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The effect of leaching on the joint toxicity of a complex metal mixture to *Folsomia candida* in relation to bioavailability in soil

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Abstract

Metal-contaminated soils generally contain mixtures rather than single metals. Laboratory toxicity tests often focus on single metals in soils freshly spiked with soluble metal salts, potentially overestimating bioavailability in field soils. This study determined the toxicity of mixtures of Cu, Zn, Cd, and Pb dosed as chloride salts to the springtail *Folsomia candida* in LUFA 2.2 soil that was either left as is after spiking or leached to remove the chloride counterion. Effects on survival, growth, and reproduction were related to total, 0.01 M calcium chloride($CaCl_2$)-extractable, and water-extractable metal concentrations in the soil and to internal concentrations in the springtails. Leaching the spiked soil adequately removed the counterion with relatively small metal losses. The sorption of Cd and Zn to the soil decreased in the presence of other metals, whereas the sorption of Cu and Pb was not changed. Metal uptake by the springtails was not affected by the other metals but decreased at high chloride concentrations. Leaching did not change metal uptake in the springtails, suggesting no direct influence of chloride competition. However, leaching reduced metal toxicity, except for Cd. Mixture toxicity showed overall antagonism for all metal fractions and all endpoints, with dose ratio- and dose level-dependent deviations from concentration addition. The relative contribution of Cd to the mixture was the most important factor associated with antagonism. Dose ratio-dependent deviations related to Cd may be explained by its high toxicity combined with the large effect of other metals on its sorption. Changes in ecotoxicological effects and metal uptake at high mixture concentrations in unleached compared with leached soils suggest that chloride contributed to the toxicity of the metal salts and may explain the dose level-dependent deviations.

Keywords springtails, mixture toxicity, bioavailability, bioaccumulation

Introduction

Soil organisms are usually exposed to mixtures of metals that vary in their toxic potential, for instance, in soils polluted by mining that contain high Cu, Zn, Cd, and Pb concentrations (see e.g., González-Alcaraz et al., 2018; Scherger et al., 2024). For ecotoxicological risk assessment, any interactions between the toxicants should be considered to fully understand this mixture exposure risk. Especially in soil, the link between total chemical concentrations and effects on organisms is not straightforward, and interactions between chemicals can occur at three levels: (a) chemical and physicochemical interactions with other soil constituents, determining sorption and bioavailability; (b) physiological interactions, affecting uptake from the soil (solution), ultimately determining the quantity available at the site(s) of action; and (c) interactions at the intoxication processes, including combination with receptors, at the target site(s; see e.g., Gong et al., 2022). Interactions at these different levels must be disentangled to understand the chemical and physiological mechanisms that lead to metal mixture toxicity in soil organisms.

The effects of metals on soil organisms are influenced by soil physicochemical properties, with pH and organic matter content being key factors influencing metal bioavailability (Lahive et al., 2023). A further factor affecting metal toxicity to soil organisms is the competition effect with other cations (e.g., Ca^{2+}, Mg^{2+}; Ardestani et al., 2015). Laboratory toxicity tests with metals are usually performed by spiking soil with water-soluble metal salts prior to the introduction of the test animals. So, the test organisms are by default exposed to the metal ions and also to the counterion, which may have either a direct or an indirect effect (Renaud et al., 2020a; Tian et al., 2017). For example, Pb toxicity to the springtail *Folsomia candida* was dependent on the metal salt used, with lead chloride being less toxic than lead nitrate (Bongers et al., 2004). The effect of the counterion on the metal

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toxicity can be exerted through several mechanisms. Organisms may be directly affected by the ions themselves (Lock et al., 2006; Schrader et al., 1998), for example, leading to a loss of osmoregulatory balance and adverse effects (Hutson, 1978). Alternatively, increased ionic strength may change metal bioavailability, which in turn may change uptake and toxicity (Tian et al., 2017), or lower pH, which then changes exposure and effects (Smolders et al., 2015).

To counter the effects of the accompanying anion, the practice of leaching spiked soil prior to exposure has been proposed (Lock et al., 2006; Smit & Van Gestel, 1998; Smolders et al., 2009). Such leaching treatment has been shown to reduce the toxicity of Pb and Zn salts to *F. candida* (Bongers et al., 2004; Smit & Van Gestel, 1998) and together with ageing, increase the threshold for Pb toxicity fourfold compared with freshly spiked soil (Oorts et al., 2021). This implies that the toxicity of freshly spiked soil with salts to soil organisms, such as *F. candida*, is at least partly related to high accompanying anion concentrations, making leaching an effective way to remove (part of) this counterion effect.

In mixture toxicity studies for metals to which chloride salts must be spiked, the counterion concentrations may reach levels that may cause direct effects or alter metal bioavailability through ionic strength or pH change-related effects (Langdon et al., 2015; Smolders et al., 2015; Van Gestel & Koolhaas, 2004). To date, the large majority of soil metal mixture studies have been conducted without considering or mitigating any anion contribution (Renaud et al., 2020a). In the absence of such studies, it remains unclear whether the patterns of response observed in such mixture studies are solely due to the metals or whether the accompanying anion may play a role. This question is especially relevant in cases where interactions are indicated through the presence of synergistic or antagonistic effects.

Only a limited number of studies have been published with a systematic approach towards mixture toxicity of metals to soil organisms (see e.g., Jonker et al., 2004; Lock & Janssen, 2002; Qiu et al., 2011; Wu et al., 2012). In most studies, however, the number of exposure concentrations is limited, and the toxicity data are not suitable for identifying interactions beyond overall antagonistic or synergistic effects, leaving interesting aspects, such as dose ratio-dependent and dose level-dependent effects on mixture interaction understudied (Jegede et al., 2020; Kilpi-Koski et al., 2020; Renaud et al., 2020b; Svendsen et al., 2026; Van Gestel & Hensbergen, 1997).

This study investigated the toxicity of mixtures of Cu, Zn, Cd, and Pb to *Folsomia candida*. Toxicity tests were performed following ISO guideline 11267 (ISO, 1999) in a natural soil spiked with individual metals, equitoxic mixtures, and a series of mixtures at different ratios, including four field ratios. To unravel the possible interactions, we studied (a) the influence of each metal on the soil-solution distribution of the other metals by determining water-extractable, $CaCl_2$-exchangeable, and total soil concentrations; (b) the uptake of each metal by the springtails in relation to its (extractable) soil concentrations and that of the other metals; and (c) the combined effects of Cu, Zn, Cd, and Pb on the survival, growth and reproduction of *F. candida* in relation to extractable concentrations in soil and internal concentrations in the animals (note that performing the studies with a wide range of concentrations and metal ratios allowed us also to assess possible dose-ratio and dose-level dependent interactions of the metals in the mixtures); and (d) the role of the chloride counterion in the mixture toxicity of Cd, Cu, Pb, and Zn to the springtail *F. candida* by performing the tests with chloride salts of all metals in soil untreated or leached to remove the chloride counterion.

Materials and methods

Test animals

Juvenile *F. candida* of similar age (10–12 days) were obtained by synchronizing the egg deposition of adult animals from a laboratory breeding stock. The animals were cultured on a layer of moist plaster of Paris mixed with activated charcoal (9:1 w/w) at 20 ± 0.1 °C, 75% relative humidity, and a 12:12-hr light: dark regime, and fed dried baker's yeast (Oetker, Veenendaal, The Netherlands).

Spiking the test soil

A natural standard soil (Landwirtschaftliche Untersuchungs- und ForschungsAnstalt, LUFA 2.2) was used, which, based on supplier analysis, had an organic carbon content of 2.19% and a cation exchange capacity of 11 $cmol_c\ kg^{-1}$. Before adding the metal solutions, the soil was dried at 40 °C to a moisture content of 6%. Copper(II) chloride dihydrate (> 99% pure, Mallinckrodt Baker, Deventer, The Netherlands), zinc chloride (> 98% pure, Merck, Darmstadt, Germany), cadmium chloride (> 99% pure, Sigma-Aldrich, Zwijndrecht, The Netherlands) and lead(II) chloride ($PbCl_2$; > 98% pure, Merck-Schuchardt, Hohenbrunn, Germany) were mixed with the soil as aqueous solutions. A few drops (0.1–0.2 ml) of 10% hydrogen chloride were added to the $PbCl_2$ solution to increase solubility; this did not affect soil pH.

