Assessment of the bioavailability and toxicity of lead polluted soils using a combination of chemical approaches and bioassays with the collembolan *Folsomia candida*

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HIGHLIGHTS

- Soils from different landscapes of a shooting range are examined.
- Bioavailability of Pb varies with soil properties especially, pH and OM.
- Collembolan reproduction was more sensitive to pH than to Pb in soils.
- Collembolans avoided acid Pb contaminated soils.

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ABSTRACT

Understanding bioavailability and toxicity is essential for effective ecological assessment of contaminated soils. Total, water and 0.01 M CaCl₂ extractable and porewater Pb concentrations and soil properties in different shooting field soils were investigated. Three artificial soils containing different pH and organic matter contents and two natural soils were included as controls. Survival, reproduction and avoidance responses of *Folsomia candida* exposed to these soils as well as internal Pb concentrations were measured. In the shooting range soils, total Pb concentrations were 47–2398 mg/kg dw, pH_{CaCl₂} 3.2–6.8 and organic matter content 3.8–7.0%. Pb concentrations in *F. candida* linearly increased with increasing Pb concentrations in the soils. Acid forest soils caused significantly higher collembolan mortality and avoidance responses and significantly lower reproduction than the neutral grassland soils, which could be attributed to differences in pH and especially CaCl₂ extractable Pb concentrations. Soil properties significantly affected bioavailability and toxicity of Pb, but overall the collembolans seemed more sensitive to pH than to Pb in soils. This study shows the importance of selecting proper reference soils for assessing the effects of field soils.

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1. Introduction

Lead-contaminated land is a global environmental problem that results not only in ecological and human health risks, but can also have large financial implications in terms of remediation costs and re-development issues [1]. Shooting ranges, used for both military training and sport, can constitute an important source of lead (Pb) contamination in terrestrial ecosystems due to the use of ammunition pellets containing Pb [2]. Because of its widespread use and its possible irreversible impairment of ecological soil processes,

environmental risks associated with Pb contamination at shooting ranges are receiving increased interest from various national and international regulatory organizations [3].

Pb availability measured using extractants such as weak salts or weak acids may provide a first indication of the potential risk [4]. However, the uncertainty associated with the performance of extractants under various environmental conditions hinders endorsement of single or sequential extraction procedures. To address the relevance of chemical extraction methods for risk assessment, the analytically extracted metal concentrations need to be interpreted in a biological context [5]. There is an increasing need to incorporate bioassays in risk assessments and hazard identification [6,7]. Bioassays with soil animals however, may also present some problems: acute tests, for instance, do not provide an

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insight into the effects of the contaminant on population dynamics while chronic tests are time-consuming and labor intensive [8]. In acute toxicity tests, some soil invertebrate species proved to be very resilient, with an “avoidance mechanism” that distorted the results toward lower toxicity values than expected [9]. As an alternative to the classic survival or reproduction tests therefore sub-lethal avoidance behavior tests have been developed as ecologically relevant, sensitive, rapid, and cost-effective first screening tools for assessing soil contamination [9–11].

Since earthworms avoid Pb treated soil (1000–10,000 mg Pb/kg dw) in a dose-related manner and prefer neutral to slightly acidic soils (pH tolerance: 4.0–9.0), they may not be suitable for testing more acidic soils [12,13]. Soil dwelling collembolans are considered ecologically relevant species for ecotoxicological tests because they represent arthropods that have a different route of exposure and have a wider pH tolerance (3.2–7.7) compared to the earthworm species commonly used in toxicity tests [12,13]. The collembolan *F. candida* is often used as bioindicator for pollution due to its widespread distribution in different soil types, flexibility of its alimentary habits, its locomotor ability, and its role in the decomposition of organic matter, regulation of microbial activity and nutrient cycling [10,14].

Terrestrial ecotoxicity not only varies between species but soil characteristics also greatly influence the effect of metals by altering bioavailability [15]. In previous studies, the effect of aging on the bioavailability of Pb was hardly taken into account. Because most toxicity tests were carried out in freshly spiked soils, the effect concentrations may be regarded as conservative [13]. When combined with environmental chemistry of naturally aged soils, bioassays (including avoidance tests) provide more complete and relevant information on contaminant bioavailability in soils and, therefore a more meaningful and holistic risk assessment of contaminated sites [9,16]. To considerably improve site-specific risk assessment of Pb-contaminated land, it is highly recommended to combine soil characterization, chemical extraction methods and bioassays [7,9,10].

