EFFECT OF SOIL PROPERTIES AND AGING ON THE TOXICITY OF COPPER FOR ENCHYTRAEUS ALBIDUS, ENCHYTRAEUS LUXURIOSUS, AND FOLSOMIA CANDIDA

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(Received 7 October 2004; Accepted 24 January 2005)

Abstract—In the present study, the effect of the heavy-metal salt copper chloride (CuCl₂·2H₂O) in soils freshly spiked (3 d) and aged (70 ± 10 d; mean ± SD) was studied in the test species Enchytraeus albidus, E. luxuriosus, and Folsomia candida. Up to nine soils were used: Besides the Organisation for Economic Co-operation and Development (OECD) artificial soil and the Agricultural Testing and Research Agency (Landwirtschaftliche Untersuchungs- und Forschungsanstalt, Speyer, Germany) 2.2 natural standard soils, the others were selected based on the EURO Soil approach, taking into account the effect of different soil parameters (pH, organic matter, grain size distribution, and carbon to nitrogen ratio). Additionally, the effect of the chloride ions was studied separately. The results revealed the following: First, a soil effect was observed; for example, in F. candida, median effective concentrations (EC50s) varied between 262 mg/kg in a sample from the same site as the original EURO Soil 5 soil and greater than 1,000 mg/kg in OECD soil. Second, an aging effect was observed, mainly in F. candida. For example, toxicity of offspring survival was increased twofold in the OECD soil and approximately eightfold with aging in the EURO Soil 7 soil, whereas the enchytraeid species did not react differently after aging. Third, an effect of chloride ions on reproduction of the animals was found; however, this effect was independent of the aging period. Fourth, species variation was seen in terms of sensitivity (EC50), decreasing in the following order: E. luxuriosus > E. albidus >> F. candida. Differences in toxicity of offspring survival between enchytraeids and F. candida might be explained by the different routes of uptake.

Keywords—Natural soils Laboratory tests EURO-Soils

INTRODUCTION

Terrestrial toxicity not only varies between species, the soil characteristics also greatly influence the effect concentration of metals by altering, for instance, the bioavailability [1]. For reasons of practicability and comparability, it is most common in toxicity testing to use the Organisation for Economic Co-operation and Development (OECD) artificial soil [2] or the standard natural Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA; Speyer, Germany) 2.2 soil. Nevertheless, current risk-assessment procedures ignore that variation in soil properties results in substantial differences regarding uptake in and effects on organisms in different soils [3]. Aging is an important issue to take into account: Laboratory tests should mimic the most realistic situation, and freshly spiked soils do not allow the equilibration time that is required to resemble the common field situation. Incorporating the effect of aging in the environmental risk assessment of metal-contaminated soils may contribute to a more realistic assessment regarding the impact of metals on terrestrial ecosystems [4]. One of the problems is in terms of feasibility of the tests: The recommended aging period is a minimum of 60 d [5], a very long period considering the urgent demand for results and the need for experimental repetition. Therefore, the assurance of the need for such periods is very relevant and should be investigated.

In the present study, we tested the effects of the heavy-metal copper on two groups of organisms, enchytraeids and collembolans. Organisms with different exposure routes, such as oligochaetes and arthropods, should be used simultaneously to assess the environmental risk of metal-contaminated soils [6]. Additionally, standardized test procedures are available for the selected test species: Enchytraeus albidus, E. luxuriosus, and Folsomia candida. The common long-term application of copper fungicides against pests results in soil contamination [7]. Despite the fact that copper is an essential metal, at high dosages it becomes toxic to soil invertebrates. Other reasons for selecting copper as a test substance include that this is a well-known substance and that results from our studies can be compared with the Dutch (see, e.g., [3]) and Belgian [8] data.

Therefore, the main aims were to study the effects of the characteristics of different soils in terms of the suitability of the test species and the influence on the toxicity of a certain toxic substance.

