



Toxicity of binary mixtures of cadmium with lead, copper, or zinc to *Folsomia candida* in relation to bioavailability in soil

Marina Bongers¹, Miranda Mesman^{1,‡}, Claus Svendsen², and Cornelis A.M. van Gestel^{1,*},

¹Amsterdam Institute for Life and Environment (A-LIFE), Faculty of Science, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands

²Centre for Ecology & Hydrology, Crowmarsh Gifford, Wallingford, Oxfordshire, United Kingdom

*Corresponding author: Cornelis A.M. van Gestel, Email: kees.van.gestel@vu.nl

[‡]Present address: National Institute for Public Health and the Environment (RIVM), P.O. Box 1, 3720 BA Bilthoven, The Netherlands.

Abstract

Metal pollution in soils usually involves mixtures, hampering a proper assessment of potential risks as metals may interact at different levels, including sorption to the soil, uptake by organisms, and interaction with target sites resulting in adverse effects. This study aimed at clarifying the interaction of metals on sorption to soil and uptake and effects in springtails. Single and combined toxicity of Cd with lead, copper, or zinc to *Folsomia candida* was studied in a natural LUFA 2.2 soil and related to total, 0.01 M calcium chloride-exchangeable and water-extractable metal concentrations in the soil and internal concentrations in the springtails. Cadmium availability in soil extracts significantly increased in the presence of another metal, and Cd also increased the availability of Pb and Zn. This, however, did not lead to increased Cd, Pb, or Zn concentrations in *F. candida*. Combined effects of the metals on springtail survival, growth, and reproduction were overall antagonistic, with some dose ratio-dependent deviations from the concentration addition reference models. Most likely, the complexation of Cd with the excess chloride introduced in the soil solution by the counterion of the lead(II) chloride, copper(II) chloride, and zinc(II) chloride salts used reduced its bioavailability. This shows the importance of taking into account all steps in the intoxication process to improve understanding of the effects of metal mixtures in soil.

Keywords metals, soil sorption, bioaccumulation, mixture toxicity, reproduction

Introduction

Metal pollution is a major concern for agricultural soils and human and environmental health (Hou et al., 2025). Metals are usually present in soil as mixtures, with combined high soil concentrations of Cd (130–285 nmol g⁻¹), Pb (2,500–>500,000 nmol g⁻¹), Cu (7,000–150,000 nmol g⁻¹), and Zn (3,800–>400,000 nmol g⁻¹) found in metal mining and smelting areas (see e.g., Anaman et al., 2024; Scherger et al., 2024). At a lower concentration range, metal mixtures in soil can also result from less obvious sources related to human activities, like manure (Cu) and fertilizer (Cd) application. Springtails live in high numbers in the top soil layer, where they play an important role in the decomposition of organic material by feeding on fungal hyphae (Hopkin, 1989). Springtails can be exposed to metal mixtures both via uptake of moisture from the soil and through feeding. Metals play different roles in animal physiology. Springtails require Cu and Zn as a micronutrient (Hopkin, 1989) and are often able to regulate internal concentrations of these metals at a rather constant level over a wide range of exposure concentrations (Bruus Pedersen et al., 2000; Van Gestel & Hensbergen, 1997). On the other hand, Cd and Pb have no known

function to springtails and are not subject to regulation (Van Gestel & Hensbergen, 1997), although springtails are able to excrete Cd and Pb via intestinal exfoliation (Van Straalen et al., 1987). In view of these facts, combinations of these metals are interesting subjects for the study of mixture toxicity.

Mixture toxicity to soil organisms is still not well investigated. This can partly be attributed to the more recent origins of soil (eco)toxicology and consequent scarcity of experiments with a systematic approach towards mixture toxicity of metals to soil organisms (e.g., Amorim et al., 2012; Gomez-Eyles et al., 2009; Kilpi-Koski et al., 2020; Posthuma et al., 1997; Svendsen et al., 2026; Van Gestel & Hensbergen, 1997; Van Loon et al., 2025). The complexity of the soil environment makes it more difficult to explain effects on soil organisms from total soil concentrations. In the uptake and effect cascade from soil to effects in organisms, metals can interact at four levels: (1) chemical and physiochemical interactions with other constituents of the soil, determining sorption and thereby bioavailability; (2) physiological interactions, affecting uptake from the soil (solution); (3) toxicokinetic interactions within organisms determining the quantity available at the site(s) of action; and (4) interactions at the

Received: December 24, 2025. Revised: March 31, 2026. Accepted: March 31, 2026

© The Author(s) 2026. Published by Oxford University Press on behalf of the Society of Environmental Toxicology and Chemistry.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<https://creativecommons.org/licenses/by/4.0/>), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.

detoxification and intoxication processes, including binding to receptors, at the target site(s), (Spurgeon et al., 2010). Therefore, to understand mixture toxicity to soil organisms, the interactions at the different levels must be disentangled.

In mixture toxicity studies, the mixture is often limited to one ratio of the individual components, usually with both chemicals added at equal fractions of their half-maximal effective concentration (EC50; equitoxic mixtures). The above-discussed interactions in a mixture can only fully be explored if more ratios and ranges of the individual metal concentrations are tested (Van Gestel et al., 2010). Most commonly, the toxicity data are only explored for antagonistic or synergistic deviations from the expected concentration-addition model. As metals are handled by different mechanisms, it is expected that the combined effect of metals depends on their proportion in the mixture. Further, dose level-dependent deviations from additivity are reported in several studies (see e.g., Sharma et al., 1999). For instance, Cd and Zn combined affected reproduction of *Folsomia candida* differently at the EC10 (additive) than at the EC50 level (antagonistic; Van der Geest et al., 2000; Van Gestel & Hensbergen, 1997). Jonker et al. (2005) provided a simple and coherent data analysis framework to detect and quantify four distinct deviation patterns from the concentration addition and independent action models in dose-response data of simple mixtures: no deviation, synergism/antagonism, dose ratio-dependent deviation, and dose level-dependent deviation. This framework will be applied to the data from our study to explore the interactions between the metals at all levels in detail and relate mixture effects to both the concentration addition and independent action reference models.

The aim of this study was to discriminate between interactions of the nonessential metal Cd with Pb (also nonessential) or the essential metals Cu or Zn on the various levels (sorption, uptake, toxicity) and to relate them to each other. Further, we wanted to obtain insight into the importance of dose ratio- and dose level-dependent deviations affecting the joint toxicity of metals. Toxicity tests were performed in a natural standard soil with the springtail *F. candida*, using concentration series of the individual metals, and their mixtures of both equitoxic and four other concentration ratios. To unravel the various possible interactions, we studied the following aspects: (a) the influence of the metals on each other's soil-solution distribution by determining water-extractable, calcium chloride (CaCl₂)-exchangeable and total soil concentrations; (b) the uptake of the metals by the springtails in relation to the (extractable) soil concentrations of both metals; and (c) the combined effects of metals on survival, growth, and reproduction of *F. candida* in relation to (extractable) concentrations in soil and internal concentrations in the animals.

Materials and methods

Test animals

Juvenile *F. candida* (Collembola, Isotomidae), of similar age (10–12 days), were obtained by synchronizing the egg deposition of adult animals (allowed to lay eggs for 48 hr and then removed) from a laboratory breeding stock (Berlin strain, cultured in our

laboratory for many years). The animals were cultured in plastic humidity chambers with a water-saturated base of plaster of Paris containing 10% activated charcoal, at 20 ± 1 °C under a 12:12-hr light: dark regime and fed with dried baker's yeast (Dr. Oetker).

Contamination of the test soil

The natural standard LUFA 2.2 soil was obtained from LUFA Speyer, Germany, and had (according to the supplier) an organic carbon content of 2.19%, and a cation exchange capacity of 11 cmol_c kg⁻¹. CdCl₂·2½H₂O (> 99% pure, Sigma-Aldrich, Zwijndrecht, The Netherlands), PbCl₂ (>98% pure, Merck-Schuchardt, Hohenbrunn, Germany), CuCl₂·2H₂O (>99% pure, Mallinckrodt Baker, Deventer, The Netherlands), and ZnCl₂ (>98% pure, Merck, Darmstadt, Germany) were mixed in with the soil as aqueous solutions. A few drops of 10% hydrogen chloride (HCl; Mallinckrodt Baker, Deventer, The Netherlands) were added to the PbCl₂ solution to increase solubility. On addition of the metal solutions or demineralized water for the controls, soil moisture content was raised to 25% (w/w), corresponding with ~50% of the maximum water-holding capacity. The soil was left for 2 weeks at room temperature in closed containers to equilibrate before starting exposures.