This study is part of a broader investigation that aimed at assessing the ecotoxicological risk of Pb in shooting range soils and deals with the effects on springtails; earlier studies described the effects on earthworms and enchytraeids [17,18]. Six natural field soils were collected from different landscapes (bullet plot, forest and grass lands) of a shooting field in the Netherlands, representing a gradient of Pb pollution but also different pH and organic matter contents. To elucidate effects of main soil properties on collembolans, three artificial reference soils with different pH and organic matter contents were used for comparison. Relationships between the survival and reproduction of *F. candida* and the total and water- and CaCl_2 -extractable and porewater Pb concentrations in soil as well as internal Pb concentrations in the surviving collembolans were determined. In addition, the behavioral response of *F. candida* to Pb in the shooting field soils was also investigated. The final aim of this study was to combine conventional chemical analysis, long-term toxicity tests and short-term avoidance tests to support the environmental risk assessment of Pb in shooting field soils.

2. Materials and methods

2.1. Soil sampling

Six natural soils were sampled from forest (F), grassland (G) and bullet plot (B) fields, which represent three landscapes of a shooting field in the Netherlands. Assuming it had similar soil properties without being contaminated, a soccer field soil (S) near the shooting range was sampled as a reference for the survival and reproduction tests. Three artificial soils (R1, R2, R3) were prepared

based on OECD artificial soil [19] in order to “mimick” the shooting field soils in pH and organic matter content, assuming these properties had more effect on Pb toxicity than soil texture. A standard artificial soil (R1) were prepared by mixing 10% finely ground sphagnum peat (<1 mm), 20% kaolin clay, and 70% quartz sand (dry weight) and adjusting nominal $\text{pH}_{\text{CaCl}_2}$ to 6.0 ± 0.5 by adding CaCO_3 . The other two artificial soils were prepared by reducing peat contents to 5% (R2) or 2.5% (R3), increasing sand content accordingly and adjusting nominal $\text{pH}_{\text{CaCl}_2}$ to 3.5 (R2) or 6.5 (R3) with CaCO_3 . The standard natural LUFA 2.2 soil (LF2.2; LUFA-Speyer, Sp 2121, Germany) was used as an additional control of the performance of the test animals.

For a full description of the methods used to analyze the soils, it can be referred to Luo et al. [17].

2.2. Survival and reproduction toxicity tests

Before starting the tests, all soils were dried completely at 50°C and subsequently moistened to 50% of their water holding capacity (WHC). *F. candida* (“Berlin strain”; VU Amsterdam) were cultured in plastic containers with a moist bottom of plaster of Paris containing 10% charcoal, at 20°C at a light/dark regime of 12/12 h. The experiment was initiated with 10–12 day old juveniles that were obtained by synchronizing the egg laying of the culture animals, fed with dried baker’s yeast (Dr. Oetker). Tests with *F. candida* were carried out according to ISO guideline 11267 [20], using 100 ml glass test containers containing 30 g moist soil each. Five replicates were prepared for each test, reference and control soil. At the start of the test, ten synchronized animals were transferred into each test jar and fed a few grains of dried baker’s yeast. The jars were incubated in a climate room at $20 \pm 1^\circ\text{C}$ and at a light/dark regime of 12/12 h. Once a week, the moisture content of the test soils was maintained by adding Milli-Q. After 28 days exposure, the content of each jar was flushed into a 300 ml beaker glass using 100 ml water. The mixture was gently stirred to let all surviving adults and juveniles produced floating on the water surface. A picture of the water surface was taken and the number of offspring was counted using digital imaging software.

Surviving adults were carefully taken from the water surface and allowed to remove excess water and empty their guts by placing them for a few minutes on a dry bottom of plaster Paris. After freeze-drying and determination of their dry weight, the animals were individually digested in a 300 μl $\text{HClO}_4/\text{HNO}_3$ mixture (1:7 v/v; Ultrex grade, Baker) and Pb concentrations in their bodies were measured using a Perkin Elmer 5100 Atomic Absorption Spectrometer equipped with a graphite furnace assembly. Quality of the analysis was controlled by analyzing certified reference material (Dolt 4). Pb recoveries were 85–115% of the certified value.

