THE EFFECT OF COUNTERION AND PERCOLATION ON THE TOXICITY OF LEAD FOR THE SPRINGTAIL FOLSOMIA CANDIDA IN SOIL

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(Received 23 October 2002; Accepted 19 June 2003)

Abstract—In standard soil toxicity tests, heavy metals are amended as water-soluble salts. The role of the counterion in metal salt toxicity is scarcely looked into. In this study, we assessed the contribution of nitrate and chloride to the toxicity of lead to Folsomia candida in a natural standard soil. Both lead salts were tested according the standard test protocol as well as after percolation of the soil with deionized water. Lead nitrate was more toxic than lead chloride for survival as well as reproduction. Percolation proved to be an effective method to remove counterions from the soil. Survival of F. candida increased for both metal salts when percolation was included. Percolation reduced the reproduction toxicity of lead, the effect of which was largest for the nitrate salt. In percolated treatments, the nitrate and chloride lead salts did not differ in toxicity. It is concluded that counterions contribute to metal toxicity and that nitrate is more toxic to F. candida than chloride.

Keywords—Folsomia candida Lead Counterion Toxicity Percolation

INTRODUCTION

One of the main objectives in the environmental policy of the European Union is the protection of soil ecosystems against the impact of toxic compounds. This policy has led to the successful development of toxicity tests for a range of soil organisms. In these tests, toxicity of heavy metals is determined by adding the metal to the soil in the form of a water-soluble salt. The toxicity is subsequently related to the metal concentration, although the counterion itself can also have a toxic effect on the test organism [1,2]. The role of the counterion is mostly overlooked, but some research has recently been done to address this problem. Peredney and Williams [3] showed that various types of counterion differ in toxicity to nematodes. A most appropriate counterion could not be given because the effect of a specific counterion may differ between metals and test organisms. For the springtail Folsomia candida, Schrader et al. [2] found that high salt concentrations inhibited egg development. A comparison between a solution of salts and an elutriate of toxic waste containing heavy metals and similar salt ions showed a clear combination of salt effects and heavy-metal effects. It is therefore likely that the choice of metal salt can bias ecotoxicity tests.

In the literature, four ways are proposed to deal with this counterion effect. First, by compensating for differences in counterion concentration between treatments with another salt of which the cation is thought to be nontoxic [1], or is abundant in the medium (e.g., potassium [4]). Second, by performing an additional experiment with the counterion, but with another, supposedly nontoxic cation to estimate the contribution of the counterion to the toxicity observed in the metal test [2]. Third, by percolating the soil with deionized water after addition of the metal salt to flush out the counterion [5]. Fourth, by using an organic counterion that is mineralized following introduction in the soil (e.g., acetate [6]).

This research aims to evaluate the role of the counterion in the toxicity of heavy metals to the springtail F. candida exposed in a natural standard soil. We selected lead as the test metal because of its low toxicity compared with other heavy metals (Cu, Zn, Cd) (e.g., [7]), therefore requiring high concentrations of lead and thus counterions in the tests. Testing high concentrations is still relevant because springtails occur at mining sites with lead concentrations as high as 11,300 mg lead/kg dry soil [8]. The study was performed in two steps. First, the toxicity of lead nitrate and lead chloride was investigated. Subsequently, the effect of percolation on the toxicity of both metal salts was assessed and thus the contribution of the counterion to the lead toxicity.

MATERIALS AND METHODS

Test animals

Juvenile F. candida Willem 1902 (Hexapoda: Collembola), of similar age (10–12 d), were obtained by synchronizing the egg deposition of adult animals from a laboratory breeding stock. Additional animals of the same size were taken from the breeding culture and used in the experiments because the synchronization cultures did not provide enough animals to carry out the toxicity tests. The F. candida breeding culture originated from arable land at an experimental farm, The Lovinkhoeve, at Marknesse, The Netherlands, and has been kept in the laboratory for about 10 years. The animals were cultured in pots with a layer of moist plaster of Paris mixed with activated charcoal (9:1 w/w) and kept in a climate room at 16 ± 0.1°C, 75% relative humidity at a 12:12-h light-dark regime. Animals were fed with dried baker’s yeast (Oetker, Veenendaal, The Netherlands).

