Determining the bioavailability and toxicity of lead contamination to earthworms requires using a combination of physicochemical and biological methods

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ABSTRACT

This study aimed at assessing the bioavailability and toxicity of lead to *Eisenia andrei* in shooting range soils representing different land uses (forest, grassland, bullet plot). Soils contained 47–2398 mg Pb/kg dry weight (dw), but also had different pH-CaCl₂ (3.2–6.8) and organic matter contents (3.8–13%). Therefore artificial soils with different pH and organic matter contents and two natural soils were included as control soils. Earthworms were exposed for 28 days and toxicity and uptake of Pb were related to total, water and 0.01 M CaCl₂ extractable and porewater Pb concentrations as well as to soil characteristics. Pb uptake in the earthworms linearly increased with increasing soil concentrations. At >2000 mg Pb/kg dw and pH 3.3–3.5, high earthworm mortality with significant weight loss and complete inhibition of reproduction were recorded. At <1000 mg/kg dw, earthworm reproduction was more related to differences in pH and other soil characteristics than to Pb.

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1. Introduction

Shooting ranges around the world represent a significant sink and potential source for trace metal pollution in soil and ground water (Van Vleet et al., 2010). Annual lead (Pb) loadings of up to six tonnes were reported on three Danish shooting ranges (Jorgensen and Willems, 1987). In Finland, a survey identified more than 2000 outdoor shooting ranges, both military and civilian, with an estimated annual deposit of approximately 500 kg Pb per range (Sorvari et al., 2006). In Switzerland, more than 2000 shooting ranges are used for the regular shooting practice of civilian militia and sports (Mozafar et al., 2002) resulting in an annual deposition of more than 400 t of Pb into soils (Johnson et al., 2005; Robinson et al., 2008). Approximately 100 t Pb used by the Norwegian armed forces were deposited in Norwegian shooting ranges from small arms ammunition (Heier et al., 2010). In the United States, 60,000 t of Pb is being deposited each year in shooting range soils (Ahmad et al., 2011). Lead accounting for 95–97% of the bullet material at shooting ranges (Cao et al., 2003) is of increasing concern in many countries (Lin, 1996; Sorvari, 2007) since it is known to be hazardous to human and environmental health.

Therefore, the United State Environmental Protection Agency has classified Pb residues in shooting range soils as hazardous materials (USEPA, 2001).

Shooting ranges increase the ecotoxicological risks for the surrounding environment by contaminating soils, surface water, ground water, terrestrial, and aquatic biota. Soil invertebrates, birds, and other animals may ingest this soil while foraging for food. Earthworms play an important role in soil ecosystems because of their role in soil structuring and decomposition processes. They are particularly prone to accumulating metals to much higher levels than in the environment (Morgan et al., 1993) and therefore may play a key role in the food chain transfer of metals from soil to higher organisms. The literature shows a lack of consensus on the toxicity of Pb to earthworms, with enhancement of reproduction at 1000 and 2000 mg Pb/kg (Reinecke and Reinecke, 1996; Reinecke et al., 1997), no observed effects on reproduction at 1810 mg Pb/kg and a significant reduction in cocoon production at 2000 mg Pb/kg (Spurgeon et al., 1994). Data on Pb uptake in earthworms show striking differences with Pb body burdens of 75.6 ± 46 mg/kg in *Lumbricus castaneus* collected from soil containing 412 to 79,963 mg Pb/kg (Terhivuo et al., 1994), and 348 ± 72 mg Pb/kg in *L. castaneus* exposed to soil containing only 200 mg Pb/kg (Svendsen et al., 1996).

Kabata-Pendias and Pendias (1992) concluded that Pb concentration in the soil solution is a better indicator of adverse effects on

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biota than total soil Pb levels, with metal ions determining toxicity (Smolders et al., 2009). In contrast, Davies (1992), Grelle and Descamps (1998) and Booth et al. (2003) found that Pb uptake in plants and earthworms was predicted best using total soil Pb concentrations. The majority of metals in natural soils at contaminated sites will be present as solids which are not bioavailable (Davies et al., 2003b). As a consequence, metal toxicity is generally less pronounced in metal-contaminated field soils than in soils freshly spiked with metal salts at similar total metal concentrations (Lock et al., 2006). It is difficult to interpret toxicological parameters derived from field studies due to co-contamination and variations in soil properties. However, results obtained are more applicable to a contaminated site because they represent realistic conditions at that site compared with using spiked artificial or uncontaminated field soils (Nahmani et al., 2007).

A variety of extraction procedures have been suggested for predicting the bioavailability of Pb in different soils, but results are inconsistent (Cook and Hendershot, 1996). Often total metal concentrations are used, taking for granted that environmental risk assessment based on the total concentrations can overestimate risks, and that organisms respond only to the fraction that is biologically available (Alexander, 2000; Ehlers and Luthy, 2003; Semple et al., 2004). Methods to assess available metal concentrations often use extraction with water or a dilute salt solution, but question is: do they really predict “biological availability”? The only way to answer that question is by comparing the results of chemical extractions with effects on organisms, preferably with simple, cost- and time-effective bioassays, covering a variety of effects across different taxa (Harmsen, 2007; Antunes et al., 2008). The combination of chemical and biological tests may provide more complete and relevant information on the bioavailability of contaminants (Van Gestel et al., 2001; Loureiro et al., 2005; Antunes et al., 2008; Alvarenga et al., 2012; Van Gestel et al., 2012). Also, the properties of the soil need to be considered when interpreting the results of tests with earthworms. Although not feasible in practice, the results of a site assessment would be more reliable if a control would be available that “mimics” the contaminated test soil in all aspects except for the contaminants being present (Alvarenga et al., 2012).