Bioaccumulation factors (BAFs) were calculated as the ratio of Pb concentrations in the collembolans and total Pb concentrations in the soil.

2.3. Avoidance test

The procedure adopted in the avoidance tests was based on International Organization for Standardization (ISO) [21] with some modifications. Cylindrical plastic boxes (diameter, 5 cm; depth, 2 cm) were divided into two equal sections by means of a card divider introduced vertically. Ten grams (dry weight equivalent) of a shooting field soil was placed in one of the sections, while the other one was filled with control soil (LF2.2) or a reference soil (R1/R2/R3). Five replicates were tested for each field soil–control/reference combination. After removing the plastic divider, 10 individuals (10–12 days old and without visible signs of damage) were added to each test vessel. To limit water

Fig. 1. Pb concentrations in body tissues (A), survival (B) and juvenile numbers (C) of *Folsomia candida* after 28 days exposure to shooting field soils. Columns with the same letter indicate no significant differences at $p > 0.05$. See [17] for the soil codes and their properties. Error bars show standard deviation ($n = 5$).

evaporation and to prevent collembolans from escaping, the containers were closed with transparent lids. All the containers were incubated at $20 \pm 2^\circ\text{C}$ and a 16/8 h light/dark photoperiod for 48 h. At the end of the test period, the two sections of each test container were divided again and emptied into different vessels. These vessels were filled with water and a few drops of blue ink were added. After gently stirring, the individuals floating on the water surface were counted. An additional combination was included containing control or reference soil on both sides of the test container to test for absence of avoidance reaction (dual-control test).

For each replicate the avoidance response was calculated as:

$$AR = \left[\frac{C - T}{N} \right] \times 100$$

where, AR = avoidance response (%), C = number of collembolans in control or reference soil, T = number of collembolans in shooting field soils, N = total number of collembolans exposed. Avoidance is indicated by a positive attraction or non-response by a negative AR value. The tested soils were considered to have limited or reduced habitat function when $\geq 80\%$ of the collembolans stayed in the control or reference soil.

2.4. Data analysis

All data were examined for both normality and homogeneity of variance (Kolmogorov–Smirnov test). One-way of ANOVA was used to make comparisons. Log transformation had to be applied

to obtain normal distribution and homogeneous variances of the data. A multiple comparison test procedure based on Tukey's HSD test was used to further determine differences among means at the 95% confidence level. Pearson's correlation analysis was conducted to test the significance of the correlations between toxicity and soil physicochemical properties and (bio)available Pb concentrations ($p < 0.05$). Multiple regressions were carried out to quantitatively analyze the relationship among collembolan bioassay endpoints (survival, reproduction, avoidance response and body residue of Pb), level of available Pb, and other soil physicochemical properties. Statistical analyses were conducted using Microsoft Excel and SPSS16.0 software.

3. Results

3.1. Chemical characteristics of shooting field soils

A full description of the soil properties and metals concentrations has been given by Luo et al. [17]. Here, only a brief summary is provided. The forest soils were most acidic with $\text{pH}_{\text{CaCl}_2}$ of 3.2–3.5. The grassland soils had the highest $\text{pH}_{\text{CaCl}_2}$ values (6.5–6.8). The bullet plot soil also was acidic with $\text{pH}_{\text{CaCl}_2}$ 3.7. Total Pb concentrations in the shooting field soils ranged from 47 to 2398 mg/kg dw, and the highest total, CaCl_2 - and water-extractable as well as pore-water Pb concentrations were found in the forest soils F1 and F3. All artificial soils (R1–R3) had identical total Pb concentrations, while they had CaCl_2 - and water-extractable Pb concentrations significantly higher than LF2.2.