The experimental design for the three binary mixture experiments (Cd-Zn, Cd-Pb, Cd-Cu) was based on the toxic unit (TU) concept, with reproduction as the endpoint ($TU = \frac{c}{EC_{50}}$, where EC50 is the median effective concentration and c is the toxicant concentration in the mixture). Reproduction EC50s used were 516 nmol Cd g⁻¹ dry soil (Van Gestel & Van Diepen, 1997), 2,606 nmol Pb g⁻¹ dry soil (Bongers et al., 2004), 5,114 nmol Cu g⁻¹ dry soil (pilot experiment), and 5,322 nmol Zn g⁻¹ dry soil (Smit & Van Gestel, 1996). Nominal concentrations of the binary mixtures were based on expected toxic strengths ($\sum TU$) of 0.25, 0.5, 1, 2, 4, and 8 TU, applying expected concentration ratios (all based on EC50s) of Cd to Pb, Cu, or Zn of 1:9, 1:3, 1:1 (equitoxic), 3:1, and 9:1 (see online [supplementary material Table S1](#)). Individual metals were tested simultaneously with each mixture at 0.125, 0.25, 0.5, 1, 2 and 4 TU.

Toxicity testing

Toxicity tests followed ISO guideline 11267 for assessing the effects of chemicals on *F. candida* (ISO, 1999). Five replicate glass jars (100 ml) filled with 30 g of moist soil were used for each treatment and 10 replicate jars for the control. Ten juvenile *F. candida* were transferred into each jar and a few grains of dried baker's yeast were added for food. Experiments were performed at 20 ± 0.1 °C, 75% relative humidity with a 12:12-hr light: dark cycle. Additional food was given to maintain ad libitum conditions. The jars were aerated three times a week and soil moisture content was kept constant by weighing the jars once a week and replenishing the water loss with deionized water.

After 4 weeks of incubation, survival and reproduction of the animals were determined as described by Van Gestel and Hensbergen (1997). For each treatment, up to 15 surviving adult animals were collected from the replicate test jars, weighed individually (wet wt) to the nearest microgram using a super

microbalance (model S4, Sartorius, Göttingen, Germany), lyophilized, weighed again to obtain the dry weight and stored at -20°C until metal analyses.

Chemical analysis

Soil was dried at 40°C . The total metal concentration was obtained by microwave digestion of 1 ± 0.05 g dry soil in a mixture of concentrated nitric acid (min. 65%, Riedel-de Haën, Seelze, Germany), HCl (min. 37%, Mallinckrodt Baker, Deventer, The Netherlands), and deionized water (4:1:1 v/v/v). At the end of the experiments, exchangeable and water-extractable metal fractions were determined by shaking 5 ± 0.05 g dry soil for 2 hr at 200 rpm with 25 ml of 0.01 M CaCl_2 (Mallinckrodt Baker, Deventer, The Netherlands) or 25 ml deionized water, respectively. After filtration over a $0.45\text{ }\mu\text{m}$ cellulose nitrate membrane filter (401196, Schleicher & Schull, Dassel, Germany), metal concentrations in the 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) was used as the reference soil for total metal concentrations. Recoveries from the reference soil were 5% to 71% for Cd, 40% to 57% for Pb, 87% to 91% for Cu and 84% to 97% for Zn. The low recoveries of Cd and Pb were due to their low concentrations in the reference soil, resulting in concentrations in the digests close to or below detection limits. The nominal and actual metal concentrations were, however, in agreement; therefore, no corrections were applied to the measured total soil metal concentrations. Detection limits for flame AAS analysis were 1.42, 6.75, 50.0, and $19.9\text{ }\mu\text{mol L}^{-1}$ for Cd, Pb, Cu, and Zn, respectively. Measurements below the detection limit were replaced with half the calculated detection limit. Chloride concentrations in the water extracts were measured on an auto-analyzer (model SA 400, Skalar, Breda, The Netherlands).

Individual animals were digested in $300\text{ }\mu\text{l}$ of a mixture of concentrated nitric acid and perchloric acid (7:1 v/v; Ultrex grade; Mallinckrodt Baker, Deventer, The Netherlands), and internal metal concentrations were measured using graphite furnace AAS. As standard reference materials, bovine liver (Cd, Cu, and Zn) and olive leaves (Pb; certified by the Community Bureau of Reference, Brussels, Belgium) were used. Recoveries were 60% to 92% for Cd, 86% to 89% for Pb, 43% to 91% for Cu, and 48% to 55% for Zn. To get a true picture of the method variation for samples in the range of the weight of individual springtails, the reference material was weighed at 0.1% of the minimum quantity certified and deviations from certified metal concentrations were therefore expected. No systematic corrections were applied to the internal metal concentrations. Detection limits for graphite furnace AAS analysis were 0.98, 10.3, 13.5, and 111 nmol L^{-1} for Cd, Pb, Cu, and Zn, respectively. Measurements below the detection limit were replaced with half the calculated detection limit.

Calculations and statistics

Sorption of metals to the test soil was described by a Langmuir isotherm:

$$C_{\text{sorbed}} = \frac{C_{\text{sorbed-max}} K_L C_{\text{diss}}}{1 + K_L C_{\text{diss}}} \quad (\text{Eq. 1})$$

where C_{sorbed} is the total soil metal concentration (nmol g^{-1} dry soil), C_{diss} is the metal concentration in the water or 0.01 M CaCl_2 extract (nmol ml^{-1}), $C_{\text{sorbed-max}}$ is the maximum sorption capacity (nmol g^{-1} dry soil), and K_L is the Langmuir sorption constant (ml nmol^{-1}); K_L may be interpreted as the inverse of the dissolved concentration at which 50% of the sorption sites is occupied. Estimates for $C_{\text{sorbed-max}}$ and K_L were obtained by nonlinear regression on log-transformed data of C_{sorbed} versus C_{diss} (GraphPad Prism 2.01, GraphPad Software, Inc., CA, USA). Controls were excluded from the analyses because (dissolved) concentrations were below detection limits. A generalized likelihood-ratio test was used to compare results from single metal and mixture treatments.

For each binary mixture and for every metal fraction, mixture effects on growth (change in biomass over the 28-day exposure period) and reproduction (number of juveniles produced per test jar) of *F. candida* were analyzed by applying the computational framework proposed by Jonker et al. (2005). The concentration-response relationships for the single-metal exposures as part of the mixture tests were modeled by fitting a three-parameter logistic curve to the experimental data by minimizing the sum of the squared residuals using the Solver Function (Newton algorithm) in Microsoft Excel (Jonker et al., 2005). This generated maximum response, slope (β), and EC50 values for each individual metal.

Mixture toxicity was modeled using the concentration addition model, so resultant predicted joint effects could be compared with observed effects. The basic procedure for fitting this descriptive model to the experimental data was the same method described for the single-metal data, but modelling both single metal and the mixture data concurrently, again minimizing the sum of the squared residuals (Jonker et al., 2005). The additional models including deviation functions for synergistic/antagonistic, dose ratio-dependent, and dose level-dependent deviations were fitted in turn to see whether the model fit could be significantly improved using the nested model testing framework delineated by Jonker et al. (2005). Full details for interpretation of parameter values are available in Jonker et al. (2005) and Gomez-Eyles et al. (2009).

Results

Soil pH, metal, and chloride concentrations

The pH- CaCl_2 of the control soil was 4.7–5.2 and the pH- H_2O was 5.2–5.7. Soil pH decreased with increasing metal concentrations, which was most pronounced in the case of Cu, single or in mixtures with Cd (max. 1.2 pH units); see online [supplementary material Figure S1](#).

Measured total metal concentrations in soil (see online [supplementary material Tables S2–S4](#)) were in agreement with nominal ones with average ($\pm\text{SD}$; $n = 23\text{--}24$) recoveries (in percentage of nominal) of 100 ± 2.97 , 96.6 ± 4.68 , and 90.4 ± 5.22 for Cd in the tests with Pb, Cu, and Zn, respectively, and of $114 \pm$

13.8, 99.4 ± 9.28 , and 98.6 ± 19.4 for Pb, Cu, and Zn, respectively. All data in this article are expressed on the basis of measured concentrations. The Ca concentrations, only measured in the Cd-Cu test, ranged between 1,218 and 1,422 mg kg⁻¹ dry soil and were independent of the added metal concentrations (see online [supplementary material Table S3](#)).

Water-extractable and CaCl₂-exchangeable metal concentrations increased with the total concentrations. Langmuir sorption parameters (K_L and $C_{sorbed-max}$) for both metals in each of the three mixtures are given in online [supplementary material Tables S5–S7](#) for the single metal treatments and for the different concentration ratios.