Contamination of the test soil

A natural standard soil (Landwirtschaftliche Untersuchungs- und ForschungsAnstalt, LUFA 2.2), was obtained from the LUFA-Institute at Speyer, Germany. The soil had an
organic carbon content of 2.27%, a pH (0.01 M CaCl$_2$) of 6.1, and a cation exchange capacity of 9 mval/100 g. PbCl$_2$ (>98% pure, Merck-Schuchardt, Hohenbrunn, Germany) or Pb(NO$_3$)$_2$ (>99.5% pure, Merck, Darmstadt, Germany) solutions were mixed in with the soil to obtain nominal concentrations of 0, 500, 1,000, 2,000, 4,000, and 8,000 mg Pb/kg dry soil. Water solubility of PbCl$_2$ is low and, as a consequence, the solutions had to be added in several steps. First, the soil was dried at 50°C to a soil moisture content of 9% (w/w). Then part of the solution was added, after which the soil was again dried at 50°C to a soil moisture content of 9% (w/w), after which the next portion was added. This was repeated five times. After the last addition, the soil was dried to retain a soil moisture content of 24% (w/w), which equals 50% of the maximum water-holding capacity. The Pb(NO$_3$)$_2$ solutions were added in one step to the soil with the right amount of deionized water to obtain the same moisture content.

The soil was left for three weeks at room temperature to equilibrate. Next, for each concentration, half of the soil was placed in 6-cm diameter polyvinylchloride tubes with a paper filter and a 0.45-µm cellulose nitrate membrane filter (401196; Schleicher & Schull, Dassel, Germany) on a gauze bottom and filter and a 0.45-

**Experimental design**

Tests followed the procedure of the International Standardization Organization Guideline 11267 [9]. Glass jars (100 ml) were filled with 30 g of moist soil. Five replicates were used for each concentration. At the start of the experiment, 10 juvenile F. candida were transferred into each of the jars and a few grains of dried baker’s yeast were added as food. The jars were placed on trays in a climate room at 20 ± 0.1°C, 75% relative humidity with a 12:12-h light:dark cycle. Additional food was given to maintain ad libitum conditions. The jars were aerated three times a week and soil moisture content was kept constant by weighing the jars once a week and replenishing the water loss with deionized water.

After four (lead chloride) or five weeks (lead nitrate) of incubation, survival, weight, and reproduction of the animals were determined in each of the soils. For lead nitrate, the tests were run for five weeks instead of four weeks because of the low number of juveniles that could be visually observed in the controls after four weeks. The contents of a jar were washed into a glass beaker using 100 ml of deionized water. The mixture was gently stirred to let all living animals float on the water surface. After the surviving animals were counted by eye, a color slide of the water surface was made. The slides were projected on a portable slide projector, and the number of offspring was counted to determine reproduction. For each treatment, a sample of surviving animals (up to a maximum of 15) was collected from the replicate test jars. Fresh weight of individual animals was determined to the nearest microgram using a supermicrobalance (model S4; Sartorius, Goettingen, Germany). The animals were lyophilized and weighed to obtain the dry weight and stored at −20°C until lead analyses.

**Chemical analysis**

Soil was dried at 50°C. The total metal fraction was obtained by microwave digestion of 1 ± 0.05 g dry soil in 6 ml of a mixture of concentrated HNO$_3$ (Riedel-de Haën, Seelze, Germany), HCl (Mallinkrodt Baker, Deventer, The Netherlands), and deionized water (4:1:1 v/v/v). Exchangeable and water-soluble fractions were determined by shaking 5 ± 0.05 g dry soil for 2 h at 200 rpm with 50 ml of a 0.01 M CaCl$_2$ (Mallinkrodt) solution or 50 ml deionized water. The suspensions were left overnight to settle, whereafter pH was determined in the solutions. After filtration through a 0.45-µm cellulose nitrate membrane filter, lead concentrations in the extracts were measured by atomic absorption spectrometry (model 1100B, Bodenseewerk Perkin-Elmer, Überlingen, Germany). Chloride concentrations in the water extracts were measured on an autoanalyzer (model SA 400; Skalar, Breda, The Netherlands). Unfortunately, nitrate levels were not measured during the experiment (later measurement is not useful because nitrate is unstable). Animals, individually or in groups of two or three, were digested in 300 µl of a mixture of HNO$_3$ and HClO$_4$ (7:1 v/v; Ultrex grade; Mallinckrodt) and internal lead concentrations were measured using graphite furnace atomic absorption spectrometry.

