Contribution of soil properties of shooting fields to lead bioavailability and toxicity to *Enchytraeus crypticus*

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ABSTRACT

Variation in soil properties may cause substantial differences in metal bioavailability and toxicity to soil organisms. In this study, lead bioavailability and toxicity to *Enchytraeus crypticus* was assessed after 21 days exposure to soils from different landscapes of a shooting range containing 47–2398 mg Pb/kg dry weight (dw). Soils had different pH_{CaCl2} (3.2–6.8) and organic matter contents (3.8–13% OM), therefore artificial soils with different pH and OM contents and two natural reference soils were included as controls. Effects on survival and reproduction and the uptake of Pb in *E. crypticus* were related to soil properties and total, water- and CaCl₂-extractable and porewater Pb concentrations in the soils. Forest soils with pH_{CaCl2} < 3.5 and total Pb concentrations ≥ 2153 mg/kg dw had the highest bioavailability and toxicity of Pb to *E. crypticus*. At pH_{CaCl2} 3.2 adult survival was inhibited and no juveniles were produced, while at pH_{CaCl2} 3.8 reproduction was also reduced. Bioaccumulation of Pb linearly increased with increasing total soil Pb concentrations. The grassland soils with pH_{CaCl2} > 6.5 and total Pb concentrations 355–656 mg/kg dw were least toxic. This study shows that *E. crypticus* was very sensitive to acidic soils with pH_{CaCl2} ≤ 3.8, suggesting that the toxic effects in the most contaminated forest soils may have been due to the low soil pH rather than the high Pb concentrations.

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1. Introduction

Soil Pb contamination is a particular challenge due to the long-term retention time of Pb in the environment from 150 to 5000 years (Kumar et al., 1995). Shooting fields often have substantial Pb pollution from the use of bullets. Worldwide, environmental protection agencies stipulate the need for assessing Pb bioavailability in shooting field soils to assist the estimation of the risk of these soils (Dayton et al., 2006). Current legislation and assessment of Pb concentrations in soils is mainly based on the total concentration of Pb present in the soil (Davies et al., 2003). However, Pb bioavailability and toxicity as well as risk depend not only on the amount of Pb in soils and the characteristics of the organisms themselves, but also on the soil characteristics (Van Gestel et al., 1995; Bradham et al., 2006; Smith et al., 2012).

Recently, there has been a shift towards the determination of “environmentally accepted endpoints” in environmental risk assessment, an approach based on the recognition that the

interactions of pollutants with the soil matrix may affect their risk (Magrissio et al., 2009). A combination of soil properties seems to govern metal bioavailability (Van Gestel et al., 1995). To assess risk, it is therefore important to accurately characterize Pb-contaminated soils with different physicochemical properties and their toxicities to different soil organisms (Adriano, 2001; Dayton et al., 2006). Bradham et al. (2006) found a huge difference in the toxicity to earthworms of Pb spiked at 2000 mg/kg dw in different field soils, which could be attributed to the difference in soil properties. Lock and Janssen (2001c) concluded that pH, cation exchange capacity, and soil organic matter content are important soil parameters affecting bioavailability of Pb to *Enchytraeus albidus*.

So far, the studies reported in the literature have given inconsistent results as to the role of different soil properties in determining the bioavailability and toxicity of Pb. In part the problem lies in the wide range of chemical forms (or species) in which Pb can be found in different soils. Furthermore, soil organisms were usually exposed to soils freshly spiked with soluble Pb salts such as Pb nitrate (Lock and Janssen, 2001c; Davies et al., 2003; Bradham et al., 2006). Pb in spiked soils is likely to be more bioavailable and hence toxic at lower concentrations than the aged mixture of Pb species

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encountered in field soils. Guideline values developed from studies using Pb-spiked artificial soil may thus overestimate the risk posed by less mobile Pb in contaminated field soils (Efroymsen et al., 1997; ESTCP-ER, 2005). Additionally, tests using clean soil spiked with soluble Pb salts do not adequately reflect the soil properties likely to be found in the field. Generally, the majority of Pb in natural soils at contaminated sites will be present as solids which are not bioavailable (Davies et al., 2003). And Pb present in shooting field soils for a prolonged period can become recalcitrant over time due to various processes (e.g., ageing, weathering, sequestration, adsorption, etc.) (Spurgeon and Hopkin, 1995; Peijnenburg and Jager, 2003; Smith et al., 2012), thus influencing its bioavailability. As a consequence, Pb toxicity is generally less pronounced in Pb-contaminated field soils than in soils freshly spiked with Pb salts at similar total Pb concentrations (Spurgeon and Hopkin, 1995; Lock et al., 2006). In order to accurately represent environmental conditions found at contaminated sites, some factors controlling Pb availability such as pH, CEC, clay content and soil organic matter content and amount of Pb available to test organisms in the soil should be considered. The effect of soil properties may be different for different organisms and therefore is not as straightforward as expected.