The present paper is part of a broader study that aimed at linking chemical and biological measures of Pb bioavailability in shooting range soils with different land uses (forest, grassland, bullet plot), containing different concentrations of Pb (47–2398 mg Pb/kg dw). Soils were tested for toxicity to earthworms and other soil invertebrates applying a combination of physico-chemical and biological assays. Since the field soils also differed in pH-CaCl₂ (3.2–6.8) and organic matter contents (3.8–13%), differently prepared artificial soils and two natural soils (soccer field soil, LUFA 2.2) were used as control soils. This paper focuses on the tests done with earthworms. Toxicity and uptake of Pb in the earthworms were related with total, water and 0.01 M CaCl₂ extractable and porewater Pb concentrations in soils as well as with soil properties.

2. Materials and methods

2.1. Sampling and experiment design

Field soils were collected from five different sites representative of different landscape types (F: forest; G: grassland; B: bullet plot) in a shooting range and a reference site (S: soccer field near the shooting range) in the Netherlands. 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. Three artificial soils (R1, R2 and R3) were prepared, based on OECD artificial soil (OECD, 1984). R1, the standard artificial soil, was prepared by mixing 10% finely ground sphagnum peat (<1 mm), 20% kaolin clay, and 70% quartz sand (dry weight), adjusted with CaCO₃ to nominal pH-CaCl₂ 6.0 ± 0.5. The other two artificial soils were prepared with peat contents of 5% (R2) or 2.5% (R3) and pH-CaCl₂ adjusted to nominal 3.5 (R2)

or 6.5 (R3) with CaCO₃ (Table 1). The standard LUFA 2.2 soil (LUFA-Speyer, Sp 2121, Germany) was used as a control (LF2.2).

2.2. Physico-chemical analysis

The soil samples were air dried, homogenised and 2 mm sieved. All soils were characterized for their cation exchange capacity (CEC) using the silver thiourea method (Dohrmann, 2006). Exchangeable cations in extracts were determined by flame atomic absorption spectroscopy (AAS; Perkin Elmer, Analyst 100). Organic matter content of the soils was determined as loss on ignition at 500 °C in an ashing oven. The organic carbon content (% OC) was determined by dividing the organic matter content by the Van Bemmelen factor (1.724; Howard and Howard, 1990). Water holding capacity (WHC) of the soils was determined by the sandbox method (ISO, 1999). Particle size distribution was determined by laser grain size analysis with laser diffraction sensors (HELOS-QUIXEL) (Konert and Vandenberghe, 1997).

To assess available Pb concentrations, soils were extracted with water and 0.01 M CaCl₂ (Houba et al., 1996), while also porewater was extracted. Water and CaCl₂ extraction are typically used to estimate metal bioavailability and may correlate with metal availability to earthworms (Davies et al., 2003b). Dried soil (5 g) was shaken with distilled water (25 mL) or 0.01 M CaCl₂ (25 mL) for 2 h at 200 rpm. After sedimentation pH was measured in the extracts using a Consort P907 pH meter. After filtration (S&S, 0.45 µm, Ø 47 mm), solutions were used to measure water and CaCl₂ extractable Pb concentrations. After saturation of the soils to 100% WHC and 1 week equilibration, porewater was collected by the double-chamber centrifugation method and filtration through a 0.45 µm membrane filter (Hobbelen et al., 2004). Immediately after centrifugation at 2056 g, solutions were analysed for Pb and dissolved organic carbon (DOC). To determine total Pb, Cu, Zn, Cd, Ca and Fe concentrations, approx. 0.1 g oven-dried soil samples were digested in 2 mL of a 4:1 mixture of nitric acid (65% p.a.; Riedel-de-Haen) and hydrochloric acid (37% p.a., Baker) in tightly closed Teflon® bombs upon heating in a destruction oven at 140 °C for 7 h. After cooling, the samples were made up to 10 mL with NANO pure water. Flame AAS (Perkin–Elmer, Analyst 100) was used to determine metal concentrations. DOC was measured by a TOC analyzer (SK¹² Skalar, Breda, The Netherlands). A certified reference soil (International Soil-Analytical Exchange, ISE sample 989) was included to control quality of the analysis. Satisfactory recoveries in triplicate analysis (mean ± SD) were obtained for Pb (93 ± 4%), Cd (89 ± 0%), Zn (83 ± 1%) and Cu (107 ± 2%), Ca (86 ± 2%) and Fe (93 ± 3%). Porewater Pb concentrations below the detection limit (0.018 mg/L) were set at the detection limit divided by two (0.009 mg/L).