**Calculations and statistics**

A t test was used to test whether percolation had an effect on pH (GraphPad Prism® 2.01, www.graphpad.com). Sorption of lead to the test soil was described by a Langmuir isotherm,

\[ C_{\text{ sorbed}} = \frac{C_{\text{ sorbed-max}} K_C C_{\text{ diss}}}{1 + K_C C_{\text{ diss}}} \]

where \( C_{\text{ sorbed}} \) is the lead concentration in the soil (mg/kg dry soil), \( C_{\text{ diss}} \) is the lead concentration in the water or 0.01 M CaCl$_2$ extract (mg/L), \( C_{\text{ sorbed-max}} \) is the maximum sorption capacity (mg/kg) and \( K_C \) is the Langmuir sorption constant (L/mg). The \( K_C \) may be interpreted as the inverse of the dissolved concentration at which 50% of the sorption sites is occupied. Estimates for \( C_{\text{ sorbed-max}} \) and \( K_C \) were obtained by nonlinear regression on log-transformed data (GraphPad Prism 2.01). Controls were excluded from the analyses because dissolved concentrations were below detection limits. A generalized likelihood ratio test [10] was used to compare results from different lead salts and percolation treatments.

The trimmed Spearman–Karber method [11,12] was used to estimate the median lethal concentration (LC50). The logistic model of Haanstra et al. [13], modified according to Van Brummelen et al. [14], was used to estimate the 10 and 50% effect concentrations (EC10 and EC50) for growth and reproduction using measured lead concentrations in soil. Estimation of the effect concentrations using lead concentrations in the animals was hampered by a lack of data for the control and lowest dose (below detection limit) and a high variation among the animals. Models were fitted to the data using the lsqcurvefit function of the MATLAB® 6.1 software package (The MathWorks, Natick, MA, USA). A generalized likelihood ratio test was used to compare results from different lead salts and percolation treatments.

**RESULTS**

**Soil analysis**

The pH-CaCl$_2$ and pH-H$_2$O of control soils were 5.3 and 5.5 for Pb(NO$_3$)$_2$-amended soils and 5.7 and 6.3 for PbCl$_2$-
amended soils. A decrease in pH-CaCl₂ and pH-H₂O with increasing lead concentrations was observed for all treatments (results not shown). This decrease was most pronounced at the highest lead dose (1–2 pH units). Over the range where the change in toxic response occurred, the pH did not decrease for Pb(NO₃)₂-amended soils, and no more than 0.8 pH unit for PbCl₂-amended soils. Percolation raised soil pH-H₂O slightly (0.2 pH unit), pH-CaCl₂ showed the same tendency, but not significantly. Without percolation, approximately 50% of the added chloride was found in the water-soluble fraction (1.4 g Cl⁻/kg dry soil for the highest lead treatment). Percolation decreased the water-soluble chloride concentrations by 57%.

Measured total lead concentrations in soil were in good agreement with nominal levels (all data in this article are expressed on the basis of measured concentrations). Percolation of the soil with deionized water only slightly affected total lead concentrations. The highest loss of lead was 8.5% for the 8,000 mg/kg PbCl₂ treatment. In all test soils, water-soluble and CaCl₂-exchangeable concentrations increased with the total lead concentrations. Availability of lead (determined as water-soluble and CaCl₂-exchangeable fractions) did not differ between PbCl₂- and Pb(NO₃)₂-spiked soils, nor did percolation affect availability of lead. Similarity in availability was confirmed by the Langmuir isotherms (Fig. 1). Langmuir sorption parameters (K_L and C_sorbed-max) are given in Table 1 for the different treatments. The sorption parameters did not differ significantly between the treatments (p > 0.05) and one overall isotherm could describe all data.

**Accumulation of lead**

Lead concentrations in the springtails increased with increasing lead concentrations in the soil (results not shown). There was no difference in internal lead concentrations between different salts and percolation treatments. Internal lead concentrations of surviving animals from the controls and 500 mg/kg treatments were below detection limits in all samples. For the other doses, lead levels in individual animals could not be measured reliably. Measurements from pooled animal samples for the 1,000- and 2,000-mg/kg treatments gave internal concentrations (± standard error) of 22 ± 2.2 (n = 9) and 80 ± 9.2 (n = 7) µg Pb/g dry body weight. Only at the percolated 4,000-mg/kg treatment of the lead chloride salt, two pooled samples could be measured: 484 and 1,460 µg Pb/g dry body weight. There were insufficient data to express toxicity on internal lead concentrations.

