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Qiu, H.

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Author: Qiu, Hao

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Chapter V

Interactions Of Cadmium And Zinc Impact Their Toxicity To The Earthworm *Aporrectodea Caliginosa*

Hao Qiu, Martina G. Vijver, Willie J.G.M. Peijnenburg

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Abstract

Individual and binary mixture effects of Cd and Zn on mortality of the earthworm *Aporrectodea caliginosa* were investigated in one soil type. Mutual, systemic interactions of Cd and Zn were assessed over a broad range of concentration combinations. Exposures were expressed as measured total concentrations in soil, porewater concentrations, and CaCl₂-extractable concentrations. The Cd-Zn and Zn-Cd interactions in soil were estimated by comparing partition coefficients (K_d) of one metal in the presence and absence of the second metal. Simple patterns of mixture toxicity (additive, antagonistic or synergistic) were evaluated by the toxic unit approach. The more complex patterns (dose ratio-dependent or dose level dependent) were quantified by the MIXTOX model. The partitioning of Cd and Zn between soil and porewater was affected neither by their concentration nor presence of the other metal, and metal partitioning remained linear at all exposure levels tested. The effect of the Cd and Zn mixtures on mortality of *A. caliginosa* was mainly antagonistic, and the magnitude of antagonism (1.1 to 2.7 toxic units) was dependent upon both the relative concentrations of Cd and Zn and the concentration magnitudes. Interactions of both metals occurred at the organism level and were manifested in terms of effects (28-day median lethal concentrations). This study highlights the importance of identifying the relative influence of various interactions from external exposure to internal assimilation in evaluating mixture toxicity.

5.1 Introduction

Cadmium (Cd) is highly toxic and has no known physiological function. Zinc (Zn) is an essential micronutrient for most organisms, but causes toxicity at high concentrations (Rüdiger and Ralf-Rainer, 2010). Cadmium and zinc have many similar physicochemical properties as Cd appears below Zn in group IIB of the periodic table. They are usually found together in nature and compete with each other for various ligands owing to structural electronic similarities (Das et al., 1997). The fact that Cd is non-essential metal and Zn essential makes this association interesting as it raises the possibility that the toxicity of one metal may be prevented or aggravated in the presence of the other. Therefore, an understanding of the mutual interactions of Cd and Zn in mixtures and the impact on organisms is important.

The toxicity of individual metals to a wide range of soil dwelling organisms has been extensively investigated and is well documented (Das et al., 1997; Fitzpatrick et al., 1996; Spurgeon et al., 2000). However, as contamination in the environment generally consists of mixtures of varying composition, investigations on singular metal effects may have little real environmental relevance. Multiple metals can interact with each other in varying degrees to produce joint toxicity, which may be similar to, stronger or weaker than expected from exposure to a single metal (Zidar et al., 2009). In addition, interactions between different components may also depend on the mixture concentration or ratios of concentrations (Jonker et al., 2005). It is unrealistic and infeasible to assess every possible mixture combination. Toxicologists have therefore developed two prominent models for predicting mixture toxicity: the concentration addition and independent action models (Altenburger et al., 2004). Concentration addition model is based on the assumption that mixture constituents with a similar mode of action will act additively, while independent action model assumes that mixture components have dissimilar action modes. Mixture effects can be quantified using these two models (Jonker et al., 2005).

In the soil ecosystem, mixture toxicity is complex to study because interactions of metals can occur at various levels (Dickson et al., 1994), e.g. the exposure level, the uptake level, the target level (Rüdiger and Ralf-Rainer, 2010) and the internal pathway of detoxification (protective effect) (Vijver et al., 2011). For the purpose of toxicity assessment of metal mixtures, it is crucial to acknowledge all relevant interaction levels.