MATERIALS AND METHODS

Test species

Two groups of organisms were used, enchytraeids and collembolans. The test species selected among the enchytraeids were E. albidus Henle 1837 and E. luxuriosus [9]. Enchytraeus albidus is one of the largest species of the genus Enchytraeus (adults reach 15–40 mm), whereas E. luxuriosus is much smaller (adults reach 8–13 mm). Both species were maintained in laboratory cultures, being bred in moist soil (50% OECD soil, 50% natural garden soil) at 20°C in the dark and fed once a week with finely ground and autoclaved rolled oats (Cimarron, Portugal). Details of the culturing process are given in Römbke and Moser [10].
Among the collembolans, F. candida Willem 1902 (Collembola: Isotomidae) is the most commonly used test species. It is a blind, unpigmented, euedaphic collembolan reproducing parthenogenetically [11]. This species is easily cultured in the laboratory in a moistened substrate of plaster of Paris/charcoal (8:1 mixture) prepared according to the method described by Usher and Stoneman [12]. Organisms were maintained in the laboratory at 20°C in the dark and were fed dried baker’s yeast (Saccharomyces cerevisae). Synchronized cultures were established for the experiments by removing egg clusters from stock cultures into new culture vessels. Two days after the start of the tests in an aqueous solution resembling the amount of soil plus food supply (finely ground and autoclaved rolled oats, 0.5 mg for E. albidus and 0.25 mg for E. luxuriosus, being half the amount supplied every week). Water was replenished weekly based on weight loss. Four replicates per treatment were used. The duration of the tests with E. albidus was six weeks: After three weeks, the adults were gently removed, and the soil was left for three additional weeks for juveniles to hatch and grow. The test duration for E. luxuriosus was four weeks, and the adults were left in the vessels until the end of the test. At the end of the test, the organisms were immobilized with alcohol and colored with Bengal red. After some hours, the organisms were colored and the soil solution was spread in a box and observed under the binocular for counting. Adult mortality and number of juveniles were evaluated for both species.

Test procedures were as described in the International Standard Organization guideline 11267 for F. candida [16]. Ten organisms with an age of 10 to 12 d were placed in each test vessel, which already contained the premoistened soil and the food supply. Four replicates per treatment were used. Vessels were covered with a parafilm layer in which a few holes for aeration were made. Food (2 mg of granulated dry yeast) and water (based on weight loss) was replenished weekly. After four weeks, the test ended, and each test vessel was filled with distilled water, which was gently mixed with a spatula. Afterward, juveniles and adults were floating on the surface. Due to the addition of a few drops of dark ink, a higher contrast between the white organisms and the black background was obtained. A digital photograph of the water surface plus the软件 program. Some replicates were randomly selected, and the pictures were checked by hand to validate the accuracy of the program. Adults and juveniles were easily distinguished by their size.

Test soils

The main properties of the test soils (pH, organic matter [OM], carbon to nitrogen ratio, cation-exchange capacity [CEC], maximum water-holding capacity, and clay, silt, and sand contents) are given in Table 1. Their selection has been described in detail by Römbke and Amorim [18].
The OECD artificial soil [2] is constituted by 69% sand, 20% kaolin clay, 10% sphagnum peat, and 0.3 to 1% of CaCO₃ for pH adjustment (6 ± 0.5; mean ± SD). The LUFA 2.2 is a natural standard soil from Speyer, Germany. Relative to the other codes given for the natural soils, ES7 means EURO Soil 7. ESo5 means that the soil is a sample from the same site as the original ES5, the numbers mean that the soil is similar to a certain ES number, and the other codes represent the first three letters of the soil original place name (Nat1 = Natzungen; Hoh2 = Hohenlimburg; Coi3 = Coimbra; Sch3 = Schmallenberg; Mon4 = Mönninghausen).

Experimental setup

The different soils were previously tested just as a control to evaluate their suitability regarding a certain test species (data not shown). Not all soils were used for the three test species because of several reasons, such as not fulfilling the validity criteria (at the end of the test, mortality should not exceed 20% of the adults for both groups, and reproduction should be a minimum of 25 individuals per test vessel in the case of enchytraeids and 100 for collembolans) (data not shown).

A first set of experiments was conducted 3 d after application of the test substance. A second set was performed 70 ± 10 d after application to allow chemicals to equilibrate. This experimental setup followed the recommendations of a workshop organized by the Society of Environmental Toxicology and Chemistry [5], which recommended that three different evaluations be made: First, 2 to 7 d after mixing the substance into the soil; second, 60 d after the 2- to 7-d initial incubation of the substance into the soil; and third, same as the second, but the soil also is leached 2 to 7 d after mixing the substance into the soil. However, because of time and resource limitations, the third option was not tested here. This 60-d period is referred to as a transformation time, and it is a trade-off between practical considerations and allowing a realistic amount of time for transformation reactions, with rapid reactions for metals typically having half-life values in the range of 1 to 100 d.

Statistical procedures

Three main hypotheses were tested as follows: First, the measured soil properties influence the toxicity of the toxic compound in two ways, either by directly altering the exposure (e.g., because of different adsorption and bioavailability) or by adding another stress factor for the organisms in addition to the chemical. Second, aging is affecting the toxicity. Third, the chloride ions are affecting the results obtained with the toxic compound.