After adding the metal solutions, soil moisture content was raised to 25% (w/w), corresponding with 50% of its maximum water-holding capacity. The soil was then left to equilibrate for 2 weeks at room temperature in closed containers. For each concentration, one half of the soil was used directly in toxicity tests. After storage for another week, the remainder of the soil was placed in 6-cm diameter polyvinylchloride tubes, which had a paper filter, and a 0.45-μm cellulose nitrate membrane filter (401196; Schleicher & Schull, Dassel, Germany) on a gauze bottom. Next, the soil in the tubes was leached by flushing with an amount of deionized water equal to two times the amount of pore water present in the soil. A volume of leachate equal to the amount of water originally present in the soil was collected, after which the leached soil was air-dried to a soil moisture content of 25% (w/w) and equilibrated in closed containers for another 2 weeks (cf. Bongers et al., 2004; Lock et al., 2006) at room temperature before being sampled for chemical analysis and used for toxicity testing.

Individual metals and their mixtures were tested simultaneously. The experimental design for the mixture experiments was based on the toxic unit (TU) concept with reproduction as the endpoint (TU = c/EC50, where EC50 is the median effect concentration and c is the toxicant concentration in the mixture). Concentrations were based on EC50s for springtail reproduction effects measured in earlier studies performed in our laboratory (Bongers et al., 2004; Smit & Van Gestel, 1996; Van Gestel & Hensbergen, 1997), being 6,531, 6,117, 516, and 6,757 nmol g^{-1} dry soil for Cu, Zn, Cd, and Pb, respectively. Nominal concentrations of the mixtures in the unleached soil were based on expected toxicity at different ratios (see online supplementary material Table S1 for the detailed test design). Alongside an equitoxic ratio, several other concentration ratios were chosen, one for each metal in which that metal dominated the mixture, one with the essential metals dominating, one with the nonessential metals dominating, and four ratios with an equal expected contribution to the toxicity of the essential and the nonessential metals but with an unequal distribution of the expected toxicity over the two essential or nonessential metals. Further, four field concentration ratios were included, representing metal ratios prevalent at three contaminated sites and a reference site (see online supplementary material Text S1, Table S1, and Table S2).

Toxicity testing

Toxicity tests were performed according to ISO-guideline 11,267 for assessing the effects of soil pollutants on *F. candida* (ISO, 1999). Five glass jars (100 ml) filled with 30 g moist soil were used for each concentration and 20 for the control. All concentrations were tested simultaneously, but for practical reasons, the experiments with the unleached and leached soils were run separately, and for each experiment, replicates were staggered in time, with half the number of replicates per concentration run 1 week after the other half. At the start of the experiments, 10 juvenile *F. candida* were transferred into each jar, and a few grains of dried baker's yeast were added for food. The jars were incubated at 20 ± 0.1 °C, 75% relative humidity with a 12:12-hr light: dark cycle. The jars were aerated three times a week; additional food was given ad libitum, and soil moisture content was kept constant by weighing the jars once a week and replenishing the water loss with deionized water.

After 4 weeks, springtail survival, weight, and reproduction were determined. The contents of a jar were washed into a glass beaker using 100 ml of deionized water and gently stirred to let all living animals float on the water surface. Surviving adults were counted by eye, and a color slide of the water surface was made to count the number of offspring produced using a portable projector. To determine fresh weight, up to 10 surviving adults were collected from replicate test jars for each treatment and weighed individually to the nearest microgram using a microbalance (model UMT2, Mettler-Toledo, Greifensee, Switzerland). The animals were lyophilized and weighed to obtain the dry weight and stored at –20 °C until metal analyses.

Chemical analysis

Soils sampled were dried overnight at 40 °C. In soils sampled at the start of the exposures, total metal concentration was obtained by microwave digestion of 1 ± 0.05 g dry soil in 10 ml of a mixture of hydrogen chloride (min. 37%, Riedel-de Haën, Seelze, Germany), concentrated nitric acid (min. 65%, Riedel-de Haën, Seelze, Germany), and deionized water (6:2:2 v/v/v). Exchangeable and water-extractable metal concentrations were determined by shaking 5 ± 0.05 g dry soil for 2 hr at 200 rpm with 25 ml 0.01 M $CaCl_2$ (Mallinckrodt Baker, Deventer, The Netherlands) or 25 ml deionized water. The suspensions were left overnight to settle, after which pH was determined. Subsequently, the suspensions were filtered over a 0.45 µm cellulose nitrate membrane filter (401196, Schleicher & Schull, Dassel, Germany). Metal concentrations in digests and extracts were measured by flame or graphite furnace atomic absorption spectrometry (AAS, model 1100B, Bodenseewerk Perkin-Elmer, Überlingen, Germany). San Joaquin soil (certified by the National Institute of Standards and Technology, Gaithersburg, MD, USA) and an uncertified reference soil SETOC (sample 2) international sediment were used as references for total metal concentrations. Recoveries for the San Joaquin soil were 90%, 98%, 14%, and 75% for Cu, Zn, Cd, and Pb, respectively. For Cd and Pb, the low recoveries are due to the low concentrations in this reference soil. The SETOC soil recoveries were 126%, 109%, 80%, and 105%, and the detection limits 0.68, 0.92, 0.053, and 0.44 nmol ml^{-1} for Cu, Zn, Cd, and Pb, respectively. Chloride concentrations in water extracts were measured on an autoanalyser (model SA 400, Skalar, Breda, The Netherlands). Loss of chloride due to leaching was calculated by comparing the measured chloride concentration with the maximum water-extractable chloride concentrations calculated from the nominal total chloride concentrations obtained by spiking the soil with the metal chloride salts. Soil pH was also measured at the end of the exposures by drying the soil followed by extraction with 0.01 M $CaCl_2$ or water, and measuring pH in the suspensions after settling as detailed above.

Individual animals were digested in 300 µl of a mixture of nitric acid and perchloric acid (7:1 v/v; Ultrex grade; Mallinckrodt Baker, Deventer, The Netherlands) following the method of Van Straalen and Van Wensem (1986), and internal metal concentrations were measured using graphite furnace AAS. For the experiment using leached soil, due to time constraints, metals in springtails could be measured only for the control, single, and equitoxic test concentrations. As standard reference material, Dolt-2 (for Zn and Cd), bovine liver (for Cu) and olive leaves (for Pb; certified by the Community Bureau of Reference, Brussels, Belgium) were used. Recoveries were 86% to 94% for Cu, 93% to 94% for Zn, 85% to 92% for Cd, and 88% to 129% for Pb. Detection limits for graphite furnace AAS analysis of the springtails were 13.5, 111, 0.98, and 10.3 nmol L^{-1} for Cu, Zn, Cd, and Pb, respectively.

Calculations and statistics

Langmuir isotherms were used to describe the sorption of the metals to the test soil and to determine potential interactions due to competition of other metal ions present in the mixture or of H^+ ions. Maximum sorption capacity ($C_{sorbed\text{-}\max}$ (nmol g^{-1} dry soil) and Langmuir sorption constant (K_L; ml $nmol^{-1}$) were estimated by nonlinear regression on log-transformed sorbed and water-extractable metal concentrations (Systat 7.0, SPSS-inc.). Controls and single treatments with the other metals were excluded because extractable concentrations were below detection limits. The significance of additional parameters was quantified using likelihood ratio statistics (X^2). Forward selection of the parameters (with threshold value $p[X^2]$ = 0.05) was used to identify competing cations.

If applicable, to assess single metal toxicity, LC50 or EC50 values were estimated by fitting three-parameter concentration-response curves to the survival, growth, and reproduction data in IBM SPSS statistics version 29. Statistical significance of differences between EC50s for unleached and leached soil was determined using likelihood ratio statistics (X^2).