2.3. Toxicity and bioaccumulation test

Eisenia andrei (Lumbricidae) were obtained from a laboratory culture at the Department of Ecological Science, VU University, Amsterdam. Earthworms were cultured in horse manure free of any pharmaceuticals at 20 ± 1 °C. Before the start of the experiment, adult earthworms with well-developed clitellum (individual mass range 333–794 mg) were acclimatized in LF2.2 soil for 24 h. The tests with earthworms followed OECD guideline 222 for determining reproduction toxicity of chemicals to earthworms (OECD, 2004). Each test soil was moistened to 50% of its WHC. Each toxicity test had five replicate test containers containing approx. 500 g soil (dry weight equivalent). At the start of the test, each batch of 10 animals was weighed and randomly assigned to a test container. After introduction of the earthworms, 5 g (dry weight) finely ground and moistened horse dung was introduced as a food source in a hole in the middle of the test soil following Van Gestel et al. (1989). Test containers were incubated at 20 °C, and constant illumination. Once a week all test containers were opened to aerate the test soils, correct for water loss and add additional food if required. After 4 weeks, surviving adults were collected by hand sorting, counted and weighed. The soils were incubated for another 4 weeks to allow the cocoons to hatch. After the second 4-week period, juveniles were extracted by placing the test containers in a water bath at 60 °C.

Surviving adult earthworms were kept on moist filter paper for approx. 24 h to void their gut contents, following Arnold and Hodson (2007), and then frozen, freeze-dried for 2 d and weighed. Wet weights of each earthworm were measured and recorded both before and after depuration. Day 28 weights (before depuration) were compared with weights recorded on day 0 to provide an additional measure of toxicity. Weight loss (WL, %) of earthworms was calculated using the equation:

$$WL = ((W_i - W_s)/W_i) \times 100$$

in which W_i is initial mean earthworm weight, and W_s is mean weight of surviving earthworms after 28 days of exposure.

Five replicate animals were collected from each replicate test soil except for F1 ($n = 4$) and analysed to obtain Pb concentrations in body issues. Dried earthworms were digested using the same acid mixture and Teflon bombs as described for soil samples. The digests were then analysed by flame AAS (Perkin Elmer, Analyst 100). The limit of detection for Pb in earthworm tissues was 4 mg/kg dry weight. All tissue analyses included procedural blanks and a certified reference material (Dolt-2) conducted in triplicate. Digested blanks contained Pb concentrations below the limit of detection. Recovery of Pb from the Dolt-2 in triplicate analysis (mean ± SD) was 97 ± 2.8%.

Table 1

Physicochemical properties and metal concentrations of field soils from different landscape types (F: forest; G: grassland; B: bullet plot) in a shooting range and a reference site (S: soccer field near shooting range) in the Netherlands and artificial soils (R) as well as a control (LF2.2: LUFA 2.2) used for assessing the toxicity and bioaccumulation of lead in *Eisenia andrei*. All values are averages with standard deviation ($n = 3$).