**Effects on survival, fresh weight, and reproduction**

Mean survival in the controls was 68%, and no animals survived at the highest dose for all experiments. Survival was higher in PbCl₂-amended soils than in Pb(NO₃)₂-amended soils and survival increased by percolation. Table 2 gives the highest lead dose at which surviving animals were found and LC₅₀ values for each treatment.

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>H₂O</th>
<th>CaCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_L (L/mg)</td>
<td>C_sorbed-max (g/kg)</td>
</tr>
<tr>
<td>PbCl₂</td>
<td>0.50 (0.20)</td>
<td>8.2 (2.4)</td>
</tr>
<tr>
<td>PbCl₂ percolated</td>
<td>0.59 (0.31)</td>
<td>8.3 (3.2)</td>
</tr>
<tr>
<td>Pb(NO₃)₂</td>
<td>0.70 (0.51)</td>
<td>7.0 (3.5)</td>
</tr>
<tr>
<td>Pb(NO₃)₂ percolated</td>
<td>0.51 (0.33)</td>
<td>8.8 (4.2)</td>
</tr>
<tr>
<td>All</td>
<td>0.57 (0.14)</td>
<td>8.0 (1.4)</td>
</tr>
</tbody>
</table>

Fig. 1. Langmuir isotherms for the sorption of lead in PbCl₂- and Pb(NO₃)₂-spiked natural standard soil, with (P) and without percolation, extracted with deionized water (A) or 0.01 M CaCl₂ (B). ▽ = Pb(NO₃)₂; ▼ = Pb(NO₃)₂-P; ■ = PbCl₂; □ = PbCl₂-P.
Table 2. Median lethal concentration (LC50) values, with 95% confidence intervals, for the effect of lead on survival of Folsomia candida exposed to different lead salts, with and without percolation, in a natural standard soil. Values are based on total lead concentrations and are expressed in g Pb/kg dry soil. The second column for each salt gives the highest dose (g/kg) at which surviving animals were found.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PbCl₂ LC50</th>
<th>Dose</th>
<th>Pb(NO₃)₂ LC50</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>No percolation</td>
<td>2.9 (0.88–1.1)</td>
<td>2</td>
<td>0.98 (0.88–1.1)</td>
<td>1</td>
</tr>
<tr>
<td>Percolation</td>
<td>2.9 (2.3–3.5)</td>
<td>4</td>
<td>2.2 (1.9–2.5)</td>
<td>2</td>
</tr>
</tbody>
</table>

95% confidence interval, be calculated, and these were 1.4 (1.2–1.7) and 2.4 (2.2–2.6) g Pb/kg dry soil, respectively. For all other treatments, no more than 50% effect on weight was observed before the animals died.

The mean number of juveniles was higher in the experiments with Pb(NO₃)₂, probably due to the longer test run. Reproduction showed a negative relationship with lead concentrations in all treatments. Percolation increased the EC50 for reproduction for both lead salts (see Fig. 2), the effect being largest for lead nitrate. In Table 3, EC50 and EC10 values for effects on reproduction are given, based on total lead concentrations in the test soils. Lead nitrate was significantly more toxic than lead chloride, and in the percolated treatments, EC50s were similar. No effect of percolation was apparent for lead chloride, and at the EC10 level, no significant difference between experimental treatments was observed.

**DISCUSSION**

In this study, we examined the role of the counterion (chloride and nitrate) in the toxicity of lead to the springtail F. candida exposed in natural standard soil. A drop in pH with increasing lead concentration was observed, but at lead doses where partial effects were observed, the pH was not below the optimal range of 4.3 to 6.2 [15]. It may therefore be assumed that the severe toxicity observed at high lead concentrations was not only due to a change in pH. Percolation did not change the total lead concentration, but chloride concentrations in the water-soluble fraction were reduced by 57% by percolating (nitrate concentrations were not measured). However, lead availability as measured in the water-soluble fraction and in the CaCl₂-exchangeable fraction was not altered (see Table 1). This indicates that there is no substantial difference in soil properties after percolation. Maximal sorbed lead concentrations, as reflected in the Langmuir constant (8,800 mg Pb/kg dry soil), were in good agreement with estimates from the cation exchange capacity (predicting a maximum binding of 9,300 mg Pb/kg dry soil). In the literature, we could not find data on internal lead concentrations for F. candida. Our data have to be regarded as indicative while values are based on only a few measurements.