Water-extractable and CaCl₂-exchangeable concentrations of Cd increased significantly in the presence of Pb, Cu, or Zn (generalized likelihood ratio test, $p < 0.001$). This is reflected by a decrease of the estimated sorption maximum ($C_{sorbed-max}$) with increasing contribution of the other metal in the mixture (see online [supplementary material Tables S5–S7](#)) as can be seen in the Langmuir isotherms ([Figure 1](#)). With increasing proportion of Pb, Cu, or Zn in the mixture, the slope of the isotherm decreased and estimates for K_L increased, indicating higher extractable concentrations of Cd. The same was true for CaCl₂-exchangeable concentrations of Pb and Zn in the presence of Cd (generalized likelihood ratio test, $p < 0.01$), but not for Cu. Cadmium did not affect the water-extractable concentrations of Pb, Cu, and Zn.

Chloride concentrations in the water extracts (see online [supplementary material Table S8](#)) increased dose-relatedly to a maximum of 31.7, 38.5, 147, 272, and 250 mg Cl L⁻¹ at the highest single Cd, Pb, Cu, and Zn doses. In the mixtures, the highest chloride concentrations were 99.4 mg Cl L⁻¹ at the highest Cd + Pb dose (4 TU) and 292 and 308 mg Cl L⁻¹ at the highest combined Cd + Cu and Cd + Zn doses (8 TU), respectively.

Metal uptake

Internal Cd and Pb concentrations in the control springtails were often below the detection limit, but increased with increasing total soil concentrations. At high exposure concentrations ($\geq 1,000$ nmol Cd g⁻¹ dry soil), in both the single and the mixture exposures, internal Cd concentrations approached a plateau of ~ 1 to 2 μmol Cd g⁻¹ dry body weight, but no such plateau was seen for internal Pb concentrations ([Figure 2](#)). Internal Cu and Zn concentrations in the springtails were regulated at the lower

soil concentrations, at average levels of 0.5–1.1 μmol Cu g⁻¹ dry body weight and 1–3 μmol Zn g⁻¹ dry body weight, respectively ([Figure 2](#)). For Cu, this was the case at concentrations $\leq 10,000$ nmol Cu g⁻¹ dry soil in the single exposures and $\leq 5,000$ nmol Cu g⁻¹ dry soil in the mixtures with Cd. For Zn, this was the case at $\leq 2,650$ nmol Zn g⁻¹ dry soil in both the single and the mixture exposures.

At equitoxic concentrations, uptake of Cd, Pb, Cu, or Zn appeared not to be affected by the presence of the other metal ([Figure 2](#)). The same pattern was found for internal Cd concentrations related to the water-extractable and CaCl₂-exchangeable Cd concentrations. Only at the highest Zn-to-Cd concentration ratio (9:1) was a slight reduction of Cd uptake in the springtails observed.

Toxicity

Springtail survival in the controls was between $84\% \pm 18\%$ and $91\% \pm 13\%$ ($\pm SD$; $n = 10$). The average number of juveniles produced per control test container ranged between 857 ± 224 and 1331 ± 229 , with coefficients of variation being 26.1%, 37.8%, and 17.2% for the tests with the mixtures of Cd with Pb, Cu, and Zn, respectively. Individual growth in the controls was between 212 ± 44 and 237 ± 20 μg ($n = 20$; see online [supplementary material Tables S9–S11](#)).

Cadmium affected survival in a concentration-related manner, with similar effects at the three highest concentrations (see online [supplementary material Tables S9–S11](#)). Because mortality in some cases did not reach 50% compared with the controls, no LC50s were calculated. Lead, Cu, or Zn did not affect springtail survival at any of the concentrations tested (see online [supplementary material Tables S9–S11](#)), but did reduce the effect of Cd, suggesting antagonistic interactions (see online [supplementary material Figure S2](#)).

Growth and reproduction of *F. candida* were negatively affected by all metals with increasing exposure concentrations. No observable effect concentrations (NOECs) and the 10% and EC50s (with corresponding 95% confidence intervals) for effects of the single metals on the growth and reproduction of the springtails are summarized in online [supplementary material Table S12](#). The results of the analysis of the toxicity data for all mixtures are summarized in [Table 1](#) for growth and [Table 2](#) for reproduction; the full outcomes of the mixture toxicity analysis

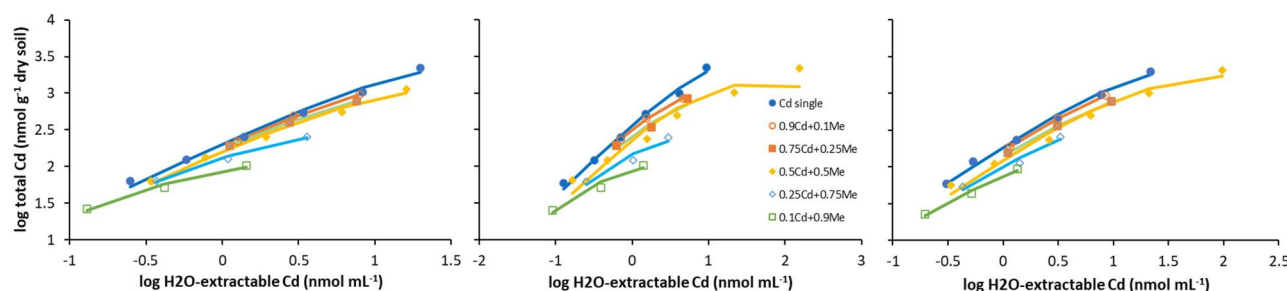


Figure 1 Langmuir isotherms for the sorption of cadmium in mixtures of cadmium chloride (CdCl₂ with PbCl₂; left), copper chloride (CuCl₂; middle), and zinc chloride (ZnCl₂; right) spiked to LUFA 2.2 soil, for different Cd-metal ratios (expressed in TU), extracted with deionized water. ● Cd 1; ○ 0.9Cd + 0.1Me; ■ 0.75Cd + 0.25Me; ◆ 0.5Cd + 0.5Me; ◆ 0.25Cd + 0.75Me; □ 0.1Cd + 0.9Me, with Me being the second metal (Pb, Cu, or Zn). See online [supplementary material Tables S5–S7](#) for the corresponding Langmuir sorption parameters.

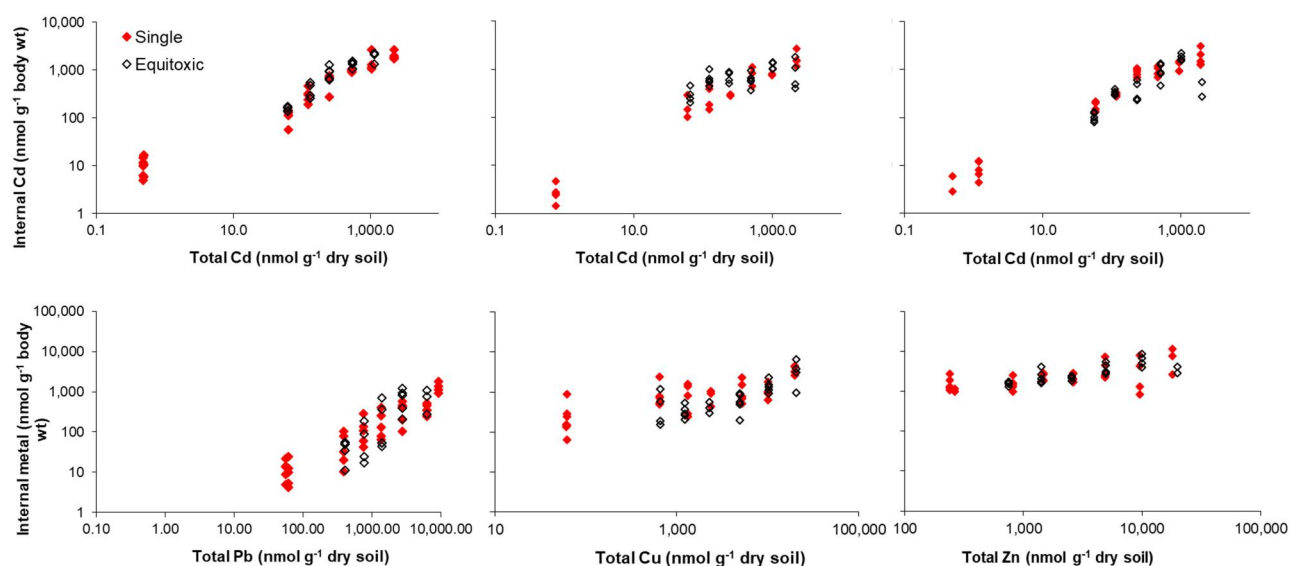


Figure 2 Concentrations of cadmium (top) and lead, copper, or (bottom) in *Folsomia candida* after 4 weeks exposure in LUFA 2.2 soil to binary mixtures of Cd with Pb (left), Cu (middle), and Zn (right), related to total soil concentrations. Results are presented for animals exposed to individual metals (Single) or to equitoxic mixtures (Equitoxic).

are shown in online [supplementary material Tables S13–S15 and S16–S18](#), respectively.