	Field soil							Artificial soil			Control
	B0	F0	F1	F3	G1	G3	S	R1	R2	R3	LF2.2
pH-H ₂ O	4.2 ± 0.11 b	3.9 ± 0.0058 a	4.0 ± 0.025 a	4.2 ± 0.021 b	7.5 ± 0.021 f	6.9 ± 0.0071 e	6.9 ± 0.014 e	5.9 ± 0.026 d	4.6 ± 0.012 c	6.8 ± 0.040 e	6.0 ± 0.021 d
pH-0.01 M CaCl ₂	3.7 ± 0.014 c	3.2 ± 0.012 a	3.3 ± 0.00 a	3.5 ± 0.014 b	6.8 ± 0.071 g	6.5 ± 0.0071 f	6.8 ± 0.00 g	5.9 ± 0.040 e	3.8 ± 0.042 c	6.4 ± 0.070 f	5.5 ± 0.010 d
WHC (%)	33 ± 0.26 a	50 ± 1.1 f	49 ± 0.52 def	44 ± 0.017 c	40 ± 0.76 b	49 ± 0.31 ef	62 ± 0.42 g	77 ± 2.0 h	39 ± 0.83 b	35 ± 0.10 a	45 ± 0.75 cde
%Clay (<8 µm) ^a	2.7 ± 1.6 a	4.2 ± 1.8 abc	4.5 ± 1.8 abc	5.9 ± 2.0 bc	3.5 ± 1.8 ab	10 ± 2.4 de	7.5 ± 2.1 cd	18.6 ± 2.5 f	25.5 ± 2.7 g	27.1 ± 2.8 g	12.2 ± 2.5 e
%Silt (8–63 µm)	6.9 ± 1.6 a	13.3 ± 1.8 c	12.1 ± 1.8 b	12.2 ± 2.0 b	10.6 ± 1.8 b	23 ± 2.4 d	11.8 ± 2.1 b	19.8 ± 2.5 c	22.2 ± 2.7 cd	23 ± 2.8 d	13.3 ± 2.5 b
%Sand (63–2000 µm)	90.5 ± 1.6 e	82.5 ± 1.8 cde	83.4 ± 1.8 cde	81.9 ± 2.0 cde	85.9 ± 1.8 de	67 ± 2.4 b	80.7 ± 2.1 cd	61.6 ± 2.5 b	52.3 ± 2.7 a	49.9 ± 2.8 a	74.5 ± 2.5 bc
OM (% LOI)	3.8 ± 0.12 b	7.0 ± 0.080 d	5.8 ± 0.078 c	6.6 ± 0.13 d	4.1 ± 0.095 b	5.3 ± 0.012 c	13 ± 0.53 f	8.8 ± 0.25 e	4.5 ± 0.10 b	2.4 ± 0.069 a	4.3 ± 0.090 b
CEC (cmolc/kg)	1.8 ± 0.088 a	2.1 ± 0.00071 a	2.1 ± 0.092 a	2.1 ± 0.11 a	5.9 ± 0.076 b	13 ± 1.8 d	21 ± 0.37 e	12 ± 0.55 d	2.8 ± 0.031 a	3.9 ± 0.027 ab	8.9 ± 0.61 c
DOC (mg/L)	703 ± 23 e	984 ± 26 f	651 ± 4.7 cde	693 ± 26 de	183 ± 4.7 a	519 ± 0.46 bc	673 ± 116 cde	945 ± 9.4 f	543 ± 31 bcd	424 ± 4.6 b	889 ± 15 f
Total Ca (mg/kg, dw)	99 ± 12 a	87 ± 27 a	130 ± 10 ab	91 ± 20 a	1179 ± 39 de	1331 ± 254 e	2467 ± 10 f	1082 ± 4.6 de	213 ± 7.5 b	495 ± 40 c	761 ± 17 cd
Total Fe (mg/kg, dw)	3332 ± 56 b	5644 ± 1728 b	4430 ± 214 bc	7703 ± 2170 c	4061 ± 259 bc	10,565 ± 910 d	11,890 ± 5004 d	596 ± 28 a	477 ± 5.6 a	497 ± 5.0 a	4313 ± 93 b
Total Pb (mg/kg, dw)	88 ± 9.0 b	47 ± 9.7 ab	2153 ± 0.0 e	2398 ± 311 e	656 ± 60 d	355 ± 54 c	36 ± 1.4 ab	42 ± 2.1 ab	45 ± 0.0 ab	50 ± 1.4 ab	15 ± 0.71 a
Total Cd (mg/kg, dw)	0.12 ± 0.042 abc	0.10 ± 0.011 abc	0.091 ± 0.0078 abc	0.068 ± 0.040 ab	0.15 ± 0.00071 bc	0.29 ± 0.071 c	0.19 ± 0.058 bc	0.021 ± 0 a	0.095 ± 0 abc	0.057 ± 0.052 ab	0.20 ± 0.055 bc
Total Zn (mg/kg, dw)	5.7 ± 0.071 b	7.4 ± 2.3 bc	7.1 ± 0.071 bc	8.9 ± 0.98 c	14 ± 0.64 d	59 ± 3.8 g	37 ± 0.14 f	3.8 ± 0.28 a	3.0 ± 0.071 a	3.2 ± 0.071 a	21 ± 0.64 e
Total Cu (mg/kg, dw)	1.2 ± 0.071 b	2.4 ± 0.42 cd	3.0 ± 0.071 cd	1.9 ± 0.38 c	8.4 ± 0.35 e	15 ± 2.5 f	12 ± 0.35 ef	0.35 ± 0.071 a	0.55 ± 0.071 a	0.50 ± 0 a	3.6 ± 0.28 d

WHC = Water holding capacity; OM = , Organic matter; CEC = Cation exchange capability; DOC = Dissolved oxygen carbon in porewater.

The same letter indicate no significant differences at $p > 0.05$, using a post-hoc Tukey HSD test.

^a The fraction < 8 µm determined with the method of Konert and Vandenberghe (1997) corresponds with the clay fraction < 2 µm.

2.4. Statistical analysis

LC50s (concentration causing 50% mortality) for the effect of Pb on earthworm survival were calculated using the trimmed Spearman–Karber method (Hamilton et al., 1977). EC10 and EC50 values and corresponding 95% confidence intervals for effects on reproduction were estimated with the log-logistic model of Haanstra et al. (1985), applying the modification described by Van Brummelen et al. (1996).

All metal concentrations, but not soil physicochemical properties and bioassay values were log-transformed before analysis to satisfy assumptions of normality. All data were checked for homogeneity of variance and normality (Kolmogorov–Smirnov test) and, when possible, subjected to one-way ANOVA. When 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 data and soil physicochemical properties ($p < 0.05$). To determine the relative importance of the different soil characteristics contributing to soil Pb toxicity and to examine the relationship between each earthworm bioassay endpoint and the combined soil properties, multiple linear regression models were constructed.

Except for the trimmed Spearman–Karber calculations, all analyses were run in the software package SPSS15.0 for Windows.

