than $Pb(NO_3)_2$. This was mainly attributed to the much higher Pb availability in $Pb(NO_3)_2$-amended soils, and therefore the higher Pb bioaccumulation in the enchytraeids. The 28d-$EC50_{reproduction}$ values were 993, 8640 and 10,246 mg Pb/kg dry soil, respectively for earthworms exposed to $Pb(NO_3)_2$-, $PbCO_3$- and PbS-spiked OECD artificial soil (Davies et al., 2003). This also confirms that the different solubility of Pb forms may have a great impact on Pb toxicity to soil invertebrates.

In this study, ageing appeared to have little influence on $Pb(NO_3)_2$ toxicity after a pre-incubation of 2 weeks. This agrees with Chen et al. (2014), who reported $EC50_{reproduction}$ values of 1085, 1163, 1667 and 1607 mg Pb/kg dry soil for the toxicity of $Pb(NO_3)_2$ to earthworms after ageing periods of 0, 7, 28 and 84 days, respectively, showing that the effect of ageing on $Pb(NO_3)_2$ toxicity mainly occurred at the beginning of the incubation. This probably is due to the high solubility of $Pb(NO_3)_2$. Thus, an inclusion of an equilibration period for freshly spiked soils before use might help to achieve a more realistic exposure situation in laboratory toxicity tests. However, Smolders et al. (2015) found that a 5-year outdoor ageing period decreased Pb^{2+} salt toxicity by a factor of 1.4–50 in 19 out of the 20 toxicity tests on plants, invertebrates and microbial processes. The decrease in toxicity could partly be explained from outdoor ageing involving leaching by rain drainage, suggesting that salt stress might be an important factor modifying Pb toxicity in freshly spiked soils. Since our study did not include such a leaching effect, further studies on the effects of percolation on $Pb(NO_3)_2$ toxicity in freshly spiked and aged soils are needed. In the present study, ageing strongly influenced PbO-spiked soil ecotoxicity with decreasing LC50s and EC50s during ageing, which might mainly be attributed to the increase of Pb availability in the soil with ageing time (Figs. S3–4). This was in agreement with Smolders et al. (2015), and also with Schreck et al. (2011) who found that a 3-month ageing period increased the toxicity of metallic Pb particles, composed of PbS, $PbSO_4$, PbO,·$PbSO_4$, α-PbO and Pb^0, to *Vibrio fisheri* by a factor of 2.0–5.1. This suggests that metallic ion dissolution in the soil solution and particle aggregation and accumulation by soil organisms may contribute to the increase of Pb toxicity during ageing. However, LC50s and EC50s reached steady levels after 6 months ageing. Ageing, therefore, affected Pb toxicity in soils and its effect was dependent on the specific metal form, suggesting that ecotoxicity tests using freshly spiked soils might not be suitable to accurately predict the risk of metals in field-contaminated soils. Laboratory toxicity tests should rather as much as possible take into account the form of the metal occurring at contaminated sites, and account for the effects of ageing and leaching.

Pb availability measured as $CaCl_2$-extractable or porewater Pb concentrations was a better descriptor of Pb toxicity (adverse effects on organisms) than total Pb concentration in soils, suggesting that toxicity might be determined by the metal ions in the soil solution (Bradham et al., 2006; Smolders et al., 2009; Zhang and Van Gestel, 2017). Although LC50s and EC50s based on total Pb concentration varied between the two Pb forms and the different ageing periods, the mortality of the worms could be explained from Pb availability in the soil or internal Pb concentration in the enchytraeids ($R^2 > 0.883$). The $CaCl_2$-extractable Pb concentration could well describe enchytraeid reproduction, independent of ageing time and Pb form (Fig. 2B, D). Body concentration was also suggested to be a predictor of metal toxicity, as the likelihood of adverse effects (mortality) to occur increases when the capacity of detoxification is exceeded, so when internal concentration reaches or exceeds the lethal body concentration (Jager et al., 2011; Vijver et al., 2004). Furthermore, in a previous study, we also observed that survival was well correlated with body Pb concentrations in *E. crypticus* exposed to different Pb salts (Zhang and Van Gestel, 2017). Eliminating effects from different routes of uptake, internal Pb concentration therefore might be a good indicator of Pb bioavailability and toxicity in soils.

5. Conclusions

In this study, Pb availability to *E. crypticus* increased with ageing for PbO but remained constant for $Pb(NO_3)_2$-spiked soils. Pb bioaccumulation in the enchytraeids did not differ between the two Pb forms when related to Pb availability in the soil. $Pb(NO_3)_2$ was more toxic than PbO in freshly spiked soils, but toxicity of PbO increased with ageing time while that of $Pb(NO_3)_2$ remained constant. Expressed on the basis of available Pb concentrations, differences in toxicity between the Pb forms and ageing periods almost disappeared, with $CaCl_2$-extraction providing the best estimate of Pb toxicity and bioaccumulation in the enchytraeids.

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Declarations of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.02.054.

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