For every metal pool, mixture effects on the survival, growth and reproduction of *F. candida* were analyzed against the concentration addition (CA) model, applying the computational framework of Jonker et al. (2005). This framework enables the quantification of four distinct deviations from the CA reference model: (1) no deviation, (2) synergism/antagonism in all mixture combinations, (3) dose ratio-dependent deviation (DR), where the deviation pattern (synergism/antagonism) depends on the relative contribution of the metals in the mixture, and (4) dose level-dependent deviation (DL), where synergism/antagonism depends on the effect level. The synergistic/antagonistic model extends the CA model with one additional parameter. The DL and DR models have two or at least two additional parameters compared with the CA model and cannot be statistically compared. For the DR model, the influence of the relative amount of each metal in the mixture on the deviation pattern can be evaluated compared with the other metals. The deviation function can be expanded with extra interaction parameters for a second, third, or fourth metal. Forward selection of the parameters (with threshold value $p[X^2]$ = 0.05) was used to identify the metals explaining most of the deviation. For the biological interpretation of the additional deviation parameters, here arbitrarily named *a* and *b*, see Jonker et al. (2005). A positive value of parameter *a* indicates antagonism, a negative value synergism. The value of parameter *b* indicates dose level-dependent deviations (b_{DL}) or dose ratio-dependent deviations (b_{DR}). All analyses were run in MATLAB 6.1 (The MathWorks, Natick, MA, USA) using the Nelder-Mead Simplex Method.

Results

Soil pH, metal, and chloride concentrations

Control soil pH-$CaCl_2$ and pH-H_2O were 4.9 and 5.5, respectively. Spiking with Zn or Cd did not affect pH-$CaCl_2$, whereas Cu and Pb caused a concentration-related decrease of up to 0.6 pH units (Figure 1). All metals affected pH-H_2O negatively, ranging in order as Cd $<$ Zn$<$Pb $<$ Cu (maximum decrease in pH of 0.2, 0.5, 1, and 1.2 units, respectively). For the equitoxic mixture, both soil pH-$CaCl_2$ and pH-H_2O dropped to 3.9 at the highest dose. For the other metal ratios, soil pH-H_2O and pH-$CaCl_2$ pH differed by less than 0.7 or 0.2 units, respectively, between the different treatments. Leaching had a marginal effect on the soil pH (Figure 1).

Measured total metal concentrations in soil agreed with nominal ones. Mean recoveries of added metal concentrations (±*SD*; *n* = 54) were 91.2 ± 7.05% for Cu, 92.5 ± 5.72% for Zn, 92.4 ± 4.79% for Cd, and 93.6 ± 3.81% for Pb (see online supplementary material Table S3). On leaching, the maximum total metal loss was 28% Cu, 63% Zn, 71% Cd, and 14% Pb at the highest equitoxic mixture concentration. Leaching losses were lower for the single metals with 11% Cu, 18% Zn, 8% Cd, and 14% Pb at the highest concentration. Leaching losses at the other mixture ratios were even lower than for the single chemicals and on average across all test concentrations of 1% Cu, 9% Zn, 14% Cd, and 2% Pb. All data in this article are expressed based on the measured concentrations reported in online supplementary material Table S3.

Chloride concentrations in the water extracts increased with treatment concentration, ranging from 1.86 mg Cl L^{-1} in the controls to 1.07 g Cl L^{-1} at the highest mixture dose (see online supplementary material Table S4). Leaching reduced chloride concentrations on average by 78% (Figure 2). Chloride concentrations in the water extracts from the leached soil still showed a dose-related increase from 0.597 mg Cl L^{-1} in the controls to 406 mg Cl L^{-1} at the highest mixture dose (see online supplementary material Table S4).

Soil-solution metal distributions

For all metals, water- and $CaCl_2$-extractable concentrations increased with the total soil concentration (Figure 3; online supplementary material Figure S1), both before and after leaching. At high doses, extractable concentrations of the metals were higher in the mixtures than in the single treatments. This effect was strongest for Cd and Zn, whereas for Cu and Pb, this pattern was only seen at very high concentrations. Table 1 shows Langmuir sorption parameters (K_L and $C_{sorbed\text{-}max}$) for all metals related to water-extractable concentrations before and after leaching. The sorption of Pb was not affected by the presence of other metals. For Cu, this was also the case before leaching, but in leached soil, Cu sorption was affected by Pb. For Zn, the fit of the sorption isotherms improved when Cu, Cd, Pb, or H^+ concentrations were included in the model (generalized likelihood ratio test, $p < .05$). For Cd, incorporating all metals in the sorption model gave the best fit of the Langmuir isotherm, both before and after leaching.

Metal uptake

Background (mean ± *SD*; *n* = 40) Cu, Zn, Cd, and Pb concentrations in the surviving springtails were 183 ± 100, 1,352 ± 273, 5.01 ± 1.62, and 19.7 ± 9.53 nmol g^{-1} dry body weight, respectively. Copper and Cd concentrations in the surviving Collembola increased with increasing total soil concentrations, whereas Zn concentrations appeared to be regulated at the lower soil concentrations. *Folsomia candida* seemed capable of regulating its internal Zn concentration between 1.4 and 2.5 µmol Zn g^{-1} dry body weight. At equitoxic concentrations, uptake of Cd and Pb was slightly higher in the presence of the other metals when based on total metal concentrations (compare single and equitoxic in online supplementary material Figures S2 and S3), but when related to water-extractable concentrations, Cd uptake in the mixtures was lower than in the single metal exposures. Tissue Cd concentrations in the highest single and equitoxic metal concentrations and Pb in the highest equitoxic concentrations did not increase with exposure concentrations. Leaching had a minimal effect on the

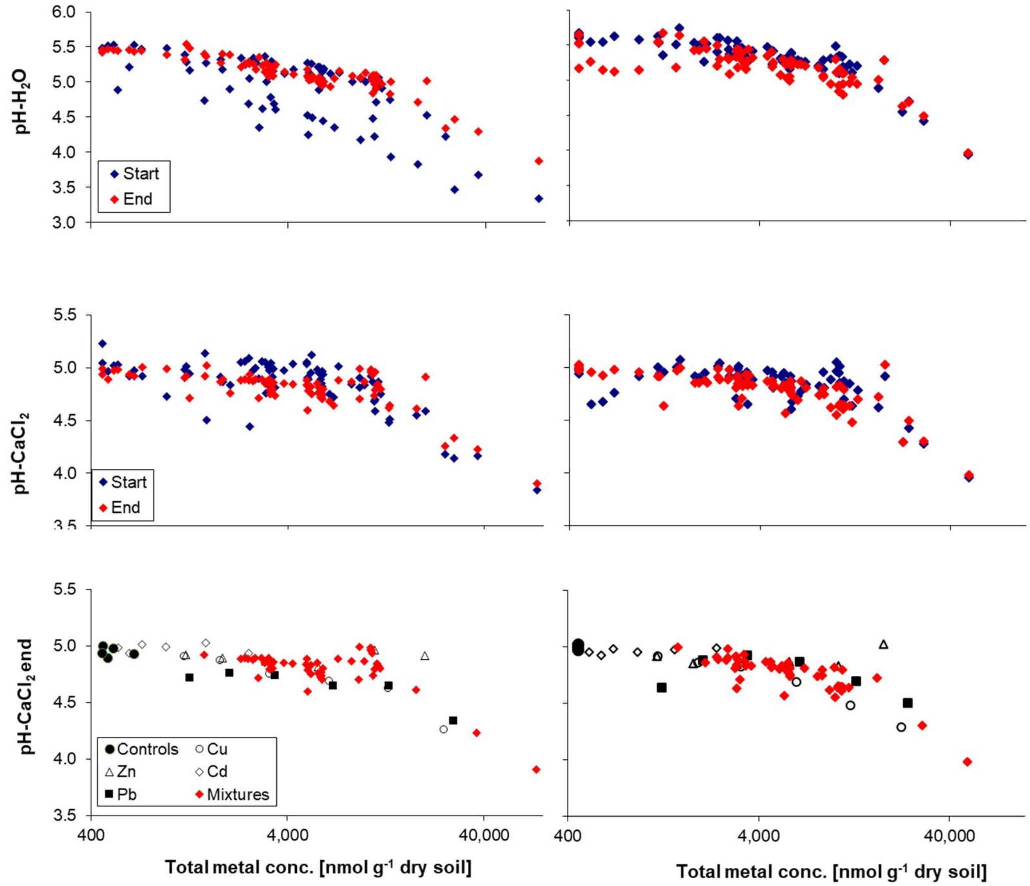

Figure 1 Effect of the addition of Cd-Cu-Pb-Zn mixtures on the pH-H_2O (top) and pH-$CaCl_2$ (middle) of LUFA 2.2 soil at the start and end, and of the metal mixtures and the individual metals (bottom) at the end of 28-day toxicity mixture toxicity tests with *Folsomia candida*. Metal concentrations are given as total mixture concentrations in nmol g^{-1} dry soil. Left graphs show the pH values for the unleached soil, right graphs for the soil leached with two pore-volumes of water to remove the chloride counterion.