Although earthworms and collembolans are recommended in various guidelines as standard test organisms for the terrestrial environment, there is a growing need for further tests with soil invertebrate species from different trophic levels to improve the risk assessment of chemicals in soil (Römbke and Moser, 2002). Enchytraeids are ecologically relevant soil organisms due to their activity in decomposition and bioturbation and their abundance in many soil types worldwide (Castro-Ferreira et al., 2012). Enchytraeidae cover an exposure route different from those of collembolans. In addition, enchytraeids are often abundant in soils where earthworms are scarce and they are sensitive to chemicals. Consequently, enchytraeids are recommended as organisms for a chronic ecotoxicological test (Weyers et al., 2002). The most commonly used species are *Enchytraeus crypticus* and *E. albidus*. *E. crypticus* seems a better model species than *E. albidus*, as it has better control performance, a shorter generation time, and higher reproduction rates, enabling reliable and faster toxicity testing (Lock and Janssen, 2001b; Van Gestel et al., 2011; Castro-Ferreira et al., 2012; Chapman et al., 2013). Only few studies have systematically examined the influence of soil parameters on the ecotoxicity of Pb to *E. crypticus*. None of the available studies allows quantification of the influence of the soil parameters on the bioavailability and ecotoxicity of Pb for *E. crypticus*.

This study aimed at gaining insight into the impact of soil characteristics on the bioavailability and toxicity of Pb to *E. crypticus*. For this purpose, six natural field soils were collected from different landscapes (bullet plot, forest and grassland) of a shooting field in the Netherlands, representing a gradient of Pb pollution but also having different pH and organic matter contents (Luo et al., 2014). To unravel the effects of main soil properties on *E. crypticus*, three reference soils with different the pH and organic matter contents were used for comparison. Effects of Pb on the survival and reproduction of *E. crypticus* were related to total, water-extractable, CaCl_2 -exchangeable and porewater concentrations as well as to internal concentrations in the surviving *E. crypticus*.

2. Materials and methods

2.1. Soil sampling and analysis

Six natural soils were collected from three landscapes (forest, grassland, bullet plot (which is an earthen dike used to capture bullets)) of a shooting field in the Netherlands. A soccer field soil

near the shooting range was sampled as a reference. At each site, a square zone (25×25 m) divided with grid pattern (5×5 m) was established. A total of 10 soil samples were collected from the cross line of the zone, using a cylindrical soil corer to a depth of 20 cm. The 10 samples from each site were pooled and mixed thoroughly to give one representative sample for each site. The soil samples were air dried, homogenised and 2 mm sieved. Three artificial soils (A1, A2, A3) were prepared to “mimic” the shooting field soils in pH and organic matter content, based on OECD artificial soil (OECD, 1984). The standard artificial soil (A1) was prepared by mixing 10% finely ground sphagnum peat (<1 mm), 20% kaolin clay, 70% quartz sand (dry weight), and some CaCO_3 to obtain a nominal $\text{pH}_{\text{CaCl}_2}$ 6.0 ± 0.5 . The other two artificial soils had peat contents of 5% (A2) or 2.5% (A3) and nominal $\text{pH}_{\text{CaCl}_2}$ levels of 3.5 (A2) or 6.5 (A3). The standard natural LUFA 2.2 soil (LUFA-Speyer, Sp 2121, Germany) was used as an additional control of the performance of the test animals (CK). For a full description of the methods used to analyse the soils, it can be referred to Luo et al. (2014).