For all metal combinations and metal fractions, overall antagonistic deviations from concentration addition were found for effects on growth (Table 1), with positive values for a . For the Cd-Pb mixture (Figure 3), the model quantifying dose ratio-dependent deviations described the data significantly better when effects were related to exchangeable and extractable metal concentrations, with slight synergism at low Cd ratios shifting to antagonism when more than 36% and 17% of the toxicity of the mixture was caused by Cd, respectively. When effects were related to internal Cd and Pb concentrations in the springtails, the model with dose-level dependent deviations gave the best fit, with a shift to synergism occurring only at concentrations above $3.5 \times \text{EC}_{50}$.

For the Cd-Cu mixture, the model with dose ratio-dependent deviations from concentration addition gave the best description in all cases, but interpretation differed depending on what exposure measure was taken (Table 1; see online [supplementary material Figure S3](#)). When effects were related to total and water-extractable soil concentrations and internal concentration in the springtails, the trend was overall antagonism with a shift to synergism only at high Cd concentrations. When related to CaCl_2 -exchangeable concentration, however, the trend was synergism at low and antagonism at high Cd level, with a shift happening around equitoxic ratios.

For the mixtures of Cd with Zn, the model with dose ratio-dependent deviations from concentration addition gave the best description when growth effects were related to total soil concentrations, with synergism at low Cd ratios shifting to antagonism when Cd caused more than 15% of the toxicity of the mixture (Table 1; see online [supplementary material Figure S4](#)). When effects were related to exchangeable and extractable concentrations in soil, both the models with dose ratio-dependent and dose level-dependent deviations gave a good description of the data. In both cases, the first model also showed synergy

only at low Cd ratios, shifting to antagonism when Cd caused more than 16% and 4% of the mixture toxicity, respectively. The second model showed overall antagonism with shift to synergism only at effect levels far above the EC_{50} . When effects were related to internal Cd and Zn concentrations in the springtails, the model with the dose level-dependent deviation best described the data. In this case, the shift to synergism occurred at concentrations above $1.8 \times \text{EC}_{50}$ (Table 1).

Effects of the Cd-Pb mixture on springtail reproduction were best described by the concentration addition model with pure synergism/antagonism deviation extension and with the positive a values indicating antagonism in all cases. Stronger antagonism was seen when effects were related to extractable compared with total metal concentrations in the LUFA 2.2 soil, and this was also the case when based on internal concentrations (Table 2; see online [supplementary material Figure S5](#)).

For the Cd-Cu mixture, the synergism/antagonism deviation model best described the data when effects were related to total and CaCl_2 -exchangeable concentrations in the soil, with the positive a values indicating antagonism (Table 2; see online [supplementary material Figure S3](#)). When effects were related to water-extractable metal concentrations, the model quantifying dose ratio-dependent deviation from concentration addition described the data significantly better, with the negative a and positive b value suggesting synergism at low Cd ratios and shifting to antagonism when Cd ratios exceeded the equitoxic ratio. When effects were related to internal Cd and Cu concentrations in the springtails, the model with dose level-dependent deviations best described the data, with a shift to synergism at concentrations above $1.7 \times \text{EC}_{50}$.

For the Cd-Zn mixture, the model with dose ratio-dependent deviations from concentration addition best described the data when effects were related to total and extractable soil concentrations, with synergism at low Cd ratios shifting to antagonism when Cd caused more than 14%, 36%, and 19% of the mixtures toxicity, respectively (Table 2; see online [supplementary](#)

Table 1 Parameter estimates and statistics of the effects of binary mixtures of cadmium with lead, copper, or zinc on the growth (in µg dry body wt) of the springtail *Folsomia candida* after 4 weeks exposure in LUFA 2.2 soil, based on total, 0.01 M CaCl₂-exchangeable, and water-extractable concentrations in the soil and internal concentrations in the surviving animals.

Exposure concentration based on				
	Total	CaCl ₂	Water	Internal
Cd + Pb				
EC50				
Cd	502	136	13.4	653
Pb	8,380	72.2	15.0	911
S/A (p)	<0.001 (a = 1.28)	<0.001 (a = 2.12)	<0.001 (a = 5.92)	<0.001 (a = 1.47)
DR (p)	0.0868	0.0099 (a = -2.41; b = 6.78)	0.0176 (a = -1.79; b = 10.8)	0.985
DL (p)	0.977	0.66	0.852	<0.001 (a = 12.1; b = 0.288)
Conclusion	Antagonism	DR: Low Cd → synergism; >36% of tox from Cd → antagonism	DR: Low Cd → synergism; >17% of tox from Cd → antagonism	DL: Antagonism, shift to synergism at 3.5*EC50
Cd + Cu				
EC50				
Cd	527	109	8.15	789
Cu	13,999	857	118	2,000
S/A (p)	<0.001 (a = 2.09)	<0.001 (a = 6.61)	<0.001 (a = 18.2)	<0.001 (a = 1.24)
DR (p)	0.005 (a = 6.96; b = -8.35)	<0.001 (a = -22.1; b = 36.8)	<0.001 (a = 61.9; b = -61.6)	0.002 (a = 1.61; b = 2.08)
DL (p)	0.977	0.419	0.379	0.042 (a = 12.6; b = 0.702)
Conclusion	DR: Low Cd → antagonism; >83% of tox from Cd → synergism	DR: Low Cd → synergism; >60% of tox from Cd → antagonism	DR: Overall antagonism (Low Cd → antagonism; only at ~100% of tox from Cd → synergism)	DR: Overall antagonism (Low Cd → antagonism; only at ~100% of tox from Cd → synergism)
Cd + Zn				
EC50				
Cd	532	37.5	3.23	1,862
Zn	10,571	858	208	6,623
S/A (p)	<0.001 (a = 2.49)	<0.001 (a = 4.39)	<0.001 (a = 9.47)	0.0401 (a = 1.68)
DR (p)	<0.001 (a = -1.10; b = 7.22)	<0.001 (a = -1.37; b = 8.52)	<0.001 (a = -0.455; b = 10.2)	0.121
DL (p)	0.654	<0.001 (a = 19.1; b = 0.200)	<0.001 (a = 30.5; b = 0.0771)	0.00276 (a = 8.99; b = 0.551)
Conclusion	DR: Low Cd → synergism; >15% of tox from Cd → antagonism	DR: Low Cd → synergism; >16% of tox from Cd → antagonism DL: Antagonism, shift to synergism at > 5.0*EC50	DR: Low Cd → synergism; >4% of tox from Cd → antagonism DL: Antagonism, shift to synergism at 13*EC50	DL: Antagonism, shift to synergism at 1.8 × EC50

Note. Values are given for calculations based on the concentration addition model and 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). Median effective concentrations (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 interpretation of the values of a and b. The p(χ²) values indicate the significance of the deviation parameters a and b compared with the reference model. See online supplementary material Tables S13–S15 for the full model output.

Table 2 Parameter estimates and statistics for the effects of mixtures of cadmium with lead, copper, or zinc on the reproduction (number of juveniles per container) of the springtail *Folsomia candida* after 4 weeks exposure in LUFA 2.2 soil, based on total, CaCl_2 -exchangeable, and water-extractable concentrations in the soil and internal concentrations in surviving animals.

		Exposure concentration based on			
		Total	CaCl_2	Water	Internal
Cd + Pb					
EC50	Cd	591	175	20.0	1,228
	Pb	6,404	46.0	11.4	1,276
	S/A (<i>p</i>)	0.000434 (<i>a</i> = 1.88)	0.00146 (<i>a</i> = 2.58)	<0.001 (<i>a</i> = 4.41)	<0.001 (<i>a</i> = 3.15)
	DR (<i>p</i>)	0.670	0.909	0.603	0.562
	DL (<i>p</i>)	0.767	0.681	0.982	0.241
Conclusion		Antagonism	Antagonism	Antagonism	Antagonism
Cd + Cu					
EC50	Cd	402	101	5.26	588
	Cu	10,054	255	151	2,057
	S/A (<i>p</i>)	<0.001 (<i>a</i> = 1.42)	<0.001 (<i>a</i> = 2.77)	<0.001 (<i>a</i> = 6.72)	0.257
	DR (<i>p</i>)	0.547	0.424	<0.001 (<i>a</i> = -15.3; <i>b</i> = 28.5)	0.919
	DL (<i>p</i>)	0.239	0.346	0.791	<0.001 (<i>a</i> = 103; <i>b</i> = 0.590)
Conclusion		Antagonism	Antagonism	DR: Low Cd → synergism; >54% of tox from Cd → antagonism	DL: Antagonism, shift to synergism at > 1.7*EC50
Cd + Zn					
EC50	Cd	451	34.7	5.08	865
	Zn	7,189	748	221	4,105
	S/A (<i>p</i>)	<0.001 (<i>a</i> = 3.14)	<0.001 (<i>a</i> = 2.62)	<0.001 (<i>a</i> = 2.72)	<0.001 (<i>a</i> = 4.89)
	DR (<i>p</i>)	<0.001 (<i>a</i> = -1.08; <i>b</i> = 7.83)	<0.001 (<i>a</i> = -3.61; <i>b</i> = 10.0)	<0.001 (<i>a</i> = -1.53; <i>b</i> = 7.85)	0.522
	DL (<i>p</i>)	0.688	0.286	0.754	0.583
Conclusion		DR: Low Cd → synergism; >14% of tox from Cd → antagonism	DR: Low Cd → synergism; >36% of tox from Cd → antagonism	DR: Low Cd → synergism; >19% of tox from Cd → antagonism	Antagonism