Different methods were used to test the hypothesis: Stepwise multiple-regression models were performed using the statistical software package SPSS 12.0 [19] to quantify the relationship of the biological data with the soil data. Data, except those for pH, were normalized using log(x + 1) transformation in the regression models. Additionally, only the two extreme categories (sand and clay, excluding silt) of the three texture classes were used because of the interdependence of the individual parameters. Nevertheless, no statistically significant regressions were obtained. Two-way analysis of variance (ANOVA) was calculated using SigmaStat 2.03 [20] to evaluate differences between each soil. Student’s t test [20] was used to analyze statistically significant differences between controls of each time of aging and between control and CtCl. The median effective concentrations (EC50s) and no-observed-effect concentrations were calculated using the ToxRatPro® program [21].

RESULTS

Freshly spiked versus aged soils

In Figures 1 to 3 and Tables 2 to 3, the results of the tests with the three species in the different soils are presented. Unfortunately, the number of invalid tests, particularly in the case of enchytraeids, for which the absolute number of tests was limited, was high.

Regarding the results for E. albidus, no significant effect of aging was observed on the adults in any of the soils tested (two-way ANOVA). In the OECD soil, a statistically significant effect of aging (two-way ANOVA: F = 62.850, df = 1, p < 0.001) on the reproduction in terms of the total number of organisms produced (maximum number of juveniles, ~300 in freshly spiked soil and ~140 in aged soil) was observed. In the LUFA 2.2 soil, a statistically significant effect of the interaction of aging and the toxic concentrations (two-way ANOVA: F₁₆ = 4.066, p = 0.003) on reproduction (the EC50 increased slightly, from 97 to 122 mg/kg) was observed. However, because the two controls were significantly different (t test: t = −3.111, df = 6, p = 0.021), the effect of aging could not be confirmed. No comparisons were made in the case of Coi3 soil because of the ambiguous results obtained after 3 d.

Comparing the soils, toxicity of offspring survival increased in the following order: OECD < Coi3 < LUFA 2.2. The EC50 value in OECD and LUFA 2.2 soil differed by a factor of at least three.

The results for E. luxuriosus were as follows: No significant effect of aging on the adults was observed in any of the soils tested (two-way ANOVA). In the OECD soil, a statistically significant effect of aging (two-way ANOVA: F = 8.625, df = 1, p = 0.005) and of the toxic substance (two-way ANOVA: F = 4.942, df = 6, p < 0.001) on reproduction (toxicity decreased by a factor of approximately three) was observed at the concentration of 320 mg/kg. Because the controls between each aging period were significantly different (t test: t = 3.526, df = 6, p = 0.012), the effect of aging could not be confirmed. No comparisons can be made in the case of LUFA 2.2, Sch3, and Coi3 soil because of invalid aging test results.

For F. candida, we found in the OECD soil a statistically significant effect of aging (two-way ANOVA: F = 505.417, df = 1, p < 0.001) and of the toxic concentrations used (two-way ANOVA: F = 5.518, df = 6, p < 0.001; 1,000/320 mg/kg ≠ control) on reproduction. Because the controls between each aging period were significantly different (t test: t = 16.23, df = 6, p < 0.001), the effect of aging could not be confirmed. In the Hoh2 soil, a statistically significant effect of aging (two-way ANOVA: F = 24.697, df = 1, p < 0.001) and of the toxic concentrations used (two-way ANOVA: F = 8.447, df = 6, p < 0.001) on reproduction was found at the concentration of 1,000 mg/kg. In the Coi3 soil, a statistically significant effect of aging (two-way ANOVA: F = 231.140, df = 1, p < 0.001) and of the toxic concentrations used (two-way ANOVA: F = 2.996, df = 6, p < 0.001; 32 mg/kg ≠ CtCl) on reproduction was found. Because the controls between each aging period were significantly different (t test: t = 3.857, df = 6, p = 0.008), the effect of aging cannot be confirmed. In the Mon4 soil, a statistically significant effect was observed to result from the interaction of aging and the toxic concentrations (two-way ANOVA: F₁₆ = 5.253, p < 0.001) on reproduction, but because the controls between each aging period were signif-
Fig. 1. Effects of copper chloride (CuCl₂·2H₂O) on the survival and reproduction of *Enchytraeus albidus* in freshly spiked and aged soils. Graphs show the average number of survivors and standard deviation (SD; n = 4). *Statistically significant differences from control. Coi3 = Coimbra (Portugal); CtCl = control spiked with KCl; LUFA 2.2 = natural standard soil (Speyer, Germany); OECD = Organisation for Economic Co-operation and Development.