2.2. Toxicity tests

E. crypticus has been cultured for several years at the VU University in agar prepared with an aqueous soil extract, fed *ad libitum* with oatmeal, and kept at 16 °C, 75% relative humidity and 16/8 h light/dark photoperiod. The toxicity tests were performed following OECD guideline 220 (OECD, 2004), using five replicate glass vials (100 mL) per test soil and control. Ten adults with white clitella and similar size were introduced into each glass vial containing 30 g of moist soil prepared previously. Then 2 mg oatmeal was supplied and vessels were closed with perforated aluminium foil. The exposures lasted 21 days at 20 °C, 75% relative humidity and 16/8 h light/dark photoperiod. Food availability and soil moisture content were checked weekly and replenished if necessary. After 3 weeks, all samples were fixated by adding 10 mL of 96% ethanol. After 2 min, 100 mL water was used to transfer the sample into a plastic container, where it was stained with 300 μL of Bengal rose solution (1% in ethanol). Then containers were tightly closed, agitated vigorously for 10 s and incubated overnight at 4 °C to achieve optimal staining of the animals. Samples were sieved over 160 μm to separate the enchytraeids from most of the soil. Subsequently, each sample was transferred into a white tray (80×50 cm²) and divided in fractions to optimize the counting of the pink-stained enchytraeids under a magnifying glass, and so assess the number of adults and juveniles per replicate. After determination of the dry weight, the animals were individually digested in a 300 μL $\text{HClO}_4/\text{HNO}_3$ mixture (1:7 v/v; Ultrex grade, Mallbaker) as described by Van Straalen and Van Wensem (1986) and Pb concentrations in their bodies were measured using a Perkin Elmer 5100 atomic absorption spectrometer (AAS) equipped with a graphite furnace assembly. Quality of the analysis was controlled by analysing certified reference material (Dolt 4); metal concentrations usually were within 15% of the certified values.

2.3. Data analysis

Data were checked for homogeneity of variance and normality (Kolmogorov–Smirnov test) and, when possible, subjected to one-way ANOVA. Whenever significant differences were found ($p < 0.05$), a post hoc Tukey HSD test was used to further elucidate differences among means ($p < 0.05$). Pearson's correlation coefficients (r) were calculated between toxicity and soil physico-chemical properties and (bio)available Pb concentrations ($p < 0.05$). Multiple regressions were carried out to quantitatively analyse the relationship among *E. crypticus* bioassay endpoints (survival,

reproduction and body residue of Pb), level of available Pb, and other soil physicochemical properties. LC50s for the effect of Pb on *E. crypticus* survival were calculated using the trimmed Spearman–Karber method (Hamilton et al., 1977). EC10 and EC50 values for effects on reproduction were estimated with a log-logistic model (Haanstra et al., 1985), applying the modification described by Van Brummelen et al. (1996). The EC10 and EC50 values were calculated using the solver option in Excel and if possible 95% confidence intervals were calculated using the IBM software package SPSS21.0 for Windows.

3. Results

3.1. Soil characteristics

Physicochemical characteristics of the shooting field soils as well as a full description of the soil properties and metal concentrations has been given by Luo et al. (2014). Here main results are briefly summarized. All forest soils were most acidic, with $\text{pH}_{\text{CaCl}_2}$ 3.2–3.5, while all grassland soils were neutral to alkaline with $\text{pH}_{\text{CaCl}_2}$ 6.5–6.8. Organic matter contents in shooting field was highest in forest soils (5.9–7.0%), followed by grassland soils (4.1–5.3%), and lowest in the bullet plot soil (3.8%). The forest soils also had the highest DOC content (651–984 mg/L), the grassland soils the lowest (183–519 mg/L). Grassland soils had the highest CEC (5.9–13 $\text{cmol}_\text{c}/\text{kg}$), followed by forest soils (2.1–2.2 $\text{cmol}_\text{c}/\text{kg}$) and the bullet plot soil (1.8 $\text{cmol}_\text{c}/\text{kg}$).

3.2. Toxicity tests

Significantly lower survival of *E. crypticus* was observed in all forest soils compared with grassland and bullet plot soils as well as artificial and CK soils ($p < 0.05$) (Fig. 1A). More than 50% of the animals died in the forest soils after 21 days of exposure. Forest soils 2 and 3 had significantly lower survival of *E. crypticus* than Forest soil 1. Survival of *E. crypticus* was 92–100% in soccer field, grassland, bullet plot and artificial soils and CK. LC50 values for the toxicity of Pb related to total, extractable and porewater Pb concentrations are shown in Table 1.