Note. Values are given for calculations based on the concentration addition model, and 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). Median effective concentrations (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 interpretation of the values of *a* and *b*. The $p(\chi^2)$ values indicate the significance of the additional deviation parameters. In some cases, no reliable fit was obtained when relating effects to internal metal concentrations in the animals. See online [supplementary material, Tables S16–S18](#) for the full model output.

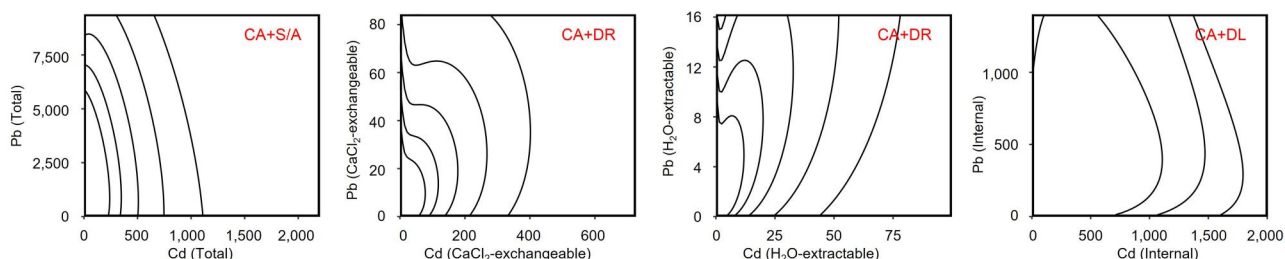


Figure 3 Isoboles of curve fits obtained using the concentration addition (CA) model for the effect of mixtures of cadmium and lead on the growth of *Folsomia candida* in LUFA 2.2 soil. The isoboles indicate from left to right 10%, 25%, 50%, 75%, and 90% reduction of growth compared with the control. Shown are, from left to right, the best fitting models for growth effects based on total, 0.01 M CaCl_2 -exchangeable and water-extractable concentrations in soil (in nmol g^{-1} dry soil), and internal concentrations in the springtails (in nmol g^{-1} dry body wt). S/A, DR, and DL = synergistic/antagonistic, dose ratio-dependent, and dose level-dependent deviations from CA, respectively.

material Figure S4). When effects were related to internal Cd and Zn concentrations in the animals, the synergism/antagonism model gave the best description, with the positive *a* value indicating antagonism.

Discussion

This study showed overall antagonism for the toxicity of binary mixtures of Cd with Pb, Cu, or Zn to the survival, growth, and

reproduction of the springtail *F. candida* in LUFA 2.2 soil when analyzed against concentration addition as the reference model. To explain the antagonism, effects were not only related to total metal concentrations in the test soil but also to available concentrations and to internal concentrations in the springtails.

Metal partitioning in the soil

In this study, Pb, Cu, or Zn affected the soil-solution distribution of Cd. In the mixtures, Cd availability was higher compared with the single metal treatment, which was most pronounced for the extraction with water (Figure 1). In the Cd-Zn mixture, log $C_{\text{sorbed-max}}$ dropped from 3.50 nmol g⁻¹ dry soil in single Cd treatments to 2.29 nmol g⁻¹ dry soil in treatments with a Cd-Zn ratio of 1:9 TU (see online supplementary material Table S7), indicating an increased availability of Cd in the soil solution. For the other mixtures, a similar drop in log $C_{\text{sorbed-max}}$ was found. At the most extreme ratio, 45 (Pb), 89 (Cu), or 93 (Zn) times as many molar units of these metals compared with Cd were added per g dry soil. The binding strength of a metal to the soil, given by the Langmuir sorption isotherm, increased in the order Cd < Zn < Cu = Pb. Thus, part of the competition between the metals in the mixtures can be explained from the differences in binding strength to the soil and another part from the proportion in which the metals are present. For Pb and Zn, a smaller but significant drop in $C_{\text{sorbed-max}}$ was found with increasing contribution of Cd to the mixture when based on CaCl₂-exchangeable concentrations (see online supplementary material Tables S5 and S7). The water-extractable fractions showed a similar but nonsignificant trend. The smaller effect of Cd on the availability of Pb and Zn can partly be explained by the lower concentrations of Cd in the mixtures (most extreme Cd-Pb and Cd-Zn ratio (9:1 [in TU]) was, when expressed in nmol g⁻¹, 1.78:1 and 1.1:1). Because molar concentrations of Cd at polluted sites are also usually lower compared with Pb or Zn concentrations (see e.g., Anaman et al., 2024; Scherger et al., 2024), this interactive effect will rarely be observed in the field.

No effect of Cd addition on extractable Cu concentrations was found. Although we used a wide variety of concentration ratios, to stay in the range of relevant toxicity, we did not test combinations in which Cd was the dominant metal in molar units (maximum Cd-Cu ratio of 1.1:1 molar units). This could partly explain the lack of interactive effects of Cd on Cu sorption. The higher $C_{\text{sorbed-max}}$ for single Cu compared with Cd further suggests that Cd is not likely to force Cu into solution. Also for Cd, the molar concentration at heavily polluted mining sites is rarely higher than that of Cu (see e.g., Anaman et al., 2024; Scherger et al., 2024). Therefore, at environmentally and toxicologically relevant situations, effects on Cu availability in soil due to the presence of Cd are not likely.

Uptake of metals

Although the availability of Cd, Pb, and Zn in soil was increased at combined exposure, their uptake into the springtails was not affected (Figure 2, compare open and closed symbols). Similar results were found by Van Gestel and Hensbergen (1997) for the combination of Cd and Zn. Thus, in general, the uptake of metals in *F. candida* was not directly determined by the metal

concentration in the soil solution as estimated with water or 0.01 M CaCl₂ extraction. This is supported by Vijver et al. (2001), who found that for Cd and Pb in field soils, total rather than extractable soil concentrations explained internal concentrations in *F. candida*. This may be explained by the biotic ligand model, which states that metals present in the soil solution as complexes may be unavailable for uptake into organisms (e.g., CdCl₂ complexes) and that only free metal ions are truly available and compete with each other and also with other ions, such as H⁺ and Ca²⁺ ions, for binding sites on biological membranes (Ardestani et al., 2014; Gong et al., 2022; Qiu et al., 2015; Van Gestel & Koolhaas, 2004). This competition might explain the absence of an increased Cd uptake in the springtails at higher concentrations of the other metals. However, because in our tests, all metals were added to the soil as chloride salts, it seems more likely that the increased water-extractable Cd was partly present as Cd-chloride complexes that could not be taken up by *F. candida*.

For the springtail *Orchesella cincta*, a significant positive correlation was found between the amounts of Cd and Pb excreted when exposed to these metals via food (Van Straalen et al., 1987). It has been suggested that Cd and Pb follow the same physiological pathway but are excreted independently, as the efficiency with which one metal is excreted was not influenced by the presence of another metal. No interaction between Cd and Pb on their accumulation in *F. candida* was found in this study, reaching similar internal concentrations at single and combined exposure concentrations. Further, the levelling off of internal Cd concentrations at higher equitoxic exposure concentrations is not reflected by reduced internal Pb concentrations. This suggests that in *F. candida* at least part of the physiological pathway differs for Cd and Pb.

For mixtures of Cd with the essential metals Cu or Zn physiological interactions were expected. Hopkin (1989) suggested that Zn, Cd, and Cu share uptake sites on the cell membrane, explaining the antagonism found between these metals in a wide range of terrestrial invertebrates. All three metals are bound to proteins on entering the cell. Exposure of the earthworm *Lumbricus rubellus* to Cd-rich soil before exposure to Cu-rich soil increased the Cu burden (Marino et al., 1998), which was explained by the binding of Cu to a metallothionein homologue induced by Cd. Our experiment, however, provided no evidence for physiological interactions during uptake of Cd and Cu in the springtails.

