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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-$CaCl_2$ (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 $CaCl_2$ 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.

1. Introduction

Shooting ranges around the world represent a significant sink and potential source for trace metal pollution in soil and ground water (Van Vleek 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-$CaCl_2$ (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 $CaCl_2$ 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 $CaCO_3$ to nominal pH-$CaCl_2$ 6.0 ± 0.5. The other two artificial soils were prepared with peat contents of 5% (R2) or 2.5% (R3) and pH-$CaCl_2$ adjusted to nominal 3.5 (R2) or 6.5 (R3) with $CaCO_3$ (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 $CaCl_2$ (Houba et al., 1996), while also porewater was extracted. Water and $CaCl_2$ 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 $CaCl_2$ (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 $CaCl_2$ 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 (SK^{12} 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%.