Hardly no juvenile *E. crypticus* were produced in all forest soils and the bullet plot soil (Fig. 1B). The Grassland soil 2 had significantly lower juvenile numbers than Grassland soil 1 which had significantly less juveniles than CK. Compared with CK, reproduction was reduced by more than 50% in all Forest soils, Bullet plot soil and Grassland soil 2, as well as in A2. Soils A1 and A3 had juvenile numbers significantly greater than A2, but significantly lower than CK ($p < 0.05$). There was no significant difference in juvenile numbers between CK and soccer field soil. Table 1 shows EC10 and EC50 values for the effect of Pb on enchytraeid reproduction, related to total, water- and CaCl_2 -extractable and porewater Pb concentrations. Because dose–response relationships were quite steep and reproduction was affected not only by Pb but also by other factors (see below), in many cases data did not allow calculation of 95% confidence intervals. In addition, steepness of the dose–response relationships resulted in only very small differences between EC50 and EC10 values (Table 1).

The data did not allow calculation of LC50 or EC50 values relating enchytraeid responses to body Pb concentrations measured in the surviving animals.

3.3. Pb bioaccumulation

Pb concentrations in *E. crypticus* increased with increasing total Pb concentrations in the shooting field soils (Fig. 1C). The lowest concentrations of Pb in enchytraeids were observed in CK and A2,

Fig. 1. Survival (A), juvenile (B) numbers and tissue Pb concentrations (C) of *Enchytraeus crypticus* after 21 days exposure to shooting field soils. Columns with the same letter indicate no significant differences at $p > 0.05$. Soils are arranged in order of increasing total Pb concentrations. See Luo et al. (2014) for soil properties and metal concentrations. Error bars show standard deviation ($n = 5$).

the highest in Forest soils 2 and 3. There were significant differences in internal Pb concentrations in enchytraeids exposed to shooting field soils: Forest soil 3 $\approx$ Forest soil 2 > Grassland soil 2 > Grassland soil 1 > Bullet plot soil > Forest soil 1 ($p < 0.05$), with internal Pb concentrations in the enchytraeids increasing with the total soil Pb concentrations. Internal Pb concentrations were significantly higher in animals from the references soils A1 and A3 than from CK ($p < 0.05$), while there was no significant difference in internal Pb concentrations between A2 and CK ($p > 0.05$).

Table 1

LC50, EC10 and EC50 values (with corresponding 95% confidence intervals) for the effects of Pb on the survival and reproduction of *Enchytraeus crypticus* exposed for 21 days to soils from a shooting range and different reference soils. Toxicity is related to total and available Pb concentrations in the soils. See Luo et al. (2014) for soil properties and metal concentrations, Fig. 1 shows the enchytraeid responses and internal concentrations in the enchytraeids.

	LC50	EC50	EC10
Total Pb (mg/kg dw)	638 (525–774)	645 (– ^a)	583 (–)
Water extractable Pb (mg/kg dw)	1.5 (1.2–1.8)	0.46 (–)	0.43 (–)
CaCl ₂ extractable Pb (mg/kg dw)	8.5 (5.6–13)	1.6 (–)	1.3 (–)
Porewater Pb (μg/L)	643 (433–955)	126 (95–157)	119 (13–225)

^a Confidence intervals very wide or not available.

3.4. Relationships between biological responses and physicochemical soil properties

Simple linear correlation coefficients for the relationships between bioassay endpoints and soil properties are given in Table 2. A significant negative relationship was observed between survival and CaCl₂-extractable Pb concentrations ($r = -0.87$, $p < 0.01$). Especially pH and Ca, but also CEC, clay and sand contents contributed most to explaining the variance of survival ($p < 0.01$). Survival was slightly, but significantly related to Fe or silt contents ($p < 0.05$). No significant correlation was found between survival and OM or DOC contents. Juvenile number was significantly and negatively correlated with all Pb concentrations, especially with porewater Pb concentrations ($r = -0.85$, $p < 0.01$), but positively correlated with Ca, pH_{CaCl2}, CEC, clay, silt and WHC ($p < 0.01$). Internal Pb concentrations in the enchytraeids were significantly correlated with soil Pb concentrations, especially with porewater ($r = 0.74$, $p < 0.01$) and total Pb concentrations ($r = 0.98$, $p < 0.01$) (Table 2).

To determine if the combined effects of multiple soil properties may modify soil Pb toxicity, a multiple linear-regression model was used to examine the relationships between enchytraeid bioassay

Table 2

Simple linear correlation coefficients relating the response of *Enchytraeus crypticus* to the physicochemical properties of different shooting range field soils and reference soils. See Luo et al. (2014) for soil properties and metal concentrations and Fig. 1 for the enchytraeid responses and internal Pb concentrations in the enchytraeids.