Specific toxic effects of Cd have been related mainly to Cd-induced oxidative damage and the interference with essential elements including Zn (Brzóska and Moniuszko-Jakoniuk, 2001; Jihen et al., 2010). Cadmium may displace Zn in a number of biological processes due to a greater affinity for S-ligands and N-donors. Zinc plays a fundamental role in various metabolic processes (Brzóska and Moniuszko-Jakoniuk, 2001). A deficiency in zinc can cause malfunctions of some organs and functions of organisms. However, excess Zn may also induce toxic effects (Rüdiger and Ralf-Rainer, 2010). It is generally agreed upon that Zn has beneficial actions against either Cd-induced oxidative stress (Jihen et al., 2009) or other Cd-induced toxic effects (Jihen et al., 2008). Increasing Zn supply may reduce Cd sorption and accumulation (Brzóska and Moniuszko-Jakoniuk, 2001), whereas Zn deficiency can intensify Cd accumulation and toxicity (Tang et al., 1998).

Mutual interactions of Cd and Zn do not only occur at the organism level. In soil, metals compete for sorption sites, resulting in competition at the exposure level and a specific distribution of interacting metals over different metal species. Previous studies have suggested that for soil organisms exposure takes place via the porewater or that uptake of metal is mediated by a porewater related route (Janssen et al., 1997; Vijver et al., 2003). The porewater hypothesis proposes that free metal ions in porewater are the major toxic species available for uptake by soil organisms (Plette et al., 1999; Van Gestel and Koolhaas, 2004).

Due to the interactions at the exposure level, bioavailability cannot be ignored in assessing mixture toxicity. It is widely recognized that total concentration is not sufficient to properly quantify metal bioavailability in soil (Sauvé et al., 2000). The bioavailable fraction is the amount of a metal in soil that is either available or potentially available for uptake by organisms (Peijnenburg et al., 2007). By using the concentration of bioavailable metal fraction for exposure assessment for soil organisms, more insight into mixture effects could be obtained. Alternative methods to establish the bioavailable metal content of a soil, like porewater collection or extraction with 0.01 M CaCl₂, seem to provide a feasible addition in this respect (Peijnenburg et al., 2007; Peijnenburg and Jager, 2003). Porewater metal concentrations are easily biological available within a short time span (Peijnenburg and Jager, 2003; Vijver et al., 2003). The fraction associated with CaCl₂ extraction is often supposed to reflect the potentially bioavailable metal concentrations, including soluble metal pools and labile metal bound to solid phases in soil matrix (Houba et al., 1996; Kalis et al., 2008). To get the full picture of the bioavailable metal fraction, a number of abiotic modifying factors (e.g. pH, competing ions, and complexing ligands) in soil should be considered as well (Peijnenburg and Jager, 2003).

This study aims to quantify the impact of Cd addition on chronic Zn toxicity with LC50 (concentration producing 50% mortality) as the endpoint for the earthworm *A. caliginosa* and vice versa, and to determine where the interactions possibly occur and their influence on the toxicity pattern of binary metal mixtures.

Soil pH is generally accepted as the dominant factor influencing metal bioavailability and toxicity (Sauvé et al., 2000). In this experiment, where pH was relatively constant, competition from Cd and Zn is presumably the main variable with respect to metal bioavailability and toxicity. It is hypothesized that competition and replacement between metals will take place at both the exposure and the organism level (sites of toxic action) (Brzóska and Moniuszko-Jakoniuk, 2001). Toxicity of Zn increases with increasing Cd concentration, because the fraction of biological action sites bound to Cd will increase. In the case of fixed Cd concentrations, a protective effect of Zn on Cd toxicity will be expected under Zn addition, as many studies have reported beneficial actions of Zn against Cd induced oxidative stress or other toxic effects (Jihen et al., 2009; 2010).

5.2 Materials and methods

Soil

Soil was sampled from a nonpolluted grassland in Boxtel, The Netherlands. The physicochemical properties of the soil sample tested are shown in Table S5.1 (Supporting Information). The soil was air dried, homogenized and passed through a 2 mm sieve before

use. It was then spiked with a wide range of concentrations of Cd alone and Zn alone, and different combinations of a mixture of Cd and Zn. The metals were added as their acetate-salts (Acros Chemicals, Leicestershire, UK; purity 96%) and allowed to equilibrate for at least two months at 35 °C to eliminate the acetate by mineralization. A pilot study showed that the net result of acetate mineralization and hydrolysis of the Cd and Zn ions released induces only marginal effects on soil pH.