Control survival was poor (68%). This could be related to the relatively small size of the springtails at the start of the experiments, making them more vulnerable to handling. The number of juveniles, however, was in the normal range, allowing reliable interpretation of the test results. The toxicity of lead nitrate in our test was more than a factor 17 higher than reported by Sandifer and Hopkin [7], who used an artificial soil with comparable characteristics, but started the tests with adult F. candida. Because the control performance was similar to that in our experiments, this difference could indicate that juveniles are more susceptible than adults are.

The effect of percolation on survival of the springtails was striking: For both lead salts, the animals survived one more dose step when the soil was percolated before testing (see Table 2). For lead chloride, this is not reflected in the LC50 due to the high variation in mortality at low lead concentrations. The effect of lead on growth is difficult to assess because, in all but one case, the animals died before 50% growth reduction was observed. For lead chloride in percolated soil, a growth reduction of more than 50% was seen and a large effect on growth was found in other experiments with lead chloride with and without percolation (M. Bongers, unpublished data).

Lead nitrate was more toxic than lead chloride both for survival and for reproduction (see Fig. 2, Tables 2 and 3). In percolated soil, this difference was not observed. The difference in toxicity cannot be explained by the difference in spiking method used for the two lead salts because there were no differences in lead availability (see Fig. 1). The information on internal concentrations is limited, but the results indicate that the animals died at different internal lead concentrations (they survived at higher total lead concentrations in percolated soil, with higher internal lead concentrations). This leads to the conclusion that the counterion must have an effect, either directly or indirectly (e.g., desiccation of the eggs or by affecting fungi growing on eggs). These results imply that nitrate is more toxic to F. candida than chloride, which is in agreement with the findings of Peredney and Williams [3] for the nematode Caenorhabditis elegans. For earthworms and aquatic ciliates, however, no difference in toxicity between chloride and nitrate salts of metals have been found [16–19].

Witteveen and Joosse [20] found that a salinity-intolerant...
springtail (*Isotoma viridis*) was reduced in growth at salinities above 4.9 g Cl−/L (25% seawater). Feeding activity and egg production were negatively affected at 25% seawater (though not significantly). Hutson [15] showed that *F. candida* had a lower fecundity at salt levels comparable with 2.6 g Cl−/L. Assuming that the chloride measured in water extracts is fully derived from the pore water, a worst-case estimate of pore-water concentrations can be derived. At a soil moisture content of 24% (w/w), the chloride concentration in the pore water at the highest lead doses was estimated to be 5.8 g Cl−/L, comparable with 30% seawater. Therefore, it is likely that *F. candida*, being a salt-intolerant springtail, was also negatively affected by the high chloride concentrations in this experiment. Due to percolation, chloride concentrations dropped to such a level that these negative effects were strongly reduced. Although nitrate concentrations have not been measured, it can be assumed that percolation also reduced the concentrations of this counterion, thus affecting toxicity.

**CONCLUSIONS**

Lead nitrate was more toxic to *F. candida* than lead chloride for both lethal and sublethal effects. Percolation of the soils partially reduced toxicity of both lead salts. In the percolated treatments, PbCl2- and Pb(NO3)2-amended soils showed no difference in toxicity. Percolation did, however, not affect total and available lead concentrations in the soil nor did it affect internal lead concentrations in the surviving animals. Therefore, the results show that the counterion contributes significantly to the toxicity of lead salts and that percolation is an effective way to get rid of unwanted effects of counterions in heavy metal testing.

Acknowledgement—This study was supported by a grant of the European Union (R&D Program: Environment and Climate: Project ENV4-CT97-0507). The authors would like to thank Rudo Verweij and Josée Kooolhaas-Van Hekezen for technical assistance. Nico van Straalen, Tjalling Jager, Ger Ernsting, and Willie Peijnenburg are acknowledged for critically reviewing earlier drafts of the manuscript.

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