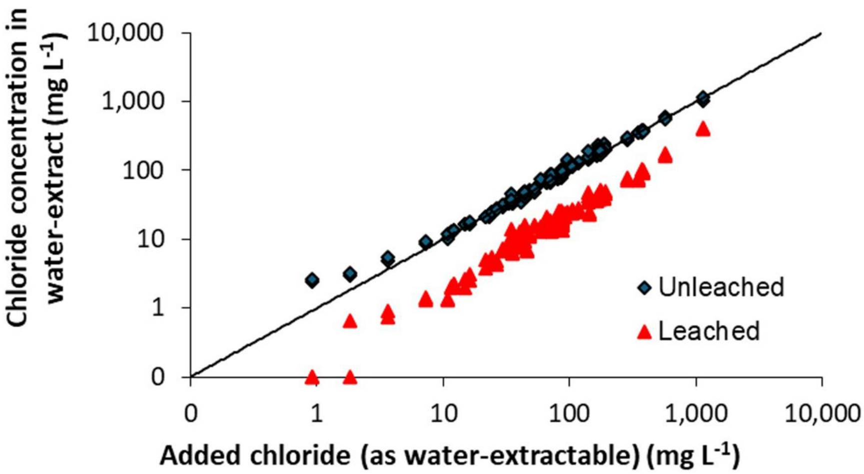

Figure 2 Water-extractable chloride concentrations in LUFA 2.2 soil spiked with chloride salts of copper, zinc, cadmium, and lead, single and in mixtures, with and without leaching treatment. The soil was leached with two pore-volumes of water. Measured water-extractable chloride concentrations are related to the maximum possible water-extractable chloride concentrations calculated from the nominal total chloride concentrations reached by spiking the soil with the metal chloride salts. The solid line represents the 1:1 line.

internal concentrations of Cu, Zn, and Cd. For Pb, uptake from the leached soil increased with increasing soil concentrations while plateauing at the highest concentrations in the unleached soil (Figure 4).

Toxicity

In the controls of the experiments with unleached and leached soil, survival was 87% ± 11% and 91% ± 14% (±*SD*; $n = 20$), mass

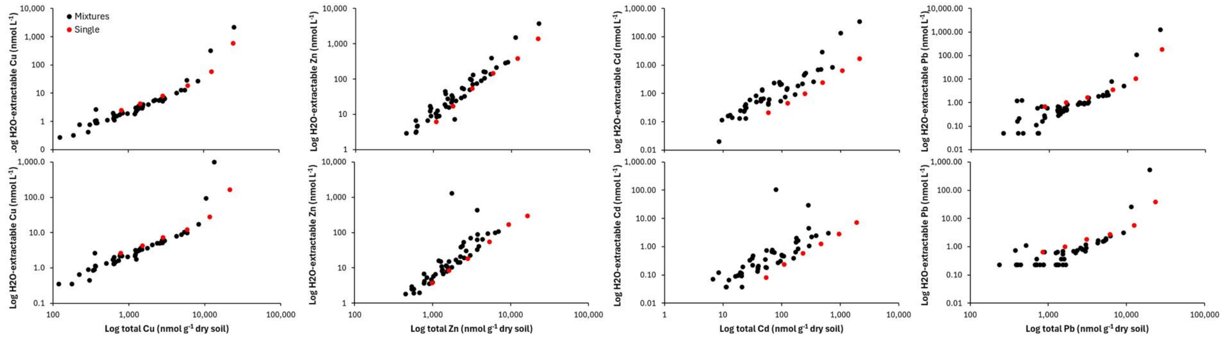

Figure 3 Water-extractable concentrations of copper, zinc, cadmium, and lead (from left to right) plotted against their total concentrations in LUFA 2.2 soil spiked with the chloride salts of the single metal or in mixtures with the other metals, before (top) and after (bottom) leaching with two pore-volumes of water to remove the chloride counterion. See Table S1 for the mixture composition. Controls are not included.

Table 1 Langmuir sorption parameters K_L (ml nmol^{-1}) and $C_{sorbed\text{-}max}$ (nmol g^{-1}) estimated for the sorption of copper, zinc, cadmium, and lead in the natural standard LUFA 2.2 soil spiked with $CuCl_2$, $ZnCl_2$, $CdCl_2$, and $PbCl_2$ in different ratios, and extracted with deionized water before (unleached) or after leaching with two pore-volumes of water to remove the chloride counterion.

Metal	Unleached				Leached			
	Significant interaction term	Log $C_{sorbed\text{-}max}$	Log K_L	SS	Significant interaction term	Log $C_{sorbed\text{-}max}$	Log K_L	SS
Cu	—*	4.243	−1.583	0.633	—	4.350	−1.693	0.849
					Pb*	4.587	−1.933	0.772
Zn	—	3.697	−1.610	1.388	—	3.725	−1.355	1.162
	Cu	3.766	−1.628	1.122	Cu	3.851	−1.400	0.637
	Cd	3.768	−1.647	1.186	Cd	3.838	−1.443	0.877
	H^+*	3.806	−0.586	1.083	Pb	3.850	−1.456	0.745
					pH	3.855	−1.016	0.840
					Cu + Pb	3.880	−1.400	0.534
					Cu + H^+	3.924	−1.229	0.534
					Cu + Pb + H^+*	3.940	−1.246	0.454
Cd	—	2.768	−0.758	4.059	—	2.701	−0.329	4.129
	Cu	3.521	−1.437	3.090	Cu	3.714	−1.259	2.813
	Zn	3.304	−1.127	2.165	Zn	3.324	−0.768	2.129
	Pb	3.398	−1.281	2.962	Pb	3.908	−1.431	2.169
	Cu + Zn	3.630	−1.399	1.704	H^+	2.900	0.252	3.309
	Cu + Zn + Pb*	3.658	−1.372	1.537	Zn + Pb	3.811	−1.209	1.252
	H^+	3.005	3.984	2.064	Zn + Pb + Cu*	3.770	−1.101	1.114
Pb	—*	4.103	−0.572	3.810	—*	4.318	−0.834	2.817

Note. Parameter values for the Langmuir sorption isotherm model are given according to forward selection of significant interaction terms. The sum of squared residuals (SS) is given for each parameter set. The most parsimonious model is indicated with an *. Isotherms for each metal were only fitted to data for soil to which this metal was added, so not including controls or the single treatments with the other metals. For the unleached soil, H^+ was only included as a separate interaction term with the metal considered, for the leached soil it also in interaction with the other metals in the mixture.

gain after 4 weeks was 265 ± 38.0 and 340 ± 45.1 µg ($n = 40$), the average number of juveniles produced per test jar 1,450 ± 215 and 1,913 ± 305 ($n = 20$), and coefficient of variation (CV) of juvenile numbers was 14.8% and 16.0%, respectively (see online supplementary material Figures S4 and S5 for unleached and leached soils, respectively). Thus, the validity criteria (> 80% survival, > 100 juveniles/test jar, CV < 30%) for the ISO (1999) Collembola test were all met.