3. Results and discussion

3.1. Physicochemical characterization of soils

Physicochemical characteristics of the field and artificial soils are summarized in Table 1. Among the soils studied, forest soils, especially F1 and F2, had the lowest pH, while grassland soil had the highest pH. Most pH–CaCl₂ values except for soil G1 fell within the tolerable pH range of *Eisenia andrei* (4.0–7.5) (Edwards, 1988;

Edwards and Bohlen, 1996). The soccer field soil had the highest and the bullet plot soil the lowest organic matter content. The forest soils also had higher DOC concentrations in the porewater than the grassland, while DOC level in soccer field soil was significantly lower and in bullet plot soil significantly higher compared to the other soils. Most field soils had lower clay content than the LF2.2 control soil. Bullet plot soil had the lowest clay content. Soil G3 had the highest silt content, bullet plot soil the lowest. Bullet plot soils had highest sand content. The soccer field soil had the highest CEC, the bullet plot soil the lowest CEC. Ca concentrations were similar in forest and bullet plot soils, but higher in grassland and soccer field soils. Fe concentrations were highest in G3 and S and did not differ much for the other field soils. Total Cu, Zn and Cd concentrations were well within the range of the background levels in Dutch soils (Cd = 0.5–0.6 mg/kg; Cu = 18–21 mg/kg; Zn = 60–70 mg/kg) (Vegter, 1995).

3.2. Availability of Pb in the soils

Total and available Pb concentrations in all test soils are shown in Fig. 1. Soils F1 and F3 had the highest total, CaCl₂ and water extractable as well as porewater Pb concentrations. Soils G1 and G3 had higher total but lower CaCl₂ extractable Pb concentrations than the bullet plot soil. Although soil B0 had similar total, CaCl₂ extractable and porewater Pb concentrations than F0, its water extractable Pb was remarkably higher than that in F0. Based on the

Fig. 1. Total (A), 0.01 M CaCl₂ (B) and water extractable (C) and porewater (D) Pb concentrations in different shooting range and reference soils. All values are mean ± SD ($n = 3$). Columns with the same letter indicate no significant differences at $p > 0.05$. Soils are arranged according to increasing total Pb concentration. See Table 1 for soil codes and soil properties.

Fig. 2. Earthworm (*Eisenia andrei*) mortality (A), weight loss (B), juvenile numbers per test container (C) and Pb concentrations in body issues (D) after 28 days exposure to different soils from a shooting range and reference soils. Columns with the same letter indicate no significant differences at $p > 0.05$. All values are mean $\pm$ SD ($n = 5$, except for soil F1 in figures B and D where $n = 4$). Soils are arranged according to increasing total Pb concentration. See Table 1 for soil codes and soil properties, and Fig. 1 for total and available Pb concentrations in these soils.

background levels of Pb in Dutch soils (Vegter, 1995), except for site F0, all shooting field soils were contaminated by Pb.

In our study, water extracted the least Pb with a maximum of about 1% of the total lead which is in agreement with previous results (Houba et al., 1996; Quevauviller, 1998). CaCl_2 extracted the highest amount of Pb with a maximum of about 10% of the total concentration (Fig. 1B), which was higher than the value of 1% reported by Davies et al. (2003a) and Singh et al. (1996), reflecting the higher availability of Pb in the shooting field soils. This high Pb availability in soils F1 and F3 may be explained from their low pH (Basta and Tabatabai, 1992; Rieuwerts et al., 2006; Farrah and Pickering, 1979). As a result of their high pH, CaCl_2 water extractable and porewater Pb concentrations were relatively low despite of relatively high total Pb concentrations in the grassland soils G1 and G3, while the opposite was seen for the acid soils F0 and B0. So the high extractability of Pb in F1 and F3 was not only because of their high total Pb concentrations but also due to their lower pH values. Also the presence of organic matter may have influenced extractability (Rieuwerts et al., 2006). Soluble organo-Pb complexes may be formed at the high DOC contents in the forest soils. So, extractability of Pb in soil F0 was remarkably high as a result of its high organic matter and DOC contents, even though it had a relatively low total Pb concentration.

3.3. Toxicity and bioaccumulation of lead in earthworms

After 28 days of exposure, high mortality of adult *E. andrei* was seen in the two most polluted soils F3 ($76 \pm 11\%$) and F1 ($66 \pm 27\%$), while no mortality occurred in the other soils (Fig. 2A). Significant earthworm mortality was first seen at concentrations of 2153 and 2398 mg/kg total Pb. Significant weight loss of the earthworms after 28 days of exposure occurred in the two most polluted soils where also high mortality was recorded (Fig. 2B). Weight loss of *E. andrei* was lowest in the soils S, F0, B0 and G3. Growth has been proposed as a sensitive indicator of exposure to contaminants (Langdon et al., 2005). Weight loss of the adult earthworms was not significantly affected at soil Pb concentrations up to 656 mg/kg dw. However, our study confirmed Emersen's suggestion (Emersen, 1994) that lead contamination at shooting ranges (for example F1 and F3) may significantly affect earthworm growth, and therefore might potentially impact on earthworm populations.