3.2. Pb uptake in collembolans

Pb concentrations in the springtails increased with increasing lead concentrations in the soils (Fig. 1A). The highest Pb concentrations in the collembolans were found at the highest concentrations of Pb in F3 soil. The collembolans exposed to forest soil F0 had the lowest internal Pb concentration, which was not significantly different from S, LF2.2, B0 and G3 ($p > 0.05$). Although internal Pb concentrations in the springtails exposed to the three most polluted soils (G1, F1, F3) were not significantly different ($p > 0.05$), they were remarkably higher than those exposed to artificial (R1–R3), soccer field (S) and LF2.2 soils ($p < 0.05$). The highest BAFs (0.2) were found in grassland soil G1 and forest soil F3, while the lowest BAF (0.07) was observed in grassland soil G3. Internal Pb concentrations in *F. candida* were significantly and positively correlated with the different measures of Pb concentration in the test soils ($p < 0.01$) (Table 1). Simple and stepwise regression analysis showed that internal Pb concentrations in the collembolans were best explained from total Pb concentrations in soil (Table 2).

3.3. Responses of the collembolans

3.3.1. Survival

Survival of *F. candida* in LF2.2, soccer field, bullet plot and grassland soils (Fig. 1B) exceeded the validity criterion of 80% set by ISO [20]. Survival of the collembolans was somewhat reduced in the forest soils (F0, F1, F3) after 4 weeks of exposure, and was significantly lower than in LF2.2 and soccer field soils ($p < 0.05$). In the other soils, survival was not significantly different from that in LF2.2 or soccer field soils ($p > 0.05$). Survival of *F. candida* was best correlated with CaCl_2 -extractable Pb concentrations in the test soils ($p < 0.01$) (Table 1), while also DOC level in porewater seemed to explain survival in the shooting field soils ($R^2 = 0.84$) (Table 2). Since mortality was never higher than 40%, no LC50 values for the effect of Pb on the survival of *F. candida* could be calculated.

3.3.2. Reproduction

After 4 weeks exposure, reproduction of *F. candida* in all soils exceeded the minimum of 100 per test container set by ISO for a test to be considered valid [20]. The juvenile numbers of *F. candida* in all forest soils including F0 were significantly lower than in LF2.2 ($p < 0.05$). The grassland soils had significantly higher juvenile numbers than the forest soils ($p < 0.05$) (Fig. 1C). The artificial soils R1 and R2, which had high OM content and low $\text{pH}_{\text{CaCl}_2}$, respectively, had lower juvenile numbers than R3 and LF2.2 (Fig. 1C). The numbers of juveniles produced were not significantly different among forest, R1 and R2 soils, and also did not differ significantly among grassland soils, bullet plot soil and soccer field soil, R3 and LF2.2.

Juvenile numbers of *F. candida* were significantly and positively correlated with especially soil pH, but negatively with Pb concentrations ($p < 0.01$) but not total Pb (Table 1). Juvenile numbers (JN) were best described by CaCl_2 -extractable Pb concentrations in the test soils ($R^2 = 0.82$) in combination with Ca, Cd, Cu, Zn contents, $\text{pH}_{\text{H}_2\text{O}}$, CEC and DOC levels (Table 2). Since reproduction was never affected by more than 50% compared to the respective control or reference soil, no EC50 values for the effect of Pb on the reproduction of *F. candida* were estimated.

3.3.3. Avoidance test

The number of dead or missing individuals during the avoidance test was zero in all treatments, fulfilling the validity criterion (less than 10% missing individuals) of the ISO Guideline 17512-2 [21]. Avoidance responses in all shooting field soils were positive when LF2.2 was used as a reference (Fig. 2). All forest soils had relatively

Fig. 2. Avoidance response (%) of *Folsomia candida* after 2 days exposure to increasing Pb concentrations in shooting field soils. See [17] for the soil codes and their properties. Values are mean $\pm$ standard deviation, $n = 5$.

high positive avoidance responses, which were not dose-related. The avoidance responses were significantly higher in the forest soils F1 and F3 than in the grassland soils G1 and G3. The grassland soils had relatively low positive avoidance responses, which did not differ from the bullet plot soil (B0). However, avoidance responses in all shooting field soils except for F0 were negative when an artificial soil was used as the reference (Fig. 2). The forest soils F1 and F3 had strong negative avoidance responses when the most acidic R2 soil was used as a reference. Grassland soil G1 had a negative avoidance response with R3 as the reference, while forest soil F1 had a positive avoidance response with R1 as the reference. Unfortunately, the amount of G3 soil available was insufficient to allow for testing the avoidance response compared to the artificial soils. Correlation analysis showed that the avoidance response was only poorly (though significantly) correlated with CaCl_2 -extractable and porewater with r values < 0.55 (Table 1). CaCl_2 -extractable Pb together with total Cd, Cu and Zn concentrations and pH of the test soils explained only part of the variation in the avoidance response (AR) data ($R^2 = 0.58$) (Table 2).