Soil physicochemical properties	Simple linear correlation coefficients (r)		
	Survival number	Juvenile number	Pb in enchytraeids (mg/kg dw)
WHC	0.025	0.40 (**)	–0.086
OM (%)	–0.061	0.27 (*)	–0.050
pH-H ₂ O	0.69 (**)	0.70 (**)	–0.12
pH-0.01 M CaCl ₂	0.70 (**)	0.75 (**)	–0.16
DOC (mg/L)	–0.25	0.075	–0.19
CEC (cmol _c /kg)	0.51 (**)	0.75 (**)	–0.20
Ca (mg/kg)	0.70 (**)	0.81 (**)	–0.18
Fe (mg/kg)	–0.30 (*)	–0.17	0.20
%Clay (<8 μm)	0.40 (**)	0.51 (**)	–0.27
%Silt (8–63 μm)	0.27 (*)	0.44 (**)	–0.20
%Sand (63–2000 μm)	–0.37 (**)	–0.50 (**)	0.26
Pb-Water (mg/kg)	–0.72 (**)	–0.73 (**)	0.66 (**)
Pb-0.01 M CaCl ₂ (mg/kg)	–0.87 (**)	–0.81 (**)	0.60 (**)
Pb-porewater (μg/L)	–0.82 (**)	–0.85 (**)	0.74 (**)
Total Pb (mg/kg)	–0.65 (**)	–0.63 (**)	0.98 (**)
Total Cd (mg/kg)	0.20	0.072	–0.012
Total Zn (mg/kg)	0.16	0.32 (*)	0.035
Total Cu (mg/kg)	0.012	0.067	0.14

**Correlation is significant at the 0.01 level (2-tailed).

*Correlation is significant at the 0.05 level (2-tailed).

endpoints and the combined soil properties. The best-fitting models are shown in Table 3. CaCl₂-extractable Pb concentration in soil was the best predictor for survival of *E. crypticus* ($R^2 = 0.93$, $p < 0.001$), porewater Pb concentration best predicted juvenile number of *E. crypticus* ($R^2 = 0.98$, $p < 0.001$) and water-extractable Pb concentration best predicted internal Pb concentration ($R^2 = 0.98$, $p < 0.001$).

4. Discussion

4.1. Impact of Pb extraction and soil properties on survival of *E. crypticus*

Ecotoxicity testing provides a direct measure of bioavailability, and the effects on survival and reproduction as well as the internal concentrations in the animals are attributable to exposure to the bioavailable fraction of metal in the soil (Smith et al., 2012). This is one of the first attempts to use enchytraeids to assess the potential toxicity of Pb-contaminated field soils. The different survival numbers in forest and grassland soils indicate that the enchytraeids exhibited a more severe toxic effect in acidic soils than in alkaline soils. The relatively high pH, CEC and organic matter contents provide the grassland soils with a higher capacity of binding Pb compared to the acid forest soils, reducing the bioavailability of Pb (Erel and Morgan, 1992; Terhivuo et al., 1994; Teutsch et al., 2001; Sauvé, 2002; Halim et al., 2005; Magrisso et al., 2009; Chapman et al., 2013). Grassland soils therefore had much higher survival than the forest soils. The correlations between survival and Pb concentrations and soil properties (Table 2) show that CaCl₂-extractable Pb concentration was the best indicator of the effects of Pb on enchytraeid survival. But also pH, CEC and Ca contents played significant roles in affecting survival of the enchytraeids (Lock and Janssen, 2001c). Since the pH optimum for *E. crypticus* is 5.9–6.5 and the pH tolerance range is 3.6–7.7 (Jänsch et al., 2005), pH_{CaCl2} values in forest soils in our study were outside the pH tolerance range. The other properties of the shooting field soils (see Luo et al., 2014) were within tolerance ranges for *E. crypticus* (1–29% clay, 1.2–42% organic matter (Kuperman et al., 2006; Van Gestel et al., 2011) and 4–80% sand (Amorim et al., 2005)). The extractable Pb concentrations in Bullet plot soil and reference soil A2, both having low but not significantly different pH_{CaCl2} values, did not lead to effects on enchytraeid survival. This shows that these concentrations, corresponding with total Pb concentrations lower than 88 mg/kg dw, were not high enough to cause enchytraeid mortality. The absence of significant mortality in soils A1, A2, A3 and CK suggests that, within the tolerance ranges of soil properties for *E. crypticus*, the differences of soil pH and OM content did not have a significant effect on enchytraeid survival. Therefore, we can conclude that the highest mortality of adult enchytraeids in Forest soils 2 and 3 was caused by a combined effect of pH values below its tolerance range and total soil Pb concentrations of 2153 mg/kg or higher (Terhivuo et al., 1994), while the relatively high mortality in Forest soil 1 was due to the low soil pH (Gonzalez et al., 2011). The best regression model in the present study confirmed that enchytraeid survival could be predicted from CaCl₂-extractable Pb concentrations and DOC concentrations in the soil porewater. This is in agreement with the findings of Lock and Janssen (2001c).