The numbers of juveniles produced were lower in soils F0, B0 and G1 than in the control soil LF2.2. No juveniles were produced in the most contaminated soils F1 and F3, while soil G3 had the highest juvenile numbers (Fig. 2C). At total Pb concentrations up to 656 mg/kg dw *E. andrei* reproduction was not affected; this

corresponded with porewater Pb concentrations <0.028 mg/L. Artificial soils R1 and R2 had similar numbers of juveniles, but significantly less than the soils LF2.2 and R3, which means that a soil pH lower than 5.9 may significantly reduce juvenile numbers. Compared to LF2.2, high concentrations of Pb (>2000 mg/kg dw) in soils did inhibit earthworm reproduction although it also has to be noted that the most contaminated soils had a low pH-CaCl₂ (3.3–3.5). Spurgeon et al. (1994) did observe a significant reduction in the cocoon production of *Eisenia fetida* at 2000 mg Pb/kg in artificial soils prepared following OECD guideline No. 207 (OECD, 1984).

The concentration of lead in the earthworms did not increase significantly until soil concentrations of 88 ± 9.0 mg Pb/kg dw (Fig. 2D). At higher total soil Pb concentrations earthworm concentrations increased in a dose-related manner. The greater availability of Pb in the most-contaminated soils F1 and F3 was reflected by higher uptake (980 mg/kg dw) and also higher mortality and weight loss and lower juvenile numbers (Fig. 2A–C).

Although different factors affected Pb toxicity in the test soils, we did try to calculate LC50, EC50 and EC10 values for the toxicity of Pb related to total and extractable soil concentrations as well as to Pb concentrations in the earthworms. These values, which are shown in Table 2, may give an indication of Pb toxicity in our soils, enable comparison with literature data and help identifying factors most affecting Pb toxicity. Because of the high earthworm mortality in the two most contaminated soils, no EC10 and EC50 values for weight loss could be determined. The 28-day LC50 for total Pb is much lower than the LC50 for soils spiked with solid PbCO₃ and PbS observed by Davies et al. (2003a,b). The EC50 for earthworm reproduction in our study is much lower than the EC50s for the soils spiked with solid PbCO₃ and PbS in the study of Davies et al. (2003b) and even lower than the EC50 for cocoon production for Pb freshly spiked into artificial soils in the study of Spurgeon et al. (1994). The latter studies used insoluble Pb forms which had a much lower availability than the Pb in our soils, explaining for the lower toxicity.

3.4. The relationship between biological responses and physicochemical soil properties

Significant negative relationships were found between earthworm survival and total and available Pb concentrations ($p < 0.01$) (Table 3). Significant positive relationships were observed between earthworm survival and pH, Ca concentrations and CEC ($p < 0.01$), although r values were lower than for Pb exposure levels. The significant correlation between earthworm survival and pH most likely is caused by the direct effect of (low) soil pH on earthworm

Table 2
LC50, EC10 and EC50 values (with corresponding 95% confidence intervals) for the effects of Pb on the survival and reproduction of the earthworm *Eisenia andrei* exposed for 28 days to soils from a shooting range and different reference soils. Toxicity is related to total and available Pb concentrations in the soils as well as to concentrations accumulated in the earthworms. See Table 1 for soil properties and Fig. 1 for the Pb concentrations; Fig. 2 shows the earthworm responses.

	LC50	EC10	EC50
Total Pb (mg/kg, dw)	1603 (1329–1939)	1377 (– ^a)	1482 (–)
Water extractable Pb (mg/kg, dw)	5.5 (3.5–8.9)	0.39 (0.096–0.69)	0.54 (0.38–0.69)
CaCl ₂ extractable Pb (mg/kg, dw)	98 (53–181)	0.44 (–)	2.2 (–)
Porewater Pb (mg/L)	5.1 (3.0–8.7)	99 (8.6–189)	130 (102–158)
Pb in earthworm (mg/kg, dw)	852 (727–999)	248 (–)	484 (133–836)

^a Very wide confidence intervals.

Table 3
Simple linear correlation coefficients relating the response of earthworms (*Eisenia andrei*) after 28 days exposure to the physicochemical properties of different shooting range field soils and reference soils. See Table 1 for soil properties, Fig. 1 for Pb concentrations and Fig. 2 for the earthworm responses.

Soil physico-chemical properties	Simple linear correlation coefficients (r)			
	Survival	Reproduction	Weight loss	Pb in earthworm
OM	–0.045	–0.15	–0.084	–0.13
pH-H ₂ O	0.50 ^a	0.67 ^a	–0.28	–0.20
pH-0.01 M CaCl ₂	0.52 ^a	0.67 ^a	–0.31 ^b	–0.27
DOC	–0.046	–0.24	–0.12	–0.40 ^a
CEC	0.37 ^a	0.49 ^a	–0.38 ^a	–0.37 ^a
Ca	0.50 ^a	0.63 ^a	–0.38 ^b	–0.33 ^b
Fe	–0.27 ^b	0.090	0.088	0.44 ^a
%Clay	0.31 ^b	0.29	–0.13	–0.59 ^a
%Silt	0.28 ^b	0.59 ^a	–0.13	–0.43 ^a
%Sand	–0.31 ^b	–0.42 ^a	0.14	0.55 ^a
Pb-water	–0.79 ^a	–0.47 ^a	0.81 ^a	0.82 ^a
Pb-0.01 M CaCl ₂	–0.83 ^a	–0.63 ^a	0.71 ^a	0.74 ^a
Pb-porewater	–0.78 ^a	–0.52 ^a	0.70 ^a	0.87 ^a
Total Pb	–0.68 ^a	–0.21	0.70 ^a	0.97 ^a
Total Cd	0.18	0.50 ^a	–0.31 ^b	0.093
Total Zn	0.088	0.54 ^a	–0.18	0.14
Total Cu	–0.012	0.35 ^b	–0.075	0.35 ^b