Toxicity tests

The earthworm *Aporrectodea caliginosa* was used in the toxicity bioassays. This endogeic earthworm lives in the upper 25 cm of the soil and feeds on decayed organic matter and bits of mineral soil (Spurgeon et al., 2000). It spreads over large areas in the Netherlands and other European countries (Spurgeon et al., 2000; Vijver et al., 2003). Amongst others, it has the ability to survive in water-saturated soils and can be used in water-only exposures. As such it is an earthworm species that is often used in laboratory testing (Khalil et al., 1996; Spurgeon et al., 2000; Vijver et al., 2003;). Mature earthworms were collected from a sandy, uncontaminated soil located in Liempde, The Netherlands. The earthworms, each weighing from 1200 to 2000 mg (5 to 8 cm in length), were kept in the laboratory for at least one week in the soil from which they originally were collected at 15 ± 2 °C. This pre-incubation period was necessary to allow the earthworms to get adapted to the exposure conditions. Thereafter they were exposed to Cd alone, Zn alone, or a mixture of Cd and Zn for 28 days. Cadmium toxicity was tested at twelve concentrations ranging from nominal 0 to 1000 mg Cd/kg soil. Fifteen concentrations of Zn were set ranging from nominal 0 to 1500 mg Zn/kg soil. All concentrations are given as dry weights. Cadmium and zinc mixtures were designed as follows: at each constant Cd concentration, different concentrations of Zn were added, or different concentrations of Cd were added at each fixed Zn concentration. Detailed concentration combinations of the spiked Cd and Zn are illustrated in Figure 5.1. Exposures were conducted in plastic boxes filled with 350 g of the different treatments of soils. Four earthworms were used in each treatment. A limited number of replicates were conducted as LC50 values of duplicates were within 8% deviation. All soils were kept at 80 % maximum water holding capacity during the experiment. Continuous illumination (1500 Lux) was used to avoid a possible escape of earthworms from the containers. Earthworms were not fed during the experiment. Mortality was checked after 28 d of exposure. Earthworms were counted as dead if they did not respond to a gentle mechanical stimulus. The number of earthworms that had survived and their body weights were recorded. In the control (no metal added), mortality of the earthworms was zero and no significant loss of weight ($p > 0.05$) was observed after 28 d of exposure.