Significantly different (t test: \( t = 9.403, df = 6, p < 0.001 \)), the effect of aging could not be confirmed. In the ESo5 soil, a statistically significant effect of aging (two-way ANOVA: \( F = 39.266, df = 1, p < 0.001 \)) and of the toxic concentrations used (two-way ANOVA: \( F = 57.610, df = 6, p < 0.001 \); CtCl/320/1000 mg/kg ≠ 100/10/control/32 mg/kg) on reproduction was observed. In the ES7, a statistically significant effect was observed to result from the interaction of aging and the toxic concentrations used (two-way ANOVA: \( F_{1,6} = 4.944, p < 0.001 \)) on reproduction, but because the controls between each aging period were significantly different (t test: \( t = 6.797, df = 6, p < 0.001 \)), the effect of aging could not be confirmed. No comparisons could be made in the case of the LUFA 2.2 and the Nat1 soils. The results concerning the adults are presented in Table 2. An effect of aging was observed in most of the tested soils, except in Mon4, causing a decrease in the absolute number of surviving offspring.

Despite the fact that some tests were not valid, the results with *F. candida* seem to indicate that toxicity of offspring survival increased after aging. For example, EC50 values were lower by a factor of approximately two in the OECD and the ESo5 soils and by a factor of eight in the ES7 soil after aging. Between soils, toxicity of offspring survival increased in the following order: OECD/Nat1/Coi3 (value > 1000 mg/kg) < LUFA 2.2 < Hoh2 < ES7 < Mon4 < ESo5. The EC50 values varied between OECD/Nat1/Coi3 soil and ESo5 (fresh-
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Fig. 2. Effects of copper chloride (CuCl₂·2H₂O) on the survival and reproduction of Enchytraeus luxuriosus in freshly spiked and aged soils. Graphs show the average number of survivors and standard deviation (SD; n = 4). *Statistically significant differences from control. Coi3 = Coimbra (Portugal); CtCl = control spiked with KCl; LUFA 2.2 = natural standard soil (Speyer, Germany); OECD = Organisation for Economic Co-operation and Development; Sch3 = Schmallenberg (Germany).

ly spiked) by a factor of approximately four and between OECD soil and ESo5 (aged) by a factor of approximately six.

Several stepwise-regression models were run. However, none gave results that allowed a significant relationship to be determined between test species and soil parameters.

Control versus CtCl

No differences were observed between the tests with water (control) and those with chloride (CtCl) in the number of adults of both enchytraeid species and F. candida. Statistically significant differences in reproduction were found for E. albidus in certain soils—LUFA 2.2 soil (freshly spiked) (t test: t = −4.130, df = 6, p = 0.006) and Coi3 aged soil (t test: t = −2.830, df = 6, p = 0.03)—with the chloride causing a positive effect. Similarly, in E. luxuriosus, in certain soils (freshly spiked)—LUFA 2.2 soil (t test: t = 4.701, df = 6, p = 0.003), Sch3 soil (t test: t = 3.502, df = 6, p = 0.013), and Coi3 soil (t test: t = 4.728, df = 6, p = 0.003)—the chloride was causing a decrease in the number of juveniles produced.

In F. candida, statistically significant differences in reproduction were found to result from the chloride ions in certain soils—Nat1 aged soil (t test: t = 5.327, df = 6, p = 0.002), Mon4 soil freshly spiked (t test: t = 9.879, df = 6, p < 0.001), and ESo5 freshly spiked (t test: t = 8.485, df = 6, p < 0.001) and aged (t test: t = 7.058, df = 6, p < 0.001)—causing a negative effect.

Comparison between species

The comparison between species is only possible in the OECD artificial and the fresh LUFA 2.2 soil. Nevertheless, in these cases, reproduction in F. candida was less sensitive than the enchytraeids. Among the enchytraeid species, E. luxuriosus was more sensitive than E. albidus.