In the present study, LC50 was lower than the value for *E. albidus* (4530 mg/kg dw) reported by Lock and Janssen (2002). The great differences in LC50s may be due to the different pH values in the studied soils. It shows the bioavailable fraction causing toxicity to *E. crypticus* was achieved at a much lower total Pb content for acidic soils relative to alkaline soils (Ming et al., 2012). LC50s based on water- and CaCl₂-extractable Pb concentrations in soils were lower for *E. crypticus* (1.5 and 8.5 mg Pb/kg dw) than for the earthworm

Table 3

Multiple-regression equations and coefficient of determination (R^2) for the relationship between the toxicity of Pb contaminated shooting range field soils to *Enchytraeus crypticus* and soil physicochemical properties. See Luo et al. (2014) for soil properties and Pb concentrations and Fig. 1 for toxicity data.

Endpoint	Multiple linear regression equation obtained	Statistics
Survival number (SN)	$SN = -3.8 (\log [Pb]_{Total}) + 0.95pH_{CaCl_2} - 0.006 DOC + 15$	$p < 0.01; R^2 = 0.82$
	$SN = -1.4 (\log [Pb]_{Water\ extractable}) - 4.0 (\log [Fe]) + 4.5 (\log [Ca]) + 1.2 pH_{CaCl_2} + 19$	$p < 0.001; R^2 = 0.89$
	$SN = -2.3 (\log [Pb]_{CaCl_2\ extractable}) - 0.002 DOC + 8.6$	$p < 0.001; R^2 = 0.93$
Juvenile number (JN)	$SN = -2.7 (\log [Pb]_{Porewater}) + 1.1 (\log [Ca]) - 0.003 DOC + 0.11 Sand - 0.67 OM + 0.17 CEC + 8.2$	$p < 0.05; R^2 = 0.92$
	$JN = -120 (\log [Pb]_{Total}) + 270 (\log [Ca]) + 216 (\log [Fe]) + 18 Clay - 1120$	$p < 0.001; R^2 = 0.89$
	$JN = 72 (\log [Pb]_{Water\ extractable}) + 675 (\log [Ca]) + 14 Clay + 0.96 DOC - 18 WHC - 1538$	$p < 0.001; R^2 = 0.94$
	$JN = -70 (\log [Pb]_{CaCl_2\ extractable}) + 22 CEC + 9.7 Clay + 0.13 DOC - 82$	$p < 0.05; R^2 = 0.98$
	$JN = -78 (\log [Pb]_{Porewater}) + 269 (\log [Ca]) + 0.22 DOC - 440$	$p < 0.001; R^2 = 0.98$
Pb concentration in <i>Enchytraeus crypticus</i> (C_w , mg/kg dw)	$\log C_w = 1.1 (\log [Pb]_{Total}) - 0.2 (\log [Cd]) - 0.021 Silt - 0.25$	$p < 0.05; R^2 = 0.98$
	$\log C_w = 0.95 (\log [Pb]_{Water\ extractable}) + 0.69 (\log [Fe]) + 0.1 pH_{CaCl_2} + 1.7$	$p < 0.001; R^2 = 0.98$
	$\log C_w = 0.66 (\log [Pb]_{CaCl_2\ extractable}) - 0.001 DOC + 0.38 pH_{CaCl_2} - 0.025 Clay - 0.082 OM + 1.2$	$p < 0.01; R^2 = 0.97$
	$\log C_w = 0.99 (\log [Pb]_{Porewater}) + 1.3 (\log [Ca]) + 0.032 Clay - 3.3$	$p < 0.001; R^2 = 0.97$

Eisenia andrei (5.5 and 98 mg Pb/kg dw) exposed to the same soils and this was also the case for LC50s based on porewater concentrations (0.643 mg/L versus 5.1 mg/L) (Luo et al., 2014).