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

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

survival (Spurgeon and Hopkin, 1996). Bradham et al. (2006) argued that the apparent correlation of earthworm survival with CEC may be caused by the strong inter-correlation between CEC and other exogenous variables such as pH, and not by a direct effect of CEC (see e.g., Criel et al., 2008). Earthworm mortality was not significantly correlated with organic matter content or DOC concentration in the porewater, which agrees with other studies (Bradham et al., 2006; Janssen et al., 1997; Peijnenburg et al., 1999). Simple and stepwise regression analysis showed that the number of surviving of adult earthworms (SN) was well predicted on the basis of the porewater Pb concentrations, CEC, silt and sand contents of the test soils (Table 4).

Reproduction expressed as juvenile numbers was significantly and positively correlated with pH, Ca, silt content, Zn, Cd and CEC, but significantly negative with sand content (Table 3). In all cases, except for pH, r values were low, suggesting these factors, although being significant, explain only a fraction of the variation in earthworm reproduction. The pH manipulated artificial soils showed an overall decrease in reproduction at pH 4.6 and 5.9 in comparison to pH 6.8 (Fig. 2C). This is in agreement with the observations in artificial soils of Arnold et al. (2003), Currie et al. (2005) and Langdon et al. (2005). Earthworm reproduction was a greatly decreased in the field soils with pH values lower than 5.9, such as F0, F1, F3 and B0 (Table 1; Fig. 2C). The correlation between reproduction and CEC was highly significant (Bradham et al., 2006), but CEC is directly related to soil pH (Sauvé, 2002; Bradham et al., 2006). In addition, Ca, Cd and Zn concentrations and silt content significantly and positively affected juvenile numbers, while a high sand content seemed to reduce juvenile numbers. Juvenile numbers were significantly and negatively correlated with water and CaCl₂ extractable and porewater Pb but not with total Pb concentrations (Table 3). Juvenile numbers (JN) were well predicted based on the porewater Pb concentration, Ca, silt, clay and OM contents and CEC of the test soils (Table 4).

Weight loss of earthworms was significantly and positively correlated with water extractable Pb concentrations, but negatively with CEC. The weight loss was significantly higher in soil R3 than in the control indicating that lower soil organic matter content could have adverse effect on earthworm growth. Other soil properties

Table 4

Multiple-regression equations and coefficient of determination (R^2) for the relationship between the toxicity of Pb contaminated shooting range field soils to the earthworm *Eisenia andrei* and physicochemical soil properties. See Table 1 for soil physicochemical properties, Fig. 1 for Pb concentrations and Fig. 2 for toxicity data.