DISCUSSION

Freshly spiked versus aged soils and comparison between soils

Despite the low number of valid tests, it seems that in the tests with the enchytraeid species, the toxicity of offspring
Fig. 3. Effects of copper chloride (CuCl₂·2H₂O) on the survival and reproduction of *Folsomia candida* in freshly spiked and aged soils. Graphs show the average number of survivors and standard deviation (SD; n = 4). *Statistically significant differences from control. Coi3 = Coimbra (Portugal); CtCl = control spiked with KCl; ESo5 = sample from the same site as the original EURO Soil 5; ES7 = EURO Soil 7; Hoh2 = Hohenlimburg (Germany); LUFA 2.2 = natural standard soil (Speyer, Germany); Mon4 = Mömninghausen (Germany); Nat1 = Natzungen (Germany); OECD = Organization for Economic Co-operation and Development; Sch3 = Schmallenberg (Germany).
Fig. 3. Continued.
significantly after spiking, whereas in our tests, only a slight decrease in pH (never larger than 0.5 and only in the highest toxic concentrations) occurred. Therefore, it is not clear whether the pore-water copper concentrations in the freshly spiked soils are higher because of the lower pH or because of the effect of aging in the spiked soils.

On the other hand, in the case of *F. candida*, aging seems to have an effect on both adults and juveniles, increasing the toxic effect at the reproduction level. In the OECD soil, an increase by a factor of two and in the ES7 soil by a factor of approximately eight occurs. Again, the lack of a complete data set hampers the generalization of this observation, but the fact that in all valid tests the toxicity of offspring survival mostly increased makes it wise to perform tests with aged soils and *F. candida*. The same recommendation was made by Smit and Van Gestel [24], who studied the effect of zinc on *F. candida* after percolating metal-contaminated soils with water and the survival of copper in freshly spiked and aged soils was nearly the same. At a maximum, toxicity of offspring survival decreased over aging by a factor of 3.5 in the test with *E. luxuriosus* in OECD soil. With *E. albidus*, no effect of aging was observed. Similarly, Lock and Janssen [22] found no effect of aging (eight weeks) on the toxicity of zinc (also an essential metal like Cu) for *E. albidus* in a test with OECD soil. The authors believe that the clay type used (kaolinite) may be responsible for this observation, because kaolinite has a low capacity for metal fixation in comparison with other clays, such as bentonite and illite [22]. However, the same authors found that despite comparable total copper concentrations, pore-water copper concentrations were significantly higher in the freshly spiked soils compared to those in the historically contaminated soils [23]. In their study, pH decreased significantly after spiking, whereas in our tests, only a slight decrease in pH (never larger than 0.5 and only in the highest toxic concentrations) occurred. Therefore, it is not clear whether the pore-water copper concentrations in the freshly spiked soils are higher because of the lower pH or because of the effect of aging in the spiked soils.

Table 2. Effect of aging (time) and CuCl₂ exposure (conc.) on the survival of *Folsomia candida* as analyzed by two-way analysis of variance and t tests*

<table>
<thead>
<tr>
<th>Soil</th>
<th>Source of variation</th>
<th>F</th>
<th>df</th>
<th>p</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>OECD</td>
<td>Time × concn.</td>
<td>2.519</td>
<td>1, 6</td>
<td>0.036</td>
<td>No effect between control effect of time confirmed</td>
</tr>
<tr>
<td>Hoh2</td>
<td>Time × concn.</td>
<td>6.936</td>
<td>1, 6</td>
<td>&lt;0.001</td>
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</tr>
<tr>
<td>Coi3</td>
<td>Time × concn.</td>
<td>9.566</td>
<td>1</td>
<td>0.004</td>
<td>No effect between control effect of time confirmed</td>
</tr>
<tr>
<td>Mon4</td>
<td>Time × concn.</td>
<td>5.441</td>
<td>1, 6</td>
<td>&lt;0.001</td>
<td>No effect between control effect of time confirmed</td>
</tr>
<tr>
<td>ESo5</td>
<td>Time × concn.</td>
<td>7.225</td>
<td>1, 6</td>
<td>&lt;0.001</td>
<td>No effect between control effect of time confirmed</td>
</tr>
<tr>
<td>ES7</td>
<td>Time</td>
<td>6.199</td>
<td>1</td>
<td>0.017</td>
<td>No effect between control effect of time confirmed</td>
</tr>
<tr>
<td></td>
<td>Concentration</td>
<td>3.059</td>
<td>6</td>
<td>0.014</td>
<td>At the concentration of 1,000 mg/kg</td>
</tr>
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</table>

*No effect between control means that no significant difference was found between fresh and aged control soils. If a significant difference was found, the t test results are shown. ESo5 is a soil sampled from the same site as the original EURO Soil 5; ES7 means EURO Soil 7. Coi3: Coimbra (Portugal); Mon4: Mönninghausen (Germany); Hoh2: Hohenlimburg (Germany); OECD = Organisation for Economic Co-operation and Development.