4. Discussion

4.1. Soil properties and availabilities of Pb

All shooting field soils except F0 had total Pb concentrations higher than the background levels in Dutch soils (55–60 mg/kg dw) [22], indicating Pb contamination in most shooting field soils. The relatively high Pb concentrations in extracts and pore water of the most polluted soils F1 and F3 [17] may be explained by the low pH and high OM and DOC levels of these soils. On the other hand, compared to the acid soils F0 and B0, the relatively low concentrations of CaCl_2 - and water-extractable and porewater Pb in the grassland soils G1 and G3 may be explained from their high pH values. In addition, the high concentrations of Ca (from CaCO_3) in soils G1 and G3 may have decreased the solubility of Pb, probably by precipitation as carbonates [23,24]. Also pH may have affected Pb availability, explaining the great difference in extractable Pb concentrations between F0 and B0 soils. See our previous paper [17] for a more detailed discussion on factors affecting Pb availability in soils.

Table 1

Simple linear correlation coefficients relating the response of *Collembola* (*Folsomia candida*) to the physicochemical properties of different shooting range field soils and reference soils. See [17] for soil properties and Pb concentrations and Figs. 1 and 2 for the collembolan responses.

Soil physico-chemical properties	Simple linear correlation coefficients (r)			
	Pb concentration in <i>Collembola</i> (mg/kg dry body weight)	Number of surviving <i>Collembola</i>	Number of juvenile collembolans produced	Avoidance response (%)
WHC	0.20	−0.15	−0.25	0.16
OM	−0.14	−0.082	−0.24	0.35
pH-H ₂ O	−0.17	0.60**	0.70**	−0.47**
pH-0.01 M CaCl ₂	−0.22	0.59**	0.67**	−0.48**
DOC	−0.30*	−0.29*	−0.50**	0.30
CEC	−0.33*	0.41**	0.32*	−0.45*
Ca	−0.29*	0.55**	0.56**	−0.46*
Fe	0.30*	−0.13	0.052	−0.086
Clay	−0.40**	0.33*	0.090	−0.19
Silt	−0.28*	0.20	0.056	−0.24
Sand	0.37**	−0.29*	−0.080	0.21
Pb-Water	0.72**	−0.60**	−0.36**	0.42*
Pb-0.01 M CaCl ₂	0.61**	−0.71**	−0.56**	0.54**
Pb-pore water	0.70**	−0.63**	−0.35**	0.53**
Total Pb	0.59**	−0.42**	−0.164	0.43**
Total Cd	−0.0090	0.32*	0.40**	−0.53**
Total Zn	0.055	0.24	0.36**	−0.40*
Total Cu	0.18	0.12	0.31*	−0.38*

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

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

4.2. Pb uptake in the collembolans

Pb concentrations in the collembolans increased linearly with increasing Pb concentration in the test soils. This was also shown by Bongers et al. [25]. At total soil concentrations of 406–49,200 mg/kg dw, a fairly constant bioaccumulation factor (BAF) for *F. candida* of 0.04–0.06 was calculated [14], also supporting the linear increase in body concentrations with increasing exposure level. In our study, the levels of Pb in *F. candida* appeared to be not significantly different for soils with total Pb concentrations less than 656 mg Pb/kg dw, corresponding with water- or CaCl₂-extractable concentrations of 0.47 and 0.18 mg Pb/kg dw, respectively or 0.13 mg Pb/L in pore water. Since F1 and F3 had similar concentrations of total, water-, 0.01 M CaCl₂-extractable and porewater Pb as well as similar soil properties [17], it is easy to understand that internal Pb concentrations in *F. candida*

exposed to the soils were not significantly different. Low contents of OM, DOC, clay, and silt and high sand content, CEC and Ca concentration may explain the increased Pb in the body of *F. candida* exposed to G1. Therefore, there was no significant difference of Pb concentrations in *F. candida* exposed to F1, F3 and G1. The BAF values in the present study (0.07–0.2) are higher than those reported by Fountain and Hopkin [14], which may be attributed to the much lower pH and consequent higher Pb availability in our test soils. The highest correlation coefficients (Table 1) and the highest determination coefficients (Table 2) for Pb concentrations in the collembolans with water extractable Pb concentrations in soils suggest that Pb uptake is mainly from the pore water. Considering that the correlations with other Pb forms were only slightly lower, ingestion of Pb-polluted soil particles by the collembolans [26–28] may however, also have contributed to exposure. In all forest soils, pH [17] was below the optimal