Endpoint	Multiple linear regression equation obtained	Statistics
Survival number (SN)	SN = $-2.06 (\log [\text{Pb}]_{\text{Total}}) - 7.52 (\log [\text{Fe}]) + 2.08 \text{ pH}_{\text{CaCl}_2} - 3.53 \text{ Clay} - 2.63 \text{ Silt} - 2.90 \text{ Sand} + 322$	$p < 0.001$; $R^2 = 0.840$
	SN = $-2.97 (\log [\text{Pb}]_{\text{Water extractable}}) - 6.05 (\log [\text{Fe}]) - 1.82 (\log [\text{Ca}]) - 0.495 \text{ Clay} + 0.362 \text{ Silt} + 0.925 \text{ pH}_{\text{H}_2\text{O}} + 27.7$	$p < 0.05$; $R^2 = 0.841$
	SN = $-2.04 (\log [\text{Pb}]_{\text{CaCl}_2 \text{ extractable}}) - 0.510 \text{ pH}_{\text{H}_2\text{O}} + 11.4$	$p < 0.05$; $R^2 = 0.696$
	SN = $-3.57 (\log [\text{Pb}]_{\text{Porewater}}) - 0.411 \text{ CEC} + 0.726 \text{ Silt} + 0.439 \text{ Sand} - 25.3$	$p < 0.001$; $R^2 = 0.851$
Juvenile number (JN)	JN = $-6.81 (\log [\text{Pb}]_{\text{Total}}) + 26.9 (\log [\text{Fe}]) + 2.62 \text{ Silt} + 3.12 \text{ CEC} - 7.34 \text{ OM} - 69.0$	$p < 0.001$; $R^2 = 0.904$
	JN = $-5.07 (\log [\text{Pb}]_{\text{Water extractable}}) + 23.0 (\log [\text{Fe}]) + 2.57 \text{ Silt} + 2.84 \text{ CEC} - 6.84 \text{ OM} - 73.1$	$p < 0.01$; $R^2 = 0.893$
	JN = $-9.40 (\log [\text{Pb}]_{\text{CaCl}_2 \text{ extractable}}) + 35.8 (\log [\text{Fe}]) + 3.36 \text{ Silt} - 2.56 \text{ OM} - 134$	$p < 0.001$; $R^2 = 0.839$
	JN = $-4.28 (\log [\text{Pb}]_{\text{Porewater}}) - 31.8 (\log [\text{Ca}]) + 3.09 \text{ Silt} - 10.2 \text{ OM} + 6.70 \text{ CEC} - 1.72 \text{ Clay} + 104$	$p < 0.05$; $R^2 = 0.910$
Weight loss (WL, %)	WL = $24.2 (\log [\text{Pb}]_{\text{Total}}) + 1.92 \text{ Clay} - 1.81 \text{ Silt} - 49.2$	$p < 0.001$; $R^2 = 0.812$
	WL = $12.0 (\log [\text{Pb}]_{\text{Water extractable}}) + 24.9$	$p < 0.001$; $R^2 = 0.645$
	WL = $13.6 (\log [\text{Pb}]_{\text{CaCl}_2 \text{ extractable}}) + 5.87 \text{ pH}_{\text{CaCl}_2} + 1.78 \text{ Clay} + 0.766 \text{ Sand} - 86.3$	$p < 0.05$; $R^2 = 0.706$
	WL = $17.0 (\log [\text{Pb}]_{\text{Porewater}}) + 1.72 \text{ CEC} + 3.30 \text{ Clay} + 1.12 \text{ Sand} - 140$	$p < 0.001$; $R^2 = 0.817$
Pb concentration in earthworms (C_w , mg/kg dw)	$\log C_w = 0.878 (\log [\text{Pb}]_{\text{Total}}) - 0.0247 \text{ Silt} + 0.280$	$p < 0.01$; $R^2 = 0.968$
	$\log C_w = 0.546 (\log [\text{Pb}]_{\text{Water extractable}}) + 0.364 (\log [\text{Fe}]) - 0.001 \text{ DOC} - 0.020 \text{ Clay} + 1.78$	$p < 0.01$; $R^2 = 0.946$
	$\log C_w = 0.548 (\log [\text{Pb}]_{\text{CaCl}_2 \text{ extractable}}) + 1.80 (\log [\text{Ca}]) + 0.619 (\log [\text{Fe}]) - 0.000451$	$p < 0.01$; $R^2 = 0.963$
	$\text{DOC} - 0.124 \text{ CEC} - 3.83$	
	$\log C_w = 0.644 (\log [\text{Pb}]_{\text{Porewater}}) + 0.800 (\log [\text{Ca}]) - 1.31$	$p < 0.001$; $R^2 = 0.908$

hardly affected earthworm weight loss, which agrees with other studies (Van Gestel et al., 1992; Currie et al., 2005). Earthworm weight loss (WL) was well predicted by porewater Pb concentrations, CEC, clay and sand contents of the test soils (Table 4).

Pb concentrations in the earthworms were positively correlated with total and available Pb concentrations in soil, sand content and Fe, but negatively with clay and silt content, DOC and CEC (Table 3), with the latter factors explaining less of the variation in earthworm Pb concentrations. Pb concentrations in the earthworms were well predicted by total Pb concentrations and silt contents of the soils (Table 4). Grelle and Descamps (1998) also found that soil total Pb concentration was a better predictor of earthworm body burden than CaCl_2 -extractable Pb levels. Previous studies demonstrated that soil physicochemical properties have a significant impact on Pb uptake by *E. andrei* (Spurgeon and Hopkin, 1995; Peijnenburg et al., 1999; Bradham et al., 2006; Smith et al., 2012). For example, soil pH has been suggested as the most important factor for controlling the bioavailability of Pb in soil, followed by organic matter content (Thomas, 1997; Peijnenburg et al., 1999). In the present study, the effect of pH was weaker for internal Pb than for reproduction (Table 3). This was not in agreement with the results observed by Peijnenburg et al. (1999), Langdon et al. (2005) and Bradham et al. (2006). Internal Pb concentrations did not correlate with soil OM content, but they significantly and negatively correlated with DOC level in the porewater. In addition, internal Pb was negatively correlated with clay content and silt content but positively with Fe and sand content, which all have been shown also by others to be modifying Pb uptake by earthworms (Janssen et al., 1997; Peijnenburg et al., 1999; Basta et al., 2005).

4. Conclusion

Most shooting range soils were contaminated with Pb at concentrations exceeding the background levels in Dutch soils. The forest soils had the highest total, water and CaCl_2 extractable and porewater Pb concentrations, but also the lowest pH. After 28-day exposure, mortality of adult earthworms occurred in the two most contaminated soils at total Pb concentrations ≥ 2153 mg/kg dw. Soil pH and organic matter contents significantly affected juvenile numbers, but did not affect mortality, weight loss and internal Pb concentrations in the earthworms. At soil total Pb concentrations > 2000 mg/kg, earthworm weight loss significantly increased,

while reproduction significantly decreased. At porewater Pb concentrations > 0.028 mg/L juvenile numbers were significantly lower. Also $\text{pH} < 5.9$ significantly reduced juvenile numbers. Earthworm survival, growth and reproduction as well as Pb uptake in the earthworms were correlated with total or available Pb concentrations and also affected by different soil properties, such as pH, CEC and OM and sand content. This study showed that a combination of physicochemical and biological assays may help determining lead toxicity and bioavailability in earthworms exposed to shooting range field soils.

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