Table 3. Median effective concentrations (EC50s) and no-observed-effect concentrations (NOECs) from tests with *Enchytraeus albidus*, *E. luxuriosus*, and *Folsomia candida* exposed to copper chloride in freshly spiked (3 d) soils and aged (70 ± 10 d) soil*

<table>
<thead>
<tr>
<th>Species</th>
<th>Soil</th>
<th>EC50 (Cu)</th>
<th>NOEC (Cu)</th>
<th>EC50 (aged)</th>
<th>NOEC (aged)</th>
</tr>
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<tr>
<td><em>E. albidus</em></td>
<td>OECD</td>
<td>&gt;320</td>
<td>&gt;320</td>
<td>≥320</td>
<td>≥320</td>
</tr>
<tr>
<td></td>
<td>LUFA 2.2</td>
<td>&gt;320</td>
<td>97</td>
<td>≥320</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Coi3</td>
<td>&gt;320</td>
<td>ND</td>
<td>≥320</td>
<td>ND</td>
</tr>
<tr>
<td><em>E. luxuriosus</em></td>
<td>OECD</td>
<td>&gt;320</td>
<td>65</td>
<td>≥320</td>
<td>3.2</td>
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<tr>
<td></td>
<td>LUFA 2.2</td>
<td>&gt;320</td>
<td>81</td>
<td>≥320</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Sch3</td>
<td>&gt;320</td>
<td>48</td>
<td>≥320</td>
<td>10</td>
</tr>
<tr>
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<td>Coi3</td>
<td>&gt;320</td>
<td>91</td>
<td>≥320</td>
<td>32</td>
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<tr>
<td><em>Folsomia candida</em></td>
<td>OECD</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>≥1000</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>LUFA 2.2</td>
<td>&gt;1000</td>
<td>987</td>
<td>≥1000</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>Nat1</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>≥1000</td>
<td>≥1000</td>
</tr>
<tr>
<td></td>
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<td>869</td>
<td>948</td>
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<td>320</td>
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<tr>
<td></td>
<td>Mon4</td>
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<td>262</td>
<td>≥1000</td>
<td>100</td>
</tr>
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<td></td>
<td>ES7</td>
<td>&gt;1000</td>
<td>794</td>
<td>≥1000</td>
<td>320</td>
</tr>
</tbody>
</table>

* Soil codes are as described in Table 1. ND = not determinable (e.g., no dose response); Mort. = mortality; OECD = Organisation for Economic Co-operation and Development; Rep. = reproduction; X = test not valid (e.g., strange behavior of the organisms toward the chemical substance. 
inclusion of an equilibration period before use. To achieve a more realistic exposure situation, these procedures should be included into laboratory toxicity tests. Nevertheless, statistical evaluation of the EC50 values (nonparametric Wilcoxon signed-rank test, test for two related samples) determined for different soil types and species for freshly spiked and aged copper contamination showed no significant differences.

Based on the EC50 values, between-soils toxicity of offspring survival increased in the following order: For E. albidus, OECD < Coi3 < LUFA 2.2; for E. luxuriosus, Coi3 < LUFA 2.2 < OECD < Sch3; and for F. candida, (OECD/Co3/Nat1) < LUFA 2.2 < Hoh2 < E57 < Mon4 < Eso5.

In the enchytraeid tests, the differences in EC50 values were relatively small in most cases. This observation is caused, at least in part, by the fact that valid tests could be performed only in a limited number of soils because of the sensitivity of enchytraeid reproduction toward soil properties. In the only soil parameter than pH, no clear influence on toxicity can be established.

Major changes, however, were obtained in the tests with F. candida. These differences probably were caused by an interaction of soil properties and copper. For example, the low pH of the ESo5 soil is the factor causing the much higher toxicity of offspring survival in this soil compared to that in the other soils. This fact is in accordance with the findings of Crommentuijn [26], who tested pH levels from 7.3 to 3.1 and found a range of EC50 values for survival of 306 to 102 μg/g of cadmium, respectively, showing a tendency for increasing toxicity with lower pH. However, Sandifer [27] did not observe an effect of pH on the reproduction of F. candida when exposed to metals (Cd, Cu, Pb, and Zn). We observed only an overall decrease in reproduction in the control samples at pH 5.0 and 4.5 in comparison to a pH of 6.0. Concerning other soil parameter than pH, no clear influence on toxicity can be established.