In plants, Zn may reduce the uptake of Cd, resulting from antagonistic interactions and competition at uptake sites (Cai et al., 2019). For the springtail *O. cincta*, Cd uptake rate from food was reduced in the presence of Zn at exposure concentrations comparable to those used in our study (Sternborg et al., 2003). In our study, no interaction of Cd and Zn on uptake patterns into *F. candida* was observed. This implies that either metals in food and soil are taken up via different routes or that the species of springtail differ in uptake patterns.

Toxicity of single metals

Although reduced by metal addition, especially by Cu, for all test concentrations, soil pH was in the optimal range for *F. candida* of 4.3–6.2 (Crouau et al., 1999; Fountain & Hopkin, 2005). Therefore, pH is not expected to have played an important role

in metal toxicity in this study. Nevertheless, it cannot be excluded that metal bioavailability increased at the lower soil pH, but the resulting higher H^+ ion concentration could have counteracted metal ion uptake by the springtails (Ardestani et al., 2014; Gong et al., 2022; Qiu et al., 2015; Van Gestel & Koolhaas, 2004). We did not make any attempts to correct for soil pH changes, because, in our experience, it is extremely difficult or even impossible to obtain exactly the same pH for all treatments, and adding chemicals (e.g., $CaCO_3$ or $NaOH$) to the test soil would also affect metal speciation and bioavailability.

No LC50s for Cd toxicity were calculated, because in all three binary mixture toxicity tests, an elevated survival was seen at the highest or two highest compared with intermediate single Cd concentrations. In all cases, this coincided with severe effects on growth and reproduction and levelling off of the uptake curve. These effects could result from changes in springtail behavior at severe Cd exposure, for example, by becoming inactive or reduce feeding, which promotes better survival but reduced energy intake and therefore affected growth and reproduction. Similar effects were observed by Van Gestel and Hensbergen (1997), with survival decreasing to almost zero at $3,560\text{ nmol g}^{-1}$ dry soil but increasing again at higher concentrations. The LC50s reported in the literature for Cd toxicity to *F. candida* range from $5,490$ – $11,220\text{ nmol g}^{-1}$ dry soil (Crommentuijn et al., 1997; Van Gestel & Van Diepen, 1997), so exceeding the highest concentration of $1,946$ – $2,229\text{ nmol g}^{-1}$ dry soil tested in this study (see online [supplementary material Tables S2–S4](#)). Lead, Cu, or Zn did not affect survival at the tested concentrations, which may also be explained by the fact that the highest exposure concentrations tested ($9,387$, $19,572$, and $18,075\text{ nmol g}^{-1}$, respectively; see online [supplementary material Tables S2–S4](#)) were below the LC50s reported in the literature of $14,000$, $> 25,179$, and $25,661\text{ nmol g}^{-1}$, respectively (Bongers et al., 2004; Bruus Pedersen et al., 2000; Smit & Van Gestel, 1998). The $EC_{50\text{growth}}$ and $EC_{50\text{reproduction}}$ values for Cd, Pb, Cu, and Zn, based on total or extractable soil concentrations or internal concentrations in the springtails (Tables 1 and 2; see online [supplementary material Tables S12](#)), generally are in agreement with the literature data (e.g., Amorim et al., 2012; Bongers et al., 2004; Bruus Pedersen et al., 2000; Crommentuijn et al., 1997; Crouau et al., 1999; Sandifer & Hopkin, 1997; Smit & Van Gestel, 1996, 1998; Van Gestel & Hensbergen, 1997; Van Gestel & Koolhaas, 2004; Van Gestel & Van Diepen, 1997).

Mixture effects

For each metal mixture, antagonism was the dominant interaction pattern compared with the concentration addition reference model irrespective of the different effect parameters and metal pools (positive α -value in Tables 1 and 2). Because of the interactions in the soil, the sorption of the metals decreased when they were combined and this effect was largest for Cd (Figure 1). This sorption interaction, and therefore the chemical availability of the metals, was strongly dependent on the dose ratio in the mixtures. This explains why the addition of a deviation parameter allowing for a dose ratio-dependent effect significantly improved the fit of the concentration addition model to the toxicity data for each of the three mixtures, when the metal concentrations were expressed on the basis of $CaCl_2$ -exchangeable or water-extractable concentrations (Table 1).

In most cases, the toxicity of the mixtures decreased when the relative contribution of Cd increased. A possible explanation for the antagonistic effects of Cd with the other metals in the mixture is the binding of the metals to metallothionein. If exposure to a metal leads to increased metallothionein expression in the exposed organism, this protein could bind part of the metal after uptake, leaving it unavailable for binding to receptors thus inducing less toxic effects. Support for this theory can be found in the positive b value seen for the dose ratio-dependent model based on internal Cd and Zn concentrations for growth, indicating that the effects of the mixture were more antagonistic if Cd was present. This was, however, not found for the other metal combinations, possibly due to the internal Cd concentration reaching a plateau as discussed in the section *Uptake of metals*. In addition, it should be noted that not all metals trigger metallothionein expression. Swain et al. (2004), for instance, demonstrated that Cd exposure induced the expression of metallothionein in *Caenorhabditis elegans* whereas Zn exposure did not. Also, metallothionein induced by Cd may not be involved in the binding of other metals. Sterenberg et al. (2003), for example, showed that Cd-induced metallothionein in the springtail *O. cincta* did not bind Zn. This means that metallothionein induction may only partly explain the dose ratio-dependent deviation from concentration addition with mostly stronger antagonism when Cd dominated in the mixture.

Another possible explanation for the antagonistic effects may be the presence of the chloride counterion that may take part in the dose ratio-dependent effects. If part of the toxicity can be attributed to chloride, then this chloride toxicity is found more in mixtures with large amounts of Pb, Cu, or Zn compared with those with large amounts of Cd, because on a molar basis, more of these metals and thus chloride had to be added relative to Cd to cause the same toxic effect. The mixture concentrations with relatively more Cd had relatively less chloride and a lower toxicity than expected. This perhaps can be explained by less toxic effects due to chloride in these mixture concentrations. However, we had no single chloride concentration range in the test, and thus, chloride concentrations could not be included in the effect modeling. In online [supplementary material Text S1](#), a brief reflection is given on the possible effects of chloride and how to cope with that in future studies.

Dose level-dependent deviations from concentration addition were found in a few cases, for example, for the water-extractable metal fraction of the Cd-Zn mixture and the internal metal fraction of the Cd-Cu mixture. In each case, the switch was from antagonism at low effect concentrations to synergism at high effect concentrations. Although it remains unclear how to explain these dose level-dependent deviations, the switch always occurred at two or more times the EC_{50} isobole. This means the analysis results more likely reflect that the degree of antagonism reduces with increasing effect levels rather than actually switches to synergy, given this would be at concentrations well above the maximum internal Cd concentration observed in this study and at not realistically high exposure levels.

Independent action

In this study, we used the concentration addition model as the reference model against which to analyze the mixture toxicity of

Cd with three other metals. The concentration addition model, however, assumes that the chemicals in the mixture have the same mode of action (Van Gestel et al., 2010). Because this may not be the case for metals, which may have several modes of action, we also analyzed the data using the independent action model as an alternative reference model, using the framework outlined in Jonker et al. (2005). The results are summarized in online supplementary material Tables S19–S20 and Figures S6–S8 for growth and reproduction effects and shown in more detail in online supplementary material Tables S21–S26. Also, based on the independent action model, binary mixtures of Cd with Pb, Cu, or Zn showed antagonism. In most cases, the best description of the data for effects on springtail growth was obtained by adding a parameter for dose ratio-dependent mainly antagonistic deviation from independent action. For reproduction effects of the Cd-Pb and Cd-Cu mixtures related to total and exchangeable soil concentrations and of the Cd-Zn mixture related to internal concentrations in the springtails, the independent action model gave the best description. This seems to confirm the different modes of action of Cd and Pb and of Cd and Cu. In most other cases, a dose ratio-dependent deviation from independent action was found for reproduction effects, confirming antagonism when Cd dominated the mixtures. This supports the conclusion of Backhaus and Faust (2012) that the concentration addition model is a suitable conservative first choice when looking at mixture toxicity, even when chemicals have different modes of action.

Mixture toxicity testing in soil

In this study, a wide range of dose ratios was tested. This created good conditions to explore dose ratio-dependent interactions, which were found to be quite common. Jonker et al. (2004), however, reported dose level-dependent deviations from concentration addition for the combined toxicity of Cd with Pb or Cu on the population growth of *C. elegans* exposed in LUFA 2.2 soil. Deviations from additivity were antagonistic at low effect levels and synergistic at high effect levels both for the exchangeable and the water-extractable metal fractions, and in case of the Cd-Pb mixture, also for the total metal pool. As the metal concentrations were in the same order of magnitude as in our study, this could be due to the smaller range of concentration ratios used in the study on nematodes. Another possibility is that the interaction patterns are species-specific or endpoint-specific, because Jonker et al. (2004) looked at population increase as the effect parameter.