Copper or Zn did not affect springtail survival at the concentrations tested. Lead affected survival only at the highest single Pb concentration (60% survival) in the leached soil but not in the unleached soil. In the unleached soil, Cd affected survival in a concentration-related manner for the lower four test concentrations. However, at the highest two test concentrations, survival was higher than at lower concentrations (46% and 46% compared with 38%). Therefore, it was not possible to calculate an LC50 for Cd due to this non-monotonic response pattern. In the leached soil, single Cd exposure reduced survival in a concentration-related manner to 14% at the highest concentration. LC50s (with corresponding 95%

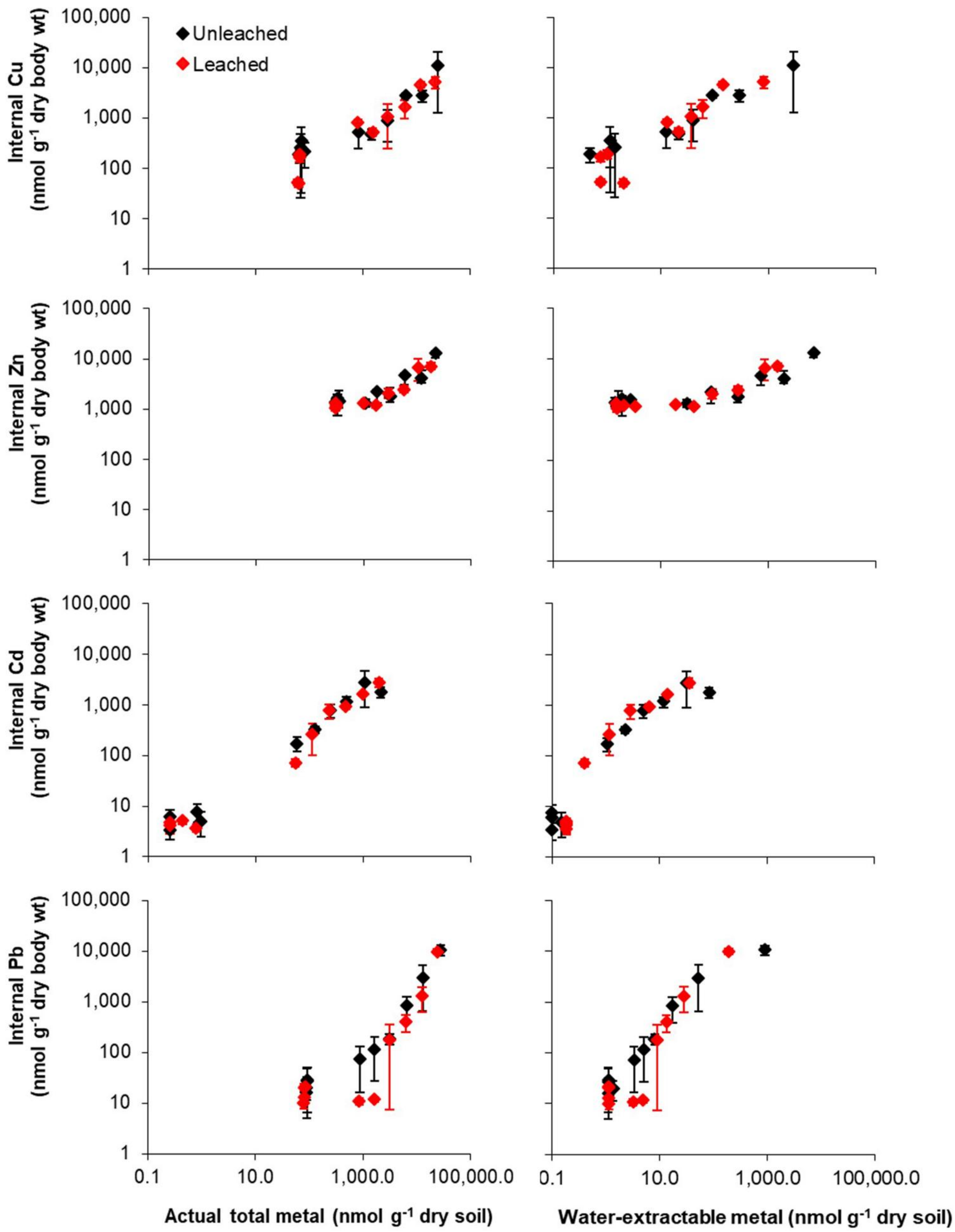

Figure 4 Concentrations of cadmium, copper, lead, and zinc in *Folsomia candida* (in nmol g^{-1} dry body wt) after 4 weeks exposure in LUFA 2.2 soil related to total (left) and water-extractable (right) soil concentrations. The soil was spiked with the chloride salts of the metals, and either used directly to expose the springtails or after leaching with two pore-volumes of water to remove the chloride counterion. Each point is the mean of 4 or 5 animals, and vertical lines indicate standard deviations. See online supplementary material Figures S2 and S3 for the metal uptake patterns following exposure to the single metals and/or the mixtures, for unleached and leached soils, respectively.

confidence interval) for single Cd toxicity were 414 (314–515), 94.7 (69.6–120), and 5.43 (3.93–6.93) nmol g^{-1} dry soil when based on total, $CaCl_2$-, and water-extractable metal pools, respectively.

In the mixture treatments, survival never dropped below 60% (at the highest equitoxic concentration). At the two highest equitoxic exposure concentrations, the springtails were observed climbing the walls of the test jars, and there was no noticeable food consumption, indicating that the organisms sought to avoid exposure to the elevated levels of metals. Survival was close to 100% in all other treatments. For the other mixture ratios, survival was only affected in the Cu:Zn:Cd:Pb ratios of 10:10:70:10 and 10:10:10:70 mixtures in the leached soil, but not in a consistent concentration-related manner. Statistical analyses confirmed the antagonistic effect of Cu, Zn, and Pb on Cd toxicity for *F. candida* survival ($p < 0.001$) when based on total and $CaCl_2$-exchangeable metal concentrations.

Growth and reproduction of *F. candida* were concentration-related reduced by all four metals and the mixtures (see online supplementary material Figures S4 and S5 for unleached and leached soil, respectively). After leaching, EC50s for effects of the single metals on springtail growth and reproduction based on total soil concentrations were higher, indicating reduced toxicity for all metals except Cd (see online supplementary material Table S5). In the cases of Pb for growth and Cu and Zn for reproduction, the EC50s differed significantly between the unleached and leached soil ($X^2_{df=1}$>3.84; $p < 0.05$). Figure 5 compares the effects of the mixtures with those of the single metals. Expressing the exposures on a TU basis suggests an overall antagonistic effect. The mixture toxicity results for growth and reproduction are summarized in Table 2 for the unleached soil and in Table 3 for the leached soil. Conclusions on the overall mixture toxicity interactions are given for each metal pool based on the parameter estimates of the most parsimonious model. For full details of the analysis, see online supplementary material Tables S6–S9.

For growth, in both soils, adding one additional deviation parameter to describe antagonism or synergism resulted in a significant improvement in data description, irrespective of the metal pool used (see online supplementary material Tables S6 and S8). The estimated value of *a* was always positive, indicating antagonism as the exposure metric (Tables 2 and 3). For the total metal pool in both soils, the $CaCl_2$-exchangeable metal pool in the unleached soil, and the water-extractable metal pool in the leached soil, the addition of a second parameter allowing for further dose level-dependent deviations and significantly improved the description of the growth data. The additional parameters in the DL model (*a* = 567, 413, 208, 130 and *b* = 0.421, 0.260, 0.196, –0.240) indicated antagonism at low effect levels and synergism at high effect levels, with the switch from antagonism to synergism occurring at concentrations of 1/*b* (i.e., between 2.4–5.1 × EC50). For the water-extractable metal fraction in the unleached soil, the $CaCl_2$-exchangeable fraction in the leached soil, and the internal metal pool in the animals, no significant dose level-dependent deviations were found. Adding an extra parameter, which allows for a dose ratio-dependent deviation to the synergism/antagonism model, significantly improved the description of the data for each metal pool; see online supplementary material Table S6 and S8 for the parameter estimates for the most parsimonious dose ratio-dependent deviation model. The relative concentration of Cd was the most influential factor, with a decrease in toxicity observed when the contribution of Cd to the mixture was higher.

For reproduction, in both soils, adding one additional deviation parameter to describe antagonism or synergism significantly improved data description in all areas, irrespective of the metal pool used (see online supplementary material Tables S7 and S9). The estimated value of *a* was always positive, indicating antagonism (Tables 2 and 3). For the total and $CaCl_2$-exchangeable metal pool in the unleached soil, adding a second parameter allowing for dose level-dependent deviation further significantly improved the description of the reproduction data. The additional parameters in