Furthermore, it is wise to reduce the amount of OM in the artificial soil to 2%, because in the original OECD soil, which contained 10% peat, the observed toxicity of offspring survival was lower than that in most field soils [28]. The OM in our soils ranged from 1.7 to 12.9%, and in this range, we could not find a significant influence (perhaps because an effect would be seen only at lower pH ranges). Accordingly, Lock and Janssen [29] as well as Lock et al. [30] found that instead of the content of clay and OM, the pH and the CEC were the most important parameters affecting zinc and cadmium ecotoxicity (i.e., toxicity decreases with increasing pH and CEC). These authors state that CEC is a better parameter with which to estimate bioavailability and ecotoxicity compared to clay and OM, because CEC is a measure of the amount of available sorption sites and, thus, incorporates the clay, metal oxyhydroxides, and OM of a soil. The question remains, however, whether this statement is similarly true for copper.

Control versus CtCl

No effect of chloride could be observed for adults in E. albidus and in E. luxuriosus, but the reproduction of the test species was affected in certain soils. Often, the toxicity of metals in soil is determined by investigating the effects of metal salts without paying attention to the influence of the anionic partner of the investigated metal. Therefore, Schrader et al. [31] evaluated the role of salt anions on the reproduction of F. candida by using a soil with a standard mixed-salt solution containing CaSO4, MgSO4, MgCl2, KCl, and NaCl applied at different concentrations. At higher salt concentrations, egg development was inhibited. Tests with single-salt solutions showed that this resulted from the inclusion of NaCl (43.5 mmol/kg dry wt soil) in the mixed-salt solution. The CaCl2 tested separately also reduced egg survival. 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. These studies indicate that chloride ions may interfere with the demonstration of toxic heavy-metal effects. The authors concluded that when chloride salts are used to determine the toxicity of metal cations, additional tests with comparable anion solutions of nontoxic cations are needed to clarify the results.

No details concerning the potential effect of potassium on enchytraeids or collembolans are known. However, in field studies with inorganic fertilizers (i.e., nitrogen–potassium–phosphorous mixtures), only indirect effects on enchytraeid populations have been found, which probably were caused by ammonium nitrate [32].

Finally, it remains an open question whether the increased toxicity after aging, as observed in several tests with F. candida, has something to do with the effects caused by the chloride ions. Of the two soils in which this increase after 70 d was observed, a very strong effect in the CtCl was visible only in ESo5 soil. This means that a combination of low pH and chloride (similar to the combination of low pH and Cu) probably is responsible for this result. On the other hand, no explanation can be given for the strong effect in the CtCl of the Nat1 soil after aging. However, this test is difficult to evaluate, because no dose–response relationship could be established.

Comparison between species

Species sensitivity to CuCl2·2H2O decreased in the following order: E. luxuriosus >> E. albidus >> F. candida. Differences in toxicity of offspring survival between enchytraeids and F. candida might be explained by the different exposure routes of uptake: Springtails probably are exposed mainly via the pore water, whereas dietary exposure also is important for worms [4]. Additionally, F. candida may have detoxification mechanisms, similar to the ones reported previously [33] for Orchesella cincta. This collembolan stores cadmium within the gut as metallothionein-bound cadmium. In this way, it was confirmed what could be expected: Enchytraeids were more affected than collembolans. Several studies show the higher sensitivity of oligochaetes toward copper in comparison to arthropods (see, e.g., Didden and Römbke [32] for enchytraeids and Spurgeon et al. [34] for earthworms).

General discussion

Only few studies have focused on the effect of different soil properties on the toxicity of copper for enchytraeids and collembolans. In 1992, Van Gestel [35] concluded that many factors affect sorption to soil and uptake in organisms and, therefore, that extrapolation between soils did not yet seem to be possible. Later, Van Gestel et al. [36], in a review of the influence on soil characteristics on the toxicity of metals to soil invertebrates, concluded that pH is the most important factor, followed by soil OM content and CEC. However, for each metal, another combination of these factors seems to determine bioavailability. Therefore, it was not possible to
derive general rules for the extrapolation of toxicity data between soils [37]. Moreover, the bioavailability of metals in soil seemed to fluctuate with time [38]. Vijver et al. [39] studied the impact of soil properties in 16 Dutch field soils on the accumulation of heavy metals in *F. candida*. Those authors confirmed that the bioavailability of metals depends on the metal, the soil properties, and the species in question. Different patterns in accumulation of metals were found for essential metals (Cu and Zn) versus cadmium and lead. The authors suggest a composite uptake. Internal body concentrations of zinc largely are unaffected by external zinc concentrations, and the same trend is found for copper. The organisms are able to maintain their internal concentration at a fixed level, independent of the external concentrations. In general terms, cadmium and lead uptake by *F. candida* is strongly associated with total metal pools and metal-binding phases of the soils, such as carbonate clay and oxyhydroxides. These findings show that biological diversity in uptake patterns exists between soil inhabitants. Solid soil phases are more important in the uptake process of springtails than expected based on formulae derived for soft-bodied oligochaete species and plants, which were influenced more strongly by pore-water characteristics. The latter results were obtained by Peijnenburg et al. [3,40,41], who found that the effects on soft-bodied species and plants were strongly associated with metal pools in the pore water and in the CaCl₂-extractable fraction. A high proportion of the variation of tissue residues was explained by pH. In conclusion, literature data regarding uptake and toxicity experiments carried out in OECD soil cannot be used in a straightforward manner to predict effects of metals in field soils.