Van Gestel and Hensbergen (1997) reported dose level-dependent deviations from concentration addition for the combined toxicity of Cd and Zn to growth, but not reproduction, of *F. candida* after 4-week exposure. At the EC10 level, the mixture acted additive but antagonistic at the EC50 level. Also Amorim et al. (2012) found dose level-dependent deviations from concentration addition for Cd-Zn toxicity to *F. candida* survival and reproduction. In our Cd-Zn mixture experiment, dose level-dependent deviations were found only for growth related to CaCl₂-exchangeable and water-extractable metal concentrations in the soil and internal concentrations in the animals (Table 1). The switch from antagonism at low dose levels to synergism at high dose levels occurred at 5.0, 13, and 1.8 times the EC50

isobole, respectively, thus not in the relevant range of concentrations. So, the nature of the interaction at low and high effect levels differed between these experiments, but in the range around the EC50 isobole, the conclusions agreed. For the effects of Cd and Zn on *F. candida* reproduction, Van Gestel and Hensbergen (1997) found additive effects, Amorim et al. (2012) reported dose level-dependent deviations, and we found dose ratio-dependent deviations with antagonistic effects when Cd dominated the mixture. The observed differences between these experiments possibly are due to differences in soil type used (OECD artificial soil versus natural LUFA 2.2 soil) or in the concentration range of the metals, affecting the focus of the effect observations and the chemical interactions. This, however, warns us to be conservative when extrapolating results from one experiment to another.

One of the objectives of this study was to relate mixture interactions in the soil and during uptake and the combined toxicity. Significant differences in Langmuir sorption parameter values between the metals, solely and in combination, indicate chemical and physicochemical interactions with other soil constituents. The sorption of the metals decreased when they were combined (Figure 1), so extractable metal concentrations increased. Because Cd and Pb, Cu, or Zn did not affect each other's uptake in the springtails, these interactions in the soil did not affect the bioavailable metal concentrations or are compensated for during uptake. The increasing uptake of Pb at combined exposure concentrations, where Cd uptake was levelling off, however, suggests that Cd and Pb did not interact during uptake. A similar conclusion is drawn for the Cd-Cu and the Cd-Zn mixtures because internal concentrations of Cu and Zn, but not Cd, were regulated. Based on internal metal concentrations, antagonistic effects were significant for reproduction and growth in all cases, which suggest that at the intoxication processes antagonistic interactions took place. But this also indicates that the interaction of the metals on their soil-solution distribution cannot predict interactions inside *F. candida* leading to toxicity.

Conclusion

In binary mixtures, the chemical availability of Cd, measured as 0.01 M CaCl₂ or water extractability, was increased by the presence of Pb, Cu, or Zn. However this did not increase its bioavailability measured as metal concentrations in the springtails. Mixture interactions were overall antagonistic for the effects of the Cd-Pb, Cd-Cu, and Cd-Zn mixtures on springtail growth and reproduction. Most likely, the complexation of Cd with the excess chloride introduced in the soil solution by the counterion of the PbCl₂, CuCl₂, and ZnCl₂ salts used reduced its bioavailability, explaining these antagonistic interactions. This shows the importance of taking into account all steps in the intoxication process to improve understanding of the effects of metal mixtures in soil.

Supplementary material

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

Data availability

The raw data produced in this research is available on Zenodo, an open-access repository for research data. The datasets can be accessed at <https://doi.org/10.5281/zenodo.16685813>.

Author contributions

Marina Bongers (Conceptualization, Formal analysis, Investigation, Methodology, Writing—original draft), Miranda Mesman (Formal analysis, Investigation, Writing—original draft), Claus Svendsen (Formal analysis, Methodology, Writing—review & editing), and Cornelis A.M. van Gestel (Conceptualization, Formal analysis, Funding acquisition, Methodology, Supervision, Writing—review & editing)

Funding

This study was supported by a grant from the European Union (R&D Program: Environment and Climate: Project NO. ENV4-CT97-0507), with data analysis partly supported by Horizon Europe agreement No 101057014.

Conflicts of interest

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

Acknowledgments

Thanks are due to R.A. Verweij and J.E. Koolhaas-Van Hekezen for technical assistance and G. Xie for preparing isoboles.

References

- Amorim, M. J. B., Pereira, C., Menezes-Oliveira, V. B., Campos, B., Soares, A. M. V. M., & Loureiro, S. (2012). Assessing single and joint effects of chemicals on the survival and reproduction *Folsomia candida* (Collembola) in soil. *Environmental Pollution*, 160, 145–152. <http://doi.org/10.1016/j.envpol.2011.09.005>
- Anaman, R., Peng, C., Jiang, Z., Amanze, C., & Ataa Fosua, B. (2024). Distinguishing the contributions of different smelting emissions to the spatial risk footprints of toxic elements in soil using PMF, Bayesian isotope mixing models, and distance-based regression. *Science of the Total Environment*, 933, 173153. <https://doi.org/10.1016/j.scitotenv.2024.173153>
- Ardestani, M. M., Van Straalen, N. M., & Van Gestel, C. A. M. (2014). The relationship between metal toxicity and biotic ligand binding affinities in aquatic and soil organisms: A review. *Environmental Pollution*, 195, 133–147. <https://doi.org/10.1016/j.envpol.2014.08.020>
- Backhaus, T., & Faust, M. (2012). Predictive environmental risk assessment of chemical mixtures: A conceptual framework. *Environmental Science & Technology*, 46, 2564–2573. <https://doi.org/10.1021/es2034125>
- Bongers, M., Rusch, B., & Van Gestel, C. A. M. (2004). The effect of counterion and percolation on the toxicity of lead for the springtail *Folsomia candida* in soil. *Environmental Toxicology and Chemistry*, 23, 195–199. <https://doi.org/10.1897/02-508>
- Bruus Pedersen, M., Van Gestel, C. A. M., & Elmegaard, N. (2000). Effects of copper on reproduction of two collembolan species exposed through soil, food and water. *Environmental Toxicology and Chemistry*, 19, 2579–2588. <https://doi.org/10.1002/etc.5620191026>
- Cai, Y., Xu, W., Wang, M., Chen, W., Li, X., Li, Y., & Cai, Y. (2019). Mechanisms and uncertainties of Zn supply on regulating rice Cd uptake. *Environmental Pollution*, 253, 959–965. <https://doi.org/10.1016/j.envpol.2019.07.077>
- Crommentuijn, T., Doornekamp, A., & Van Gestel, C. A. M. (1997). Bioavailability and ecological effects of cadmium on *Folsomia candida* (Willem) in an artificial soil substrate as influenced by pH and organic matter. *Applied Soil Ecology*, 5, 261–271. [https://doi.org/10.1016/S0929-1393\(97\)00003-6](https://doi.org/10.1016/S0929-1393(97)00003-6)
- Crouau, Y., Chenon, P., & Gisclard, C. (1999). The use of *Folsomia candida* (Collembola, Isotomidae) for the bioassay of xenobiotic substances and soil pollutants. *Applied Soil Ecology*, 12, 103–111. [https://doi.org/10.1016/S0929-1393\(99\)00002-5](https://doi.org/10.1016/S0929-1393(99)00002-5)
- Fountain, M. T., & Hopkin, S. P. (2005). *Folsomia candida* (Collembola): A “standard” soil arthropod. *Annual Review of Entomology*, 50, 201–222. <https://doi.org/10.1146/annurev.ento.50.071803.130331>
- Gomez-Eyles, J. L., Svendsen, C., Lister, L., Martin, H., Hodson, M. E., & Spurgeon, D. J. (2009). Measuring and modelling mixture toxicity of imidacloprid and thiacloprid on *Caenorhabditis elegans* and *Eisenia fetida*. *Ecotoxicology and Environmental Safety*, 72, 71–79. <https://doi.org/10.1016/j.ecoenv.2008.07.006>
- Gong, B., Qiu, H., Romero-Freire, A., Van Gestel, C. A. M., & He, E. (2022). Incorporation of chemical and toxicological availability into metal mixture toxicity modelling: State of the art and future perspectives. *Critical Reviews in Environmental Science and Technology*, 52, 1730–1772. <https://doi.org/10.1080/10643389.2020.1862560>
- Hopkin, S. P. (1989). *Ecophysiology of metals in terrestrial invertebrates. Pollution monitoring series*. Elsevier.
- Hou, D., Jia, X., Wang, L., McGrath, S. P., Zhu, Y.-G., Hu, Q., Zhao, F.-J., Bank, M. S., O'Connor, D., & Nriagu, J. (2025). Global soil pollution by toxic metals threatens agriculture and human health. *Science*, 388, 316–321. <https://doi.org/10.1126/science.adr5214>
- International Standardization Organization (ISO). (1999). Soil quality-inhibition of reproduction of Collembola (*Folsomia candida*) by soil pollutants. ISO 11267.
- Jonker, M. J., Sweijen, R. A. J. C., & Kammenga, J. E. (2004). Toxicity of simple mixtures to the nematode *Caenorhabditis elegans* in relation to soil sorption. *Environmental Toxicology and Chemistry*, 23, 480–488. <https://doi.org/10.1897/03-29>
- Jonker, M. J., Svendsen, C., Bedaux, J. J. M., Bongers, M., & Kammenga, J. E. (2005). Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio-dependent effects in mixture dose-response analysis. *Environmental Toxicology and Chemistry*, 24, 2701–2713. <https://doi.org/10.1897/04-431R.1>
- Kilpi-Koski, J., Penttinen, O.-P., Väisänen, A. O., & Van Gestel, C. A. M. (2020). Toxicity of binary mixtures of Cu, Cr and As to the