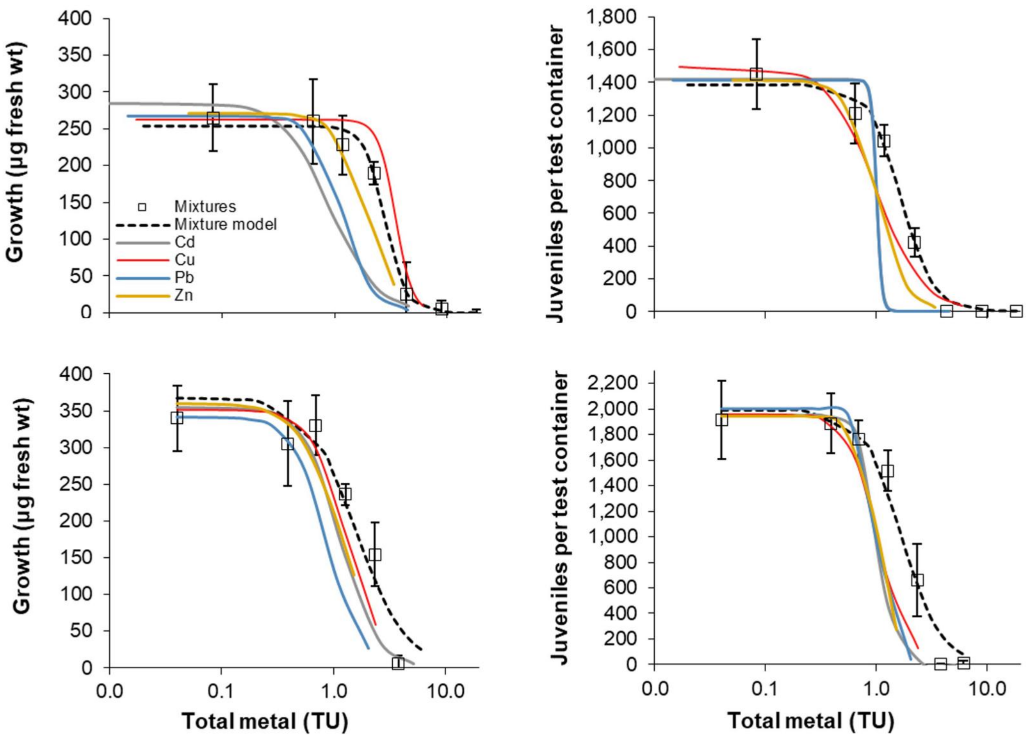

Figure 5 Effects of copper, zinc, cadmium, and lead and their mixtures on the growth (left) and reproduction (right) of *Folsomia candida* after 4 weeks exposure in LUFA 2.2 soil spiked with chloride salts of the metals. Total soil concentrations are given, expressed as toxic units (TU) calculated using the EC50s for reproduction for the single metals obtained from this study. Top graphs show the effects of the metals in unleached soil, the bottom graphs in the soil leached with two pore-volumes of water to remove the chloride counterion. The dashed lines show the dose-response curves for all mixtures; data points are only given for the equitoxic mixtures. Each data point is the mean of 10 animals (growth) or five test jars (reproduction), and vertical lines indicate *SD*s. Lines for the single metals are modelled dose-response curves; see online supplementary material Figures S4 and S5 for the full data with corresponding variations and Table S5 for the EC50 values derived from the dose-response curves for the individual metals.

Table 2 Summary of parameter estimates and statistics of the mixture model analyses of the copper-zinc-cadmium-lead effect on the growth and reproduction of *Folsomia candida* exposed in LUFA 2.2 soil, based on total, $CaCl_2$-exchangeable and water-extractable concentrations in the soil and internal concentrations in the springtails.

		Unleached soil: Exposure concentration based on			
		Total	**$CaCl_2$-exchangeable**	**Water-extractable**	**Internal**
Growth					
EC50	**Cu**	1,270	763	383	4,850
	Zn	1,280	5,770	2,440	6,810
	Cd	696	214	32.0	3,010
	Pb	9,670	106	23.1	1,650
S/A (*p*)		<0.001 (*a* = 152)	<0.001 (*a* = 242)	<0.001 (*a* = 329)	<0.001 (*a* = 129)
DR (*p*)		0.0270 (*a* = 89.8; *b* = 232^{Cd})	0.00538 (*a* = 16.4; *b* = 618^{Cd})	<0.001 (*a* = −189; *b1* = 2,390^{Cd}; *b2*=-1,370^{Cu})	<0.001 (*a* = −15.4; *b* = 665^{Pb})
DL (*p*)		<0.001 (*a* = 567; *b* = 0.421)	0.00950 (*a* = 413; *b* = 0.260)	0.514	0.483
Conclusion		DL: low effect level → antagonism, high effect level → synergism; switch at 1/b = 2.4 x EC50	DR: high Cd → decreased toxicity DL: low effect level → antagonism, high effect level → synergism; switch at 1/b = 3.8 x EC50	DR: high Cd → decreased toxicity, high Cu → increased toxicity	DR: high Pb → decreased toxicity
Reproduction					
EC50	**Cu**	5,430	109	89.0	2,720
	Zn	7,030	2,530	994	3,690
	Cd	543	165	22.9	$1.01*10^6$
	Pb	6,670	56.5	17.6	1,300
S/A (*p*)		<0.001 (*a* = 179)	<0.001 (*a* = 166)	<0.001 (*a* = 126)	<0.001 (*a* = 110)
DR (*p*)		0.124	0.168	<0.001 (*a* = −19.1; *b* = 657^{Cd})	0.0057 (*a* = 431; *b*=−4,380^{Cd})
DL (*p*)		<0.001 (*a* = 429; *b* = 0.315)	0.00485 (*a* = 480; *b* = 0.351)	0.379	0.360
Conclusion		DL: low effect level → antagonism, high effect level → synergism; switch at 1/*b* = 3.2 × EC50	DL: low effect level → antagonism, high effect level → synergism; switch at 1/*b* = 2.8 × EC50	DR: high Cd → decreased toxicity	DR: high Cd → increased toxicity

Note. Values are given for calculations based on the concentration addition model (CA), on the model extended with a deviation parameter (*a*) for synergism/antagonism (S/A) or with an additional deviation parameter (*b*) for dose ratio-dependent (DR) or dose level-dependent deviations (DL). In the case of DR deviation, more than one additional parameter (b_1, b_2, etc.) could be introduced into the model if more than one metal ratio appeared to be significantly influencing the mixture toxicity. The most parsimonious DR model is shown. Next to the value for *b*, the metal to which *b* is related is given. The EC50s are given in nmol g^{-1} dry soil or in nmol g^{-1} dry body weight, based on the best fitting model. See Jonker et al. (2005) for the interpretation of the parameters *a* and *b*. For significance levels note that S/A is compared with CA, and both DR and DL are compared with S/A, unless S/A was not significantly different from CA. See Tables S6 and S7 for the full results of the mixture toxicity analysis for the growth and reproduction effects, respectively.

the DL model (*a* = 429, 480 and *b* = 0.315, 0.351) indicated antagonism at low effect levels and synergism at high effect levels, switching from antagonism to synergism at concentrations of 1/*b* (i.e., between 2.9–3.2 × EC50). No significant DL deviations were found for the water-extractable metal pool in the soil and the internal metal pool. Adding an extra parameter for DR deviation significantly improved the data description for the water-extractable and internal metal pools but not for the total and $CaCl_2$-exchangeable pools. The parameter estimates for the most parsimonious DR model are given in Table 2 for each metal pool. For the leached soil, the addition of the parameter for dose ratio-dependent deviation further significantly improved the model fit for all metal pools (Table 3). The relative concentration of Cd was the most important factor, with decreased toxicity at a high contribution from Cd to the mixture effect and smaller concentration of high Pb or Zn concentrations. Adding an extra parameter allowing for dose level-dependent deviation did not significantly improve the model fit for any metal pool.

Discussion

This study assessed the toxicity of a complex mixture of four metals, added as chloride salts, to the growth and reproduction of the springtail *F. candida* in a natural soil, before and after

Table 3 Summary of parameter estimates and statistics of the model analyzing the copper-zinc-cadmium-lead mixture effect on the growth and reproduction of *Folsomia candida* exposed in LUFA 2.2 soil spiked with the metals as chloride salts and leached with two pore-volumes water to remove the chloride counterion, based on total, $CaCl_2$-, and water-extractable concentrations in the soil.