Lock and Janssen [1] assessed the influence of soil type on cadmium (CdCl₂·2H₂O) availability in a standard artificial soil, a sandy field soil, and a loamy field soil. The authors could not evaluate the influence of soil parameters on the bioavailability of cadmium neither based on the conducted experiments nor by including literature data. *Enchytraeus albidus* was the most sensitive species, followed by *F. fetida* and *F. candida*. Furthermore, those authors revealed that the acute ecotoxicity of copper was determined mainly by pH and OM content (significant effect) and by CEC (highly correlated). They also could show that the lethal concentration causing 50% mortality for *E. albidus* exposed to metals varied over more than two orders of magnitude, depending on the composition of the artificial soil [8,30]. Later, they studied the influence of copper (CuCl₂·2H₂O) and other metals on *E. albidus* in OECD soil [25], and they observed that the concentration–response relationships were steeper for the essential elements (Zn and Cu) than for those obtained with the nonessential elements (Cd and Pb). Finally, Peijnenburg et al. [3] tested *E. crypticus* in 20 Dutch field soils contaminated with cadmium, copper, lead, and zinc. Multivariate expressions that describe uptake rate constants and bioaccumulation factors as a function of soil characteristics were derived. The pH and CEC were the most important parameters, but these differed with each metal. Unfortunately, relatively to copper, concentrations on organisms after exposure did not differ significantly from body concentrations in the culture, and in view of this apparent regulation, the copper data were considered to be irrelevant in the present study. Hence, no relation to soil properties could be established for copper. Thus, it seems doubtful whether reliable correction factors can be derived for the influence of soil properties on the toxicity of copper to soil invertebrates similar to the ones for cadmium in agricultural soils [28]. Nevertheless, studies by Sauvé et al. [42,43] show that copper does have a high affinity for OM. It is even possible to develop a model in which copper activities are predicted on the basis of pH, total OM in the solid phase, and total amount of copper in the solid phase. The underlying mechanism would be preferential sorption of copper to OM, modulated by pH.

**CONCLUSION**

Our results confirm that soil properties affect the toxicity of copper on enchytraeids and collembolans (soil effect). The EC₅₀ values varied in each species from soil to soil, reaching factors of approximately three in *E. albidus* and approximately six in *F. candida*, although no relation with any specific soil parameter among the tested soils was found. Accordingly, the precautionary values for copper as laid down in the German Soil Protection Ordinance [44] which are different according to the three soils texture classes (20, 40, and 60 mg/kg total concentration), were set as a threshold for a “no-effect-level” in sandy, silt, and clay soils. These values have been extrapolated based on a relatively small literature review in the mid-1990s [45]. Microbial lowest-observed-effect concentration in sandy soils were in the range of 10 to 50 mg/kg, the lowest no-observed-effect concentration from an earthworm test was 32 mg/kg, and plants started to react between 25 and 30 mg/kg. From these test results, the precautionary value were extrapolated by expert knowledge, knowing that more than 80% of the German soils have copper background values of less than 20 mg/kg. Results for *F. candida* results showed an important effect of aging, increasing the toxicity of offspring survival to a maximum of a factor of eight in the ES7 soil. The effect of the chloride ions, added simultaneously with the copper, caused effects in the reproduction of *E. luxuriosus* and *F. candida* in certain soils and, interestingly, changed with aging in certain cases. Additionally, species sensitivities differed, with enchytraeids being more sensitive than *F. candida*. A literature search relative to the influence of soil properties on the toxicity of metals shows no conclusive answers relative to these issues. Apparently, toxicity changes with the soil, the metal, and the species.

**Acknowledgement**—The authors thank António Nogueira for his advice regarding statistics. The present study was sponsored by Fundação para a Ciência e Tecnologia, Portugal, through a PhD grant to Mónica Amorim (SFRH/BD 1348/20000).

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Effect of soil type and aging on terrestrial toxicity

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