- earthworm *Eisenia andrei*. *Ecotoxicology*, 29, 900–911. <https://doi.org/10.1007/s10646-020-02240-1>
- Marino, F., Sturzenbaum, S. R., Kille, P., & Morgan, A. J. (1998). Cu-Cd interactions in earthworms maintained in laboratory microcosms: The examination of a putative copper paradox. *Comparative Biochemistry and Physiology Part C*, 120, 217–223. [https://doi.org/10.1016/S0742-8413\(98\)00043-7](https://doi.org/10.1016/S0742-8413(98)00043-7)
- Posthuma, L., Baerselman, R., Van Veen, P. M., & Dirven-Van Breemen, E. M. (1997). Single and joint effects of copper and zinc on reproduction of *Enchytraeus crypticus* in relation to sorption of metals in soil. *Ecotoxicology and Environmental Safety*, 38, 108–121. <https://doi.org/10.1006/eesa.1997.1568>
- Qiu, H., Vijver, M. G., He, E., Liu, Y., Wang, P., Xia, B., Smolders, E., Versieren, L., & Peijnenburg, W. J. G. M. (2015). Incorporating bioavailability into toxicity assessment of Cu-Ni, Cu-Cd, and Ni-Cd mixtures with the extended biotic ligand model and the WHAMF(tox) approach. *Environmental Science and Pollution Research International*, 22, 19213–19223. <https://doi.org/10.1007/s11356-015-5130-2>
- Sandifer, R. D., & Hopkin, S. P. (1997). Effects of temperature on the relative toxicities of Cd, Cu, Pb, and Zn to *Folsomia candida* (Collembola). *Ecotoxicology and Environmental Safety*, 37, 125–130. <https://doi.org/10.1006/eesa.1997.1536>
- Scherger, L. E., Luengo, C. V., Lafont, D., Lexow, C., & Avena, M. J. (2024). Fractionation and leaching of Cd, Cu, Fe, Pb, and Zn from smelter residues of an old environmental liability in Argentina. *Chemosphere*, 364, 143019. <https://doi.org/10.1016/j.chemosphere.2024.143019>
- Sharma, S. S., Schat, H., Vooijs, R., & Van Heerwaarden, L. M. (1999). Combination toxicology of copper, zinc, and cadmium in binary mixtures: Concentration-dependent antagonistic, nonadditive, and synergistic effects on root growth in *Silene vulgaris*. *Environmental Toxicology and Chemistry*, 18, 348–355. <https://doi.org/10.1002/etc.5620180235>
- Smit, C. E., & Van Gestel, C. A. M. (1996). Comparison of the toxicity of zinc for the springtail *Folsomia candida* in artificially contaminated and polluted field soils. *Applied Soil Ecology*, 3, 127–136. [https://doi.org/10.1016/0929-1393\(95\)00078-X](https://doi.org/10.1016/0929-1393(95)00078-X)
- Smit, C. E., & Van Gestel, C. A. M. (1998). Effects of soil type, pre-percolation, and ageing on bioaccumulation and toxicity of zinc for the springtail *Folsomia candida*. *Environmental Toxicology and Chemistry*, 17, 1132–1141. <https://doi.org/10.1002/etc.5620170621>
- Spurgeon, D. J., Jones, O. A. H., Dorne, J. L. C. M., Svendsen, C., Swain, S., & Sturzenbaum, S. R. (2010). Systems toxicology approaches for understanding the joint effects of environmental chemical mixtures. *Science of the Total Environment*, 408, 3725–3734. <https://doi.org/10.1016/j.scitotenv.2010.02.038>
- Sterenberg, I., Vork, N. A., Verkade, S. K., van Gestel, C. A. M., & van Straalen, N. M. (2003). Dietary zinc reduces uptake but not metallothionein binding and elimination of cadmium in the springtail, *Orchesella cincta*. *Environmental Toxicology and Chemistry*, 22, 1167–1171. <https://doi.org/10.1002/etc.5620220528>
- Svendsen, C., Spurgeon, D. J., McClennan, D., Green Etxabe, A., & Van Gestel, C. A. M. (2026). Interactive toxicity of two commonly co-occurring metals zinc and cadmium to earthworms in a natural soil. *Environmental Toxicology and Chemistry*, 45, 438–447. <https://doi.org/vu-nl.idm.oclc.org/10.1093/etjnl/vgaf295>
- Swain, S. C., Keusekotten, K., Baumeister, R., & Stürzenbaum, S. R. (2004). *C. elegans* metallothioneins: New insights into the phenotypic effects of cadmium toxicosis. *Journal of Molecular Biology*, 341, 951–959. <https://doi.org/10.1016/j.jmb.2004.06.050>
- Van der Geest, H. G., Greve, G. D., Boivin, M. E., Kraak, M. H. S., & Van Gestel, C. A. M. (2000). Mixture toxicity of copper and diazinon to larvae of the mayfly (*Ephoron virgo*) judging additivity at different effect levels. *Environmental Toxicology and Chemistry*, 19, 2900–2905. <https://doi.org/10.1002/etc.5620191208>
- Van Gestel, C. A. M., & Hensbergen, P. J. (1997). Interaction of Cd and Zn toxicity for *Folsomia candida* Willem (Collembola: Isotomidae) in relation to bioavailability in soil. *Environmental Toxicology and Chemistry*, 16, 1177–1186. <https://doi.org/10.1002/etc.5620160612>
- Van Gestel, C. A. M., Jonker, M., Kammenga, J. E., Laskowski, R., & Svendsen, C. (2010). *Mixture toxicity: Linking approaches from ecological and human toxicology*. SETAC.
- Van Gestel, C. A. M., & Van Diepen, A. M. F. (1997). The influence of soil moisture content on the bioavailability and toxicity of cadmium for *Folsomia candida* Willem (Collembola: Isotomidae). *Ecotoxicology and Environmental Safety*, 36, 123–132. <https://doi.org/10.1006/eesa.1996.1493>
- Van Gestel, C. A. M., & Koolhaas, J. E. (2004). Water-extractability, free ion activity, and pH explain cadmium sorption and toxicity to *Folsomia candida* (Collembola) in seven soil-pH combinations. *Environmental Toxicology and Chemistry*, 23, 1822–1833. <https://doi.org/10.1897/03-393>
- Van Loon, S., Xie, G., Svendsen, C., Kraak, M. H. S., De Jeu, L., Schut, N. C., Sprokkereef, E., Hurley, R., Van Wezel, A. P., & Van Gestel, C. A. M. (2025). Microplastics and PFAS as ubiquitous pollutants affect potencies of highly toxic chemicals in mixtures. *Journal of Hazardous Materials*, 500, 140493. <https://doi.org/10.1016/j.jhazmat.2025.140493>
- Van Straalen, N. M., Burghouts, T. B. A., Doornhof, M. J., Groot, G. M., Janssen, M. P. M., Joosse, E. N. G., Van Meerendonk, J. H., Theeuwes, J. P. J. J., Verhoef, H. A., & Zoomer, H. R. (1987). Efficiency of lead and cadmium excretion in populations of *Orchesella cincta* (Collembola) from various contaminated forest soils. *Journal of Applied Ecology*, 24, 953–968. <https://doi.org/10.2307/2403992>
- Vijver, M., Jager, T., Posthuma, L., & Peijnenburg, W. (2001). Impact of metal pools and soil properties on metal accumulation in *Folsomia candida* (Collembola). *Environmental Toxicology and Chemistry*, 20, 712–720. <https://doi.org/10.1002/etc.5620200404>