4.2. Impact of Pb extraction and soil properties on reproduction of *E. crypticus*

Artificial soil A2 and reference soil CK had low but not significantly different total Pb concentrations (≤ 45 mg Pb/kg dw) while the pH of A2 ($pH_{CaCl_2} = 3.8$) was significantly lower than that of CK ($pH_{CaCl_2} = 5.5$), but still within the tolerance range for *E. crypticus* (Jänsch et al., 2005). The lower reproduction in A2 suggests that $pH_{CaCl_2} 3.8$ had a significant effect on enchytraeid reproduction. Almost no juveniles appeared in forest and bullet plot soils indicating that the enchytraeids were unable to reproduce in acidic soils with $pH_{CaCl_2} 3.2$ – 3.7 . *E. albidus* was reported to be unable to reproduce in sandy soils with pH lower than 4.5 (Lock and Janssen, 2001b). Since Forest soils 2 and 3 had the highest total and extractable Pb concentrations and pH values below the enchytraeid's tolerance range, both total Pb concentration ≥ 2153 mg/kg dw and $pH \leq 3.5$ in soils contributed to the effects on the reproduction of *E. crypticus*. Although the Forest soil 1 and Bullet plot soil had total, $CaCl_2$ -extractable and porewater Pb concentrations significantly lower than the Forest soils 2 and 3, also in these soils hardly any juveniles were produced. This probably can be attributed to the very low pH_{CaCl_2} (3.2–3.7) of Forest soil 1 and Bullet plot soil, which also suggests that it was not Pb but rather the low pH that affected reproduction of *E. crypticus*. The pH of Grassland soil 2 ($pH_{CaCl_2} = 6.8$) approached the optimum for *E. crypticus* (5.9–6.5) while its total Pb concentration was significantly higher than that of Grassland soil 1. The relatively low levels of organic matter, CEC, Ca and Fe increased the bioavailability of Pb in Grassland soil 2 (Lock and Janssen, 2001a; Amorim et al., 2005; Bradham et al., 2006; Chapman et al., 2013). Therefore, the significantly lower reproduction of *E. crypticus* in Grassland soil 2 than in Grassland soil 1 was caused by the significantly higher total and available (porewater) Pb concentrations. The correlations between reproduction and soil properties (Table 2) showed that the most important soil properties modifying reproduction were porewater Pb concentration, Ca, pH and CEC. Compared to these four factors, the effect of other soil properties was less important. The regression models relating juvenile numbers with available Pb concentrations and soil properties (Table 3) also demonstrated that porewater Pb concentration best predicted *E. crypticus* reproduction in the test soils.

Based on the differences between survival and reproduction in Forest soil 1 and the artificial soils A1, A2 and A3, it may be concluded that juvenile number was more sensitive to the low soil pH than survival. This implies that for any tier of risk assessment,

the selection of test species should not only depend on its sensitivity to the contaminant of concern, but also on its tolerance to the soil properties of the site being assessed. It should also be noted that, in this study, dose–response relationships for survival and reproduction were quite similar. As a consequence EC50 and LC50 values were the same when expressed on the basis of total soil Pb concentrations and differed no more than a factor of 3.3–5.3 when expressed on the basis of water- and $CaCl_2$ -extractable or porewater Pb concentrations (Table 1).

Although we could not find any document reporting EC50_{reproduction} for the effect of Pb on *E. crypticus* in different soils, the EC50s in present study were lower than those for *Eisenia fetida* (Spurgeon and Hopkin, 1995) and *Folsomia candida* (Sandifer and Hopkin, 1996, 1997; Bongers et al., 2004). EC50_{reproduction} values based on total Pb concentrations were lower for *E. crypticus* (645 mg Pb/kg dw) in the present study and the earthworm *E. andrei* (1482 mg Pb/kg dw) reported by Luo et al. (2014), while EC50 values related to water and $CaCl_2$ -extractable and porewater concentrations were similar for both test organisms (see Luo et al., 2014).

4.3. Pb bioavailability in relation to soil properties

Although internal Pb concentrations increased with increasing Pb concentrations in the shooting field soils, it was not possible to judge whether the uptake of Pb in the enchytraeids reached steady-state level. Some regulation of the uptake of Pb seems to occur at low contamination levels with Pb uptake being limited at Pb concentrations of ≤ 3000 mg Pb/kg soil (Davies et al., 2003). Meanwhile, environmental conditions and organism-specific uptake routes play a crucial role in determining metal bioavailability (Janssen et al., 1997; Peijnenburg et al., 1999b; Teutsch et al., 2001; Peijnenburg, 2002).