		Leached soil: Exposure concentration based on		
		Total	$CaCl_2$-exchangeable	Water-extractable
Growth				
EC50	**Cu**	1,290	590	173
	Zn	1,540	5,350	1,230
	Cd	532	159	12.0
	Pb	1,100	161	22.2
S/A (*p*)		<0.001 (a = 141)	<0.001 (a = 173)	<0.001 (a = 165)
DR (*p*)		<0.001 (a = −16.4; $b = 592^{Cd}$)	<0.001 (a = −442; $b1 = 1{,}320^{Cd}$; $b2 = 629^{Zn}$)	<0.001 (a = −296; $b1 = 1{,}610^{Cd}$;
DL (*p*)		0.0223 (a = 208; b = 0.196)	0.555	0.0410 (a = 130, b = −0.240)
Conclusion		DR: high Cd → decreased toxicity DL: low effect level → antagonism, high effect level → synergism; switch at 1/b = 5.1 x EC50	DR: high Cd → decreased toxicity, high Zn → decreased toxicity	DR: high Cd → decreased toxicity DL: antagonism but magnitude dependent on effect level
Reproduction				
EC50	**Cu**	10,400	335	119
	Zn	13,100	4,360	1,100
	Cd	518	167	12.0
	Pb	13,000	238	29.0
S/A (*p*)		<0.001 (a = 151)	<0.001 (a = 130)	<0.001 (a = 209)
DR (*p*)		<0.001 (a = −83.6; $b1 = 766^{Cd}$; $b2 = 137^{Pb}$)	<0.001 (a = −553; $b1 = 1{,}510^{Cd}$; $b2 = 781^{Zn}$)	<0.001 (a = −325; $b = 1{,}710^{Cd}$)
DL (*p*)		0.253	0.351	0.351
Conclusion		DR: high Cd → decreased toxicity, high Pb → decreased toxicity	DR: high Cd → decreased toxicity, high Zn → decreased toxicity	DR: high Cd → decreased toxicity

Note. Values are given for calculations based on the concentration addition model (CA), on the model extended with a deviation parameter (a) for synergism/antagonism (S/A), or with an additional deviation parameter (b) for dose ratio-dependent deviation (DR) or dose level-dependent deviation (DL). In the case of dose ratio-dependent deviation, more than one additional parameter (b_1, b_2, etc.) could be introduced into the model if more than one metal ratio appeared to be significantly influencing the mixture toxicity. The most parsimonious DR model is shown. Next to the value for b, the metal to which b is related is given. The EC50s are given in nmol g^{-1} dry soil or in nmol g^{-1} dry body weight. See Jonker et al. (2005) for the interpretation of parameters a and b in each column. For significance levels note that S/A is compared with CA, and both DR and DL are compared with S/A, unless S/A is not significantly different from CA, in which case they are compared with CA. Full results of the mixture toxicity analysis for growth and reproduction effects are shown in Tables S8 and S9.

leaching, to remove the chloride counterion. The metals affected each other's sorption to the soil, leading to a higher availability, especially of Cd and, to a lesser extent, Zn, when high concentrations of the other metals were present. This effect, however, did not result in higher metal uptake in mixtures when related to available concentrations. Removal of the chloride by leaching did not affect metal uptake in the springtail, although it did reduce toxicity for all metals except for Cd. In the experiments conducted before and after leaching, the main pattern of joint mixture effect was predominantly antagonistic, with dose-ratio and dose-level dependent deviations from concentration addition also seen when exposure was assessed based on some metal pools.

Soil solution distribution

Leaching of the spiked soil successfully reduced the soil chloride concentrations (by 78% on average) without excessive metal loss (limited to between 1%–14%). Although largely successful in reducing soil chloride levels, the leaching efficiency (i.e., proportion of total added chloride removed) decreased with increasing exposure concentrations (Figure 2). This may be related to the formation of metal-chloride precipitates, especially for Pb, which may at least partly have remained undissolved during leaching, especially at high concentrations of this metal.

Metal losses following leaching were lower in this experiment than those observed in other studies (see e.g., Smit & Van

Gestel, 1998; Renaud et al., 2020b). This pattern may be explained by the use of different soils and the lack of an equilibration period before leaching. The losses of Cu, Zn, and Cd were all generally higher at the highest concentrations in the mixtures than in the single metal treatments. The loss of Pb was not affected or even reduced by the presence of other metals, probably due to its complexation with the additional added chloride reducing its availability for leaching.

Compared with the unleached soils, the Langmuir isotherms for leached soils had slightly higher log $C_{sorbed\text{-max}}$ for all metals, whereas log K_L was lower for Cu, Zn, and Pb and higher for Cd (Table 1). The higher log $C_{sorbed\text{-max}}$ after leaching can be explained by the reduced presence of metals as complexes with chloride ions, resulting in greater metal binding to the soil and lower extractable metal concentrations.

The soil-solution distribution of Cd was affected by all other metals in soils both with and without leaching. For Zn, soil solution concentrations were influenced by pH shifts with minimal effects of other metal addition. For Cu and Pb, the effect of the other metals was also minimal (Table 1). Jonker et al. (2004), testing binary mixtures, also found that the soil-solution distribution of Cu and Pb was hardly affected by the less strong binding metals Cd and Zn. In the mixtures, Cd and Zn were found in relatively higher concentrations in water and $CaCl_2$ extracts, than the single metal treatment (Figure 3).

Uptake of Cu, Zn, Cd, and Pb

Copper, Cd, and Pb body concentrations in *F. candida* compared with the single metal generally increased with soil metal concentrations in both the unleached and leached soil (Figure 4), with the exception of Zn at lower concentrations, where internal concentration was initially regulated. In the equitoxic mixture, there was a clear decrease in body concentrations compared with the lower treatments at higher equitoxic mixture exposure concentrations of Cd and Pb. This effect pattern was probably due to the avoidance behavior of the springtails seen at the highest, but not the lowest, exposure concentrations. Dai et al. (2018) reported a significant avoidance of *F. candida* of soil spiked with 7,240 nmol Pb g^{-1} or 444 nmol Cd g^{-1} (and onwards), consistent with the avoidance pattern in our experiments. For Pb, the lower uptake may also be further linked to the low water-solubility of the chloride salt. Given the maximum water solubility of $PbCl_2$ of 35.6 mM, at the highest equitoxic concentration without leaching (25.2 mM Pb + 603 mM Cl), it is likely that part of the added Pb precipitated as $PbCl_2$, thereby reducing its availability. After leaching, the estimated pore-water concentrations at the highest equitoxic concentration dropped to 10.6 mM Pb and 229 mM Cl, reducing the extent of any precipitation. This resulted in a lower reduction in availability consistent with the high accumulation found for springtails at the highest Pb levels. For Cd, breakpoints in bioaccumulation occurred at water-extractable chloride concentrations 8.15–15.7 mM Cl in the unleached soil and 4.77–11.5 mM Cl in the leached soil. The similarity of these values suggests a change in Cd bioavailability at the prevailing water-extractable chloride levels that ultimately affected uptake. Cadmium, however, forms chloride complexes not available for uptake (Van Gestel & Koolhaas, 2004), which, especially in the unleached soil, could explain the limitations to Cd uptake.

In the equitoxic mixture exposures, internal Zn and Cd concentrations were elevated compared with the single metal exposure based on total but not water-extractable soil concentrations (see online supplementary material Figures S4 and S5). This pattern was not seen for Cu or Pb, most likely due to their stronger sorption and consequently lower bioavailability. The Langmuir sorption isotherms showed increased water-extractable Zn and Cd concentrations in the presence of other metals, explaining the higher internal metal concentrations in mixture exposures. The magnitude of the internal concentration increase was, however, always below that of the water-extractable metal concentrations. This suggests that the additional water-extractable metal concentrations were either not completely bioavailable to *F. candida* or, more likely, that the increased extractable metal was counteracted by increased competition with H^+ or other metal cations consistent with the biotic ligand model theory (Ardestani et al., 2015; Gong et al., 2022; Qiu et al., 2015).

Similar patterns of bioaccumulation were found in both leached and unleached soils (Figure 4), indicating that leaching had only a minor effect on the extent of accumulation for all metals. The general similarity is consistent with the results of previous studies (e.g., for Zn see Smit & Van Gestel, 1998).

Toxicity of single metals

The EC50s for the effects of the single metals on springtail growth and reproduction based on total metal concentrations in both soils (see online supplementary material Table S5) are similar to reported literature values (see e.g., Bongers et al., 2004; Bruus Pedersen et al., 2000; Bur et al., 2010; Dai et al., 2020; Smit & Van Gestel, 1996, 1998; Van Gestel & Hensbergen, 1997; Van Gestel & Koolhaas, 2004). Based on total metal concentrations, the EC50s for Pb effects on growth and for Cu, Zn, and Pb effects on reproduction were higher in leached than in unleached soil but similar for Cd (see online supplementary material Table S5). The general reduction in toxicity by leaching could be related to the alleviation of additional osmotic stress. *Folsomia candida* had a lower fecundity at salt levels exceeding 9 g NaCl L^{-1}, comparable with 2.6 g Cl L^{-1}, with a strong reduction in egg hatching rates, explained from egg desiccation at high salinity (Hutson, 1978). Assuming all chloride measured in water extracts is fully derived from the pore water, at a soil moisture content of 25% (w/w), a chloride concentration in the pore water of 2.6 g Cl L^{-1} compares with 130 mg Cl L^{-1} (=3.67 mM) in the extract. For the higher single and mixture test concentrations of Cu, Zn, and/or Pb, this level was reached in the unleached soil, suggesting a contribution of chloride to the observed effects. After leaching, chloride concentrations exceeding 130 mg Cl L^{-1} in the extract were found only for the two highest equitoxic mixture concentrations.