Table 2

Multiple-regression equations and coefficient of determination (R^2) for the relationship between the toxicity of Pb contaminated shooting range field soils to the *Collembola* (*Folsomia candida*) and soil physicochemical properties. See [17] for soil physicochemical properties and Pb concentrations and Figs. 1 and 2 for toxicity data.

Endpoint	Multiple linear regression equation obtained	Statistics
Pb concentration in <i>Collembola</i> (C_w , mg/kg dw)	$\log C_w = 0.55 (\log [Pb]_{Total}) - 0.046 \text{ Silt} - 0.032 \text{ CEC} + 0.99$ $\log C_w = 0.60 (\log [Pb]_{Water \text{ extractable}}) + 0.44 (\log [Fe]) - 0.001 \text{ DOC} + 0.39$ $\log C_w = 0.39 (\log [Pb]_{CaCl_2 \text{ extractable}}) - 0.001 \text{ DOC} + 2.0$ $\log C_w = 0.71 (\log [Pb]_{Porewater}) + 0.78 (\log [Ca]) + 0.027 \text{ Clay} - 2.4$	$p < 0.05$; $R^2 = 0.71$ $p < 0.05$; $R^2 = 0.79$ $p < 0.01$; $R^2 = 0.72$ $p < 0.05$; $R^2 = 0.78$
Survival number (SN)	$SN = -0.65 (\log [Pb]_{Total}) + 0.66 \text{ pH}_{H_2O} + 6.1$ $SN = -0.98 (\log [Pb]_{Water \text{ extractable}}) - 0.0020 \text{ DOC} + 9.5$ $SN = -0.69 (\log [Pb]_{CaCl_2 \text{ extractable}}) - 0.0020 \text{ DOC} + 9.2$ $SN = -0.63 (\log [Pb]_{Porewater}) + 1.1 (\log [Cd]) - 0.0020 \text{ DOC} + 12$	$p < 0.01$; $R^2 = 0.74$ $p < 0.05$; $R^2 = 0.75$ $p < 0.05$; $R^2 = 0.84$ $p < 0.05$; $R^2 = 0.77$
Juvenile number (JN)	$JN = -45 (\log [Pb]_{Total}) - 242 (\log [Ca]) + 139 (\log [Cd]) + 185 \text{ pH}_{H_2O} + 409$ $JN = 267 (\log [Pb]_{Water \text{ extractable}}) - 489 (\log [Ca]) + 1460 (\log [Cd]) + 17 (\log [Cu]) - 1117 (\log [Zn]) + 457$ $\text{pH}_{H_2O} + 34 \text{ CEC} + 1.2 \text{ DOC} + 982$ $JN = 196 (\log [Pb]_{CaCl_2 \text{ extractable}}) + 48 (\log [Ca]) + 1299 (\log [Cd]) - 311 (\log [Cu]) - 558 (\log [Zn]) + 395$ $\text{pH}_{H_2O} - 6.2 \text{ CEC} + 0.90 \text{ DOC} - 322$ $JN = 175 (\log [Pb]_{Porewater}) + 1.4 (\log [Ca]) + 1126 (\log [Cd]) - 418 (\log [Cu]) - 514 (\log [Zn]) + 276$ $\text{pH}_{H_2O} + 22 \text{ CEC} + 0.55 \text{ DOC} + 15$	$p < 0.05$; $R^2 = 0.79$ $p < 0.05$; $R^2 = 0.81$ $p < 0.05$; $R^2 = 0.82$ $p < 0.05$; $R^2 = 0.80$
Avoidance response (AR, %)	$AR = 7.2 (\log [Pb]_{Total}) - 42 (\log [Cd]) + 30 (\log [Cu]) - 11 (\log [Zn]) - 9.4 \text{ pH}_{H_2O} + 21$ $AR = 5.6 (\log [Pb]_{Water \text{ extractable}}) - 41 (\log [Cd]) + 32 (\log [Cu]) - 9.1 (\log [Zn]) - 8.8 \text{ pH}_{H_2O} + 28$ $AR = 5.8 (\log [Pb]_{CaCl_2 \text{ extractable}}) - 37 (\log [Cd]) + 30 (\log [Cu]) - 13 (\log [Zn]) - 5.5 \text{ pH}_{H_2O} + 17$ $AR = 6.3 (\log [Pb]_{Porewater}) - 34 (\log [Cd]) + 28 (\log [Cu]) - 5.8 (\log [Zn]) - 7.7 \text{ pH}_{H_2O} + 11$	$p < 0.05$; $R^2 = 0.53$ $p < 0.05$; $R^2 = 0.53$ $p < 0.05$; $R^2 = 0.58$ $p < 0.05$; $R^2 = 0.57$