Soil acidity is the most important solid-phase characteristic modulating the availability of Pb (Peijnenburg et al., 1999a, 1999b; Ming et al., 2012), and internal Pb levels in *E. crypticus* increased linearly with Pb concentrations in soils with pH_{CaCl_2} below 3.9 (Peijnenburg et al., 1999a, 1999b). Therefore, Pb uptake was expected to be high on the acidic Forest soils 2 and 3 ($pH_{CaCl_2} = 3.3$ – 3.5) with high total and extractable Pb concentrations. The good correlations between Pb uptake by *E. crypticus* and water-extractable Pb concentrations in soil (Table 2) indicated that the water-extractable fraction was the bioavailable “pool” for the enchytraeids (Davies et al., 2003; Hobbelen et al., 2006). Water extracted less Pb from the shooting field soils than $CaCl_2$, indicating that the shooting field soils had low bioavailability of Pb to *E. crypticus*. Previous studies suggest that the Pb found in the soil solution, a measure of the portion available to biota, is a more reliable indicator of the threat posed to the environment than total

Pb (Kabata-Pendias and Pendias, 1992). The current findings provide evidence in support of water-mediated uptake of free Pb ions by the enchytraeids. This also explains why the enchytraeids showed similar Pb concentrations in the Forest soil 1, Bullet plot soil and the grassland soils, which had similar water-extractable but different CaCl_2 -extractable and porewater Pb concentrations (see Luo et al., 2014).

Although correlations between internal Pb concentrations in the enchytraeids and the other soil properties were overshadowed by total Pb concentrations in the soil (Table 2), pH and Fe were included in the best regression model on the basis of water-extractable Pb concentrations (Table 3). This indicates that pH and Fe were the most important soil properties affecting Pb partitioning between the soil solid phase and the soil solution and therefore indirectly affect Pb accumulation in the enchytraeids. Furthermore, also the equations describing internal Pb concentrations in relation to water-extractable Pb concentrations in soil were significantly improved by adding pH and Fe content (Table 3).

The order of chronic toxicity identified by bioassays: Forest soil 3 > Forest soil 2 > Forest soil 1 > Bullet plot soil > Grassland soil 2 > Grassland soil 1, was completely different from the results obtained by chemical methods, such as total and extractable Pb concentrations in the shooting field soils. This shows that an environmental assessment based on total Pb concentrations can overestimate the risks for neutral or alkaline grassland soils but underestimate the risks for acidic soils like Forest soil 1 and Bullet plot soil. Therefore, as already pointed out by other authors (Amorim et al., 2008; Udovic and Lestan, 2010; Luo et al., 2014), a combination of chemical analysis with bioassays is needed to provide a more complete and relevant assessment of the bioavailability of Pb in shooting field soils. This study also shows that soil properties need to be considered when interpreting the toxicity of shooting field soils, and that enchytraeids may be suitable test organisms for assessing contaminated field soils. The results obtained in the present study are more applicable and reliable for site-specific assessment of shooting field soils because they represent realistic soil properties at a shooting field and included some additional artificial reference soils to “mimic” the soil properties of shooting field in all aspects except for Pb concentrations.

5. Conclusion

Forest soils from a shooting field with $\text{pH}_{\text{CaCl}_2} \leq 3.5$ and total Pb concentrations ≥ 2153 mg/kg dw showed high Pb bioavailability and toxicity to *E. crypticus*. Clean forest soil with $\text{pH}_{\text{CaCl}_2} \leq 3.2$ however, also significantly reduced survival and reproduction of the enchytraeids, while in the bullet plot soil at $\text{pH}_{\text{CaCl}_2}$ 3.7 reproduction was almost completely inhibited. Bioaccumulation of Pb in *E. crypticus* linearly increased with increasing total Pb concentrations in soils. The grassland soils with $\text{pH}_{\text{CaCl}_2} > 6.5$ and total Pb concentrations of 355–656 mg/kg dw had lowest extractable Pb concentrations and the lowest toxicity to *E. crypticus*. This study shows that *E. crypticus* was very sensitive to acidic soils with $\text{pH}_{\text{CaCl}_2} \leq 3.8$, suggesting that the toxic effects seen in the most contaminated forest soils may have been due to the low soil pH rather than the high Pb concentrations.

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