Mixture effects

Although both single Cd and Pb showed a negative effect on the survival of *F. candida*, very limited mortality was observed in the mixtures, suggesting antagonism. Antagonism was also found for growth and reproduction for Cd and Pb for the mixture analyses conducted based on all metal pools in both the leached and unleached soil. This dominantly antagonistic effect is consistent with previous published results, including for mixtures of Cu, Zn, Cd, and Pb on the reproduction of the potworm *Enchytraeus albidus* (Lock and Janssen (2002), complex metal mixtures in the earthworm *Eisenia fetida* (Spurgeon & Hopkin, 1995), and for 90% of metal mixtures tested in *Oppia nitens* (Jegede et al., 2020). This similar finding suggests antagonism, as the most common response pattern for metal mixtures in aquatic bioassays was also found by Vijver et al. (2011). The study of Renaud et al. (2020b), however, reported that *F. candida* responses to the mixtures of Pb, Cu, Ni, Zn, and Co followed additivity, indicating that antagonism is not always the dominant mixture response in all cases.

Dose ratio-dependent deviations were detected for effects of metals on growth in models based on all measured pools. For reproduction, this was also the case for water-extractable and internal metal pools in the unleached soil and for all metal pools in leached soil. Detailed assessment indicated a reduction in toxicity in the complex mixtures with increasing relative Cd concentrations. This dominant contribution of Cd to dose ratio-dependent deviations can be explained by its effects on metal sorption in the mixtures. Langmuir isotherms showed that the sorption of Cd was most affected by the presence of other metals. Thus, the same toxic effect was induced by relatively higher concentrations when related to extractable compared with total concentrations. Because internal Cd concentrations were unrelated to extractable concentrations, the expected effects based on available Cd concentrations are overestimated, suggesting decreasing toxicity at higher Cd concentrations. Because this decreasing toxicity at relatively higher Cd concentrations was also found for the total metal pool, there appears to be a real antagonistic effect of Cd on metal mixture toxicity. This is supported by the negative estimates for parameter *a* in the DR-dependent models (Tables 2 and 3), which implied synergism between the metals not included in the *b* parameter(s), namely, Zn and Cu or Pb. This suggests that adding Cd (or Pb) to the mixture reduced its toxicity. Bur et al. (2012) found that a mixture of Cd and Pb had a lower than expected toxicity, suggesting antagonism. Zinc and Cd have also been reported to act in an antagonistic manner when exposed as a mixture (Jegede et al., 2020; Renaud et al., 2020b; Svendsen et al., 2026). An explanation for this observed antagonism could be that the presence of Cd induces the production of metallothionein as a protective metal binding protein (Swart et al., 2022; Nakamori et al., 2010).

The fact that Cd-dominated dose ratio-dependent interactions were conserved across the leached and unleached soils suggests that the antagonistic effect is independent of the presence of chloride. More likely, it can be attributed to interactions between Cd and the other metals at the level of bioavailability, toxicokinetics, or toxicodynamics (Spurgeon et al., 2010, Van Gestel et al., 2010).

Dose level-dependent deviations were found for effects on growth and reproduction when effects were calculated based on both the total and $CaCl_2$-exchangeable metal pools in the unleached soil. Parameter values indicated that the mixture was antagonistic at low effect levels and synergistic at high effect levels, with the switch from antagonism to synergism occurring between 2.4 and 3.8 times the EC50. Because only the single metal and equitoxic concentrations were tested at toxic strengths higher than 2 TU, this effect could only be seen in the equitoxic mixture. The high chloride concentrations in the high (equitoxic) mixture concentrations could probably explain this effect in treatments with salt levels exceeding the critical level of 2.6 g Cl L^{-1} (Hutson, 1978; comparable with 130 mg Cl L^{-1} in the extract) at both 2 and 4 TUs Cu, Zn, and Pb, the four higher equitoxic mixture levels and 12 of the 14 other ratio mixtures. This exceedance suggests a clear potential for a chloride counterion contribution to the observed toxicity.

Limitations of this study

Due to the workload required to undertake this extensive study, the exposure could not be started simultaneously for all replicates. Staggering the start date was required to cope with the logistical challenges of the study. The staggered start date meant that the soil for some replicates was aged a few days longer than for others. Because we aged our soils for at least 2 weeks after percolation instead of the more commonly applied 1-week ageing period (see e.g., Bongers et al., 2004; Lock et al., 2006), this difference in ageing period is unlikely to have affected the objectives of our study to understand ageing and leaching effects on metal mixture bioavailability and toxicity. This assumption is supported by the differences in growth or reproduction between replicates, which did not exceed the normal variation usually seen in springtail toxicity tests.

Metal exposure in the field will generally not take place in the presence of a significant counterion concentration. Hence, where possible inclusion of a leaching step into a metal spiking protocol is recommended to improve the relevance of mixture studies (Smolders et al., 2009) and further to use measured concentrations to account for leaching losses when modeling mixture effects (Renaud et al., 2020a). Alternatively, metal oxides could be used instead of chloride salts to obtain more realistic metal exposure conditions and limit the possible salt effects (see e.g., Renaud et al., 2020a, 2020b). However, given that few studies have yet used oxides and most soil ecotoxicity data currently available comes from studies in which soil is spiked with soluble metal salt solutions, it still remains important to understand how counterion behavior may affect the results of such bioassays.

This study used only a single soil in a controlled laboratory setting, limiting the translation of results to other soil types. One way to further progress understanding would be to include metal speciation modeling, which may allow translating results to other natural soils. Speciation modeling was beyond the scope of this study. However, such approaches may not account for the variation in exposure that will be encountered in field soils, where heterogeneity is rule rather than exception. Expanding future work to include aged soils, alternative metal sources, or more complex soil matrices would help to test the robustness and ecological relevance of the antagonistic interactions seen here.

Conclusion

In the complex mixtures, Cd and Zn, but not Cu and Pb availability, as indicated by extractable concentrations were altered in the presence of other metals. Metal uptake from soil into the exposed springtails was seemingly not affected by the presence of other metals in the mixture. Uptake was, however, decreased by the presence of high chloride concentrations resulting from its addition as a counterion in chloride salts. Leaching the spiked soil adequately removed the counterion at the expense of only small metal losses. Metal uptake in *F. candida* in the leached soils was similar to the unleached soils except at some of the highest unleached exposure concentrations, in which chloride complexes and precipitation to reduce availability occurred. Metal toxicity was reduced by leaching, explainable partly by a reduced additional salt stress. The complex metal mixture acted mainly antagonistic in both unleached and leached soils, a pattern consistent with many but not all previous reports of metal antagonisms. The presence of counterions may in some cases have affected the interactions between metals, indicating the importance of conducting prior leaching in studies of metal mixture effects. Joint effect model fitting indicated that Cd was the most important metal linked to the observed antagonistic interactions, a role that was more pronounced after leaching, indicating a stronger interactive effect of Cd in the absence of excess chloride. The role of Cd, as a strong inducer of metallothionein that may also bind other metals, may be the driver of this antagonistic interaction.

Supplementary material

Supplementary material is available at *Environmental Toxicology and Chemistry* online.

Data availability

All data are available in a Zenodo repository: 10.5281/zenodo.17011179.

Author contributions

Marina Bongers (Conceptualization, Formal analysis, Investigation, Methodology, Writing—original draft), David J. Spurgeon (Writing—review & editing), Susana Loureiro (Writing—review & editing), and Cornelis A.M. van Gestel (Conceptualization, Formal analysis, Funding acquisition, Methodology, Supervision, Writing—review & editing)

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Conflicts of interest

The authors have no relevant financial or nonfinancial interests to disclose.

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