value of 6.0 for *F. candida* [12], which also increased Pb bioavailability for the collembolans. Therefore, the low pH may amplify the ecological risk caused by bioaccumulation of Pb in acid soils [26,29].

4.3. Ecotoxicity of shooting field to collembolans

4.3.1. Survival assessment

Since no dose-related decrease of collembolan survival was seen and no 50% effect observed, collembolan survival was not sensitive to Pb contamination. This was also shown by other studies [30–32]. Fountain and Hopkin [14] observed that *F. candida* feeding on yeast containing up to 49,200 mg Pb/kg exhibited no significant changes in survival. Joosse and Verhoef [33] also noticed no significant effect of Pb on the growth of the collembolan *Orchesella cincta*. Since total Pb concentrations in F1 and F3 soils were very close to the LC50 value (2900 mg/kg dw) reported for collembolans [25], mortality in these most polluted forest soils might also be related to Pb contamination.

4.3.2. Reproduction assessment

No dose-related decreasing reproduction was seen and 50% effect was only observed in the acid forest soils F0, F1 and F3 (Fig. 1C). Based on high correlation coefficients (Table 1) and determination coefficients (Table 2), reproduction was better described by CaCl_2 -extractable Pb concentrations than by total, water-extractable or porewater Pb concentrations. Similar results were also observed by other studies [26–28]. Total Pb concentrations in all our test soils were lower than the LOEC (lowest observed effects concentration) of 2400 mg Pb/kg dw for reproduction of *F. candida* in a soil with 2% OM, 37.2% clay and $\text{pH}_{\text{H}_2\text{O}}$ 8.2. The total Pb concentrations in F1 and F3 were not only higher than the LOEC of 1200 mg Pb/kg dw for reproduction in a soil with 1.6% OM, 24.8% clay and $\text{pH}_{\text{H}_2\text{O}}$ 6.1, but also significantly higher than the LOEC of 300 mg Pb/kg dw for reproduction in an acidic soil with 16.5% OM, 19.4% clay and $\text{pH}_{\text{H}_2\text{O}}$ 4.5 [26]. It was obvious that soil properties, especially pH and OM content, influenced Pb toxicity to *F. candida*. An EC50 of 2970 mg/kg dw was reported for the effect of Pb on the reproduction of collembolans after 28 days exposure [34], and no offspring was produced by *Paronychiurus kimi* at 3000 mg/kg dw [35]. Lock et al. [36] found that Pb concentrations of 3877 mg/kg dw or higher always caused a significant decrease in collembolan reproduction. On the other hand, Menta et al. [30] reported an evidently negative effect on collembolan reproduction by Pb already at 1000 mg/kg dw. A Pb concentration of 1312 mg/kg dw may cause extinction of populations of *F. candida* [35]. Bongers et al. [25] found an $\text{EC50}_{\text{reproduction}}$ for *F. candida* of 1900 mg Pb/kg dw. Nursita et al. [32] were not able to determine an $\text{EC50}_{\text{reproduction}}$ for *F. candida* at soil Pb concentrations up to 3000 mg/kg dw. Therefore, in the present study, it is unlikely that the effect on collembolan reproduction seen in the forest soils was entirely caused by Pb. Although there were significant differences in Pb concentrations between the forest soils F0, F1 and F3, and between the grassland soils G1 and G3, no significant differences were found in juvenile numbers among the forest soils and between grassland soils. The relatively low juvenile number in the clean soil F0 suggests that the pH in this soil reached the lower limit of the pH tolerance range ($\text{pH} = 3.2\text{--}7.7$) for *F. candida* [12]. The effect of the low soil pH on reproduction was also confirmed by the significantly lower juvenile numbers in soil R2 compared to LF2.2. The significant correlation between juvenile number and pH also reveals that pH probably was more important than Pb in affecting collembolan reproduction. Sandifer and Hopkin [31] reported $\text{EC50}_{\text{reproduction}}$ values for the effect of Pb on *F. candida* in soils with different pH values: 2970 mg/kg dw at pH 6.0; 1360 mg/kg dw at pH 5.0 and 3160 mg/kg dw at pH 4.5. Although Sandifer and Hopkin [31] did

not find any relationship between effects on reproduction and soil pH, their lowest pH was still higher than that of the most acid soils in the present study. Therefore, a pH of 4.5 seems to be a critical threshold, below which the composition of the soil fauna clearly changes. Artificial soil R1, which had high organic content, also had lower juvenile numbers than LF2.2 (Fig. 1C), suggesting that natural soils seem more beneficial for collembolan reproduction than artificial soils.

4.3.3. Avoidance behavior assessment

Applying the 80% avoidance criterion [37], no field soil presented an impaired habitat quality when compared to LF2.2 and the reference soils. The high avoidance in the forest soils F0, F1 and F3 compared to LF2.2 probably was caused by the combination of high Pb concentrations (particularly the CaCl_2 -extractable fraction) and low pH. High correlation and determination coefficients between avoidance responses and soil properties (Tables 1 and 2) also indicated that CaCl_2 -extractable Pb concentrations seemed to give better predictions of avoidance behavior than other Pb concentrations and soil properties. Short-term microcosm experiments using compartmented boxes showed that some species dispersed from an acid source to a neutral soil heavily polluted with metals [38,39]. The relatively high negative avoidance responses for the forest soils (F1 and F3) with R2 as a reference suggest that artificial soil with both low pH ($\text{pH}_{\text{CaCl}_2} = 3.8$) and low Pb concentration (45 mg/kg dw) may have higher toxicity than acid forest soils ($\text{pH}_{\text{CaCl}_2} = 3.3\text{--}3.5$) with relatively high concentrations of Pb (2153–2398 mg/kg dw). However, avoidance of the acid forest soil F0, which had low Pb concentration (47 mg/kg dw), with R1 as reference further suggested that also soil organic matter content was an important factor for determining collembolan avoidance behavior. It therefore is important to highlight a characteristic of this bioassay, already pointed out by other authors [8,11] and the ISO Guideline [21]: soil properties need to be considered when interpreting the results of bioassays on metal-contaminated soils. The influence of soil properties (e.g., pH, organic matter content and texture) upon the avoidance response of collembolans could mask a possible effect of the contaminant. In the present study, correlation and regression analyses indicated that avoidance response was also affected by Cd, Cu, Zn, Ca, DOC and CEC. The effects of these soil properties on the performance of the test species therefore should be considered during the assessment of Pb contaminated soils. It also seems important to select test species that have a broad tolerance range for relevant soil properties, like soil pH.

5. Conclusion

Acid forest soils from a shooting field with total Pb concentrations of 2153–2398 mg/kg dw and $\text{pH}_{\text{CaCl}_2}$ values of 3.5–3.8 had higher toxicity to springtails than grassland soils with total Pb concentrations of 355–656 mg/kg dw and $\text{pH}_{\text{CaCl}_2}$ values of 6.5–6.8. Differences in toxicity were caused by the differences in both pH and Pb concentrations between the forest and grassland soils and seemed more affected by low soil pH than by the high Pb concentrations. The survival, reproduction and avoidance responses of the collembolans were best described by 0.01 M CaCl_2 -extractable Pb concentrations in the test soils. Internal concentrations of Pb in *F. candida* linearly increased with increasing concentrations of Pb in the soils. Soil properties significantly affected collembolan survival, reproduction and avoidance response as well as the bioavailability of Pb. Therefore, care must be taken in the selection of reference soils for bioassays with Collembola. A combination of chemical approaches and toxicity tests could help characterizing the risk of Pb-contaminated soils.

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