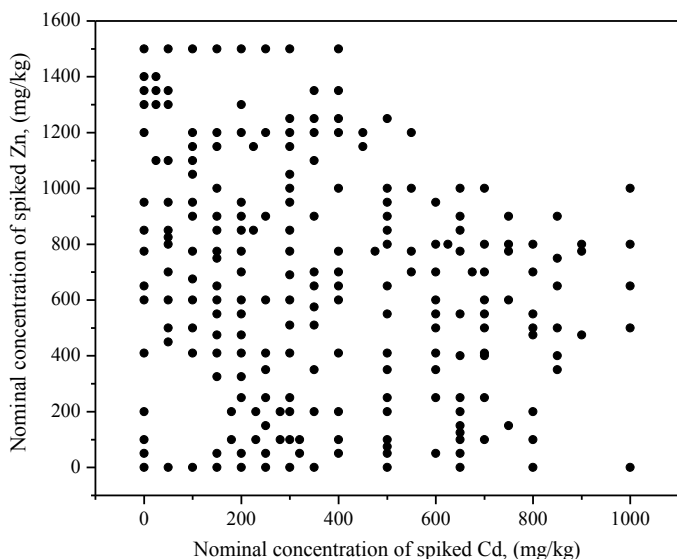


Figure 5.1 Concentration combinations of Cd and Zn spiked for mixture exposures

Chemical analysis

After 28 d of exposure, soil samples were dried at 40°C and sieved (< 2 mm). Total metal concentration of the soil was measured after digestion with *aqua regia* in a microwave oven (Milestone ETHOS900, Italy). For quality assurance purposes, the certified reference material CRM 483 (the Community Bureau of Reference, BCR, Brussels, Belgium) was used for each of 30 samples. Recovery was $95\% \pm 14\%$. Organic matter content was determined by heating soil that had been dried for 3 h at 100 °C to 550 °C for 3 h. Clay content and cation exchange capacity (CEC) were determined by the hydrometer method and the ammonium acetate method respectively (Pansu and Gautheyrou, 2006). After toxicity tests, porewater was sampled by means of centrifugation and subsequent filtration through 0.45 µm acetate filter of soil samples at 80% maximum water holding capacity (15 °C). Some soil samples were taken before, during and after the tests. No noteworthy differences in metal contents of porewater were observed among different sampling periods. Soil pH was measured in the porewater samples using a Metrohm 691 pH Meter equipped with a combination pH glass electrode (Metrohm AG, Herisau, Switzerland). Dissolved organic carbon (DOC) in the porewater was measured with a TOC/DOC analyzer (TOC-VCSH, Shimadzu, Kyoto, Japan). Chemical extraction was performed using 10 g of soil in 10 ml of 0.01 M CaCl₂ and the suspension was shaken for 24 h. Metal concentrations were determined by inductively coupled plasma atomic emission spectroscopy (Spectro Analytical Instruments, Kleve, Germany). The detection limits for Zn and Cd were 0.2 µg/L and 0.1 µg/L, respectively.

Data analysis

Median lethal concentrations (LC50) were calculated by probit analysis using software SPSS 16.0 (IBM, Chicago, USA). In case no exact LC50 could be calculated because of a very steep dose-response relationship, a binominal approach was used in which the LC50 was set equal to the arithmetic average of the maximum concentration causing 0% mortality and the minimum concentration causing 100% mortality. Relationships between Cd and Zn concentrations in total soil, porewater and in CaCl₂-extracts were determined by means of general linear regression using software GraphPad Prism 5.0 (GraphPad Software Inc., California, USA).

To address the toxic effects in the mixtures, the observed effect was compared to the expected effect of mixtures that calculated from the single metal exposure toxicities. This procedure was based on the concentration addition model:

$$TU_{\text{mix}} = \sum_{i=0}^n \frac{c_i}{EC_{xi}} \quad (5-1)$$

where c_i (mg/kg) is the concentration used for metal i in the mixture and EC_{xi} (mg/kg) is the standard effect concentration of metal i . TU_{mix} is therefore a dimensionless ratio that can be regarded as the sum of each toxic unit (TU). In the present study, mortality was selected as the endpoint in the binary mixtures of Cd and Zn, so the above equation can be expressed as:

$$TU_{\text{mix}} = \frac{c_{\text{Cd}}}{\text{LC50 of single Cd}} + \frac{c_{\text{Zn}}}{\text{LC50 of single Zn}} \quad (5-2)$$

where c_{Cd} and c_{Zn} are the experimental concentrations at which we observed 50% mortality. In a mixture of Cd and Zn, strict additivity occurs when the $TU_{\text{mix}}=1$. Values of TU_{mix} exceeding 1 describe antagonism, while values below 1 denote synergy. When manually calculating TU_{mix} , additivity was allowed to deviate up to 10% before the effect of a mixture was assigned as being either antagonistic or synergistic. This deviation was the conservative choice considering the actual experimental condition.

More complex calculations using the concentration addition model including dose ratio (DR) and dose level (DL) were analyzed using the MIXTOX modules (Jonker et al., 2005). Hence, deviation from additivity could then be quantified using significance tests. The binary TU_{mix} model was generalized to

$$\frac{c_1}{f_1^{-1}(Y)} + \frac{c_2}{f_2^{-1}(Y)} = \exp(G) \quad (5-3)$$

Where c_1 and c_2 indicate the concentration of individual metals in the mixture, f_1^{-1} and f_2^{-1} indicate the inverse dose-response functions of the single metal in the mixture. Y defines the biological response and G was an extent function used to quantify deviation from additivity. Extra parameters a and b were introduced into the model using a stepwise approach to describe deviation. The model was fitted to the data using the method of maximum likelihood while minimizing the sum of the square residuals. The statistical significance of the improvement in fit from the extended parameters was obtained through Chi-square (χ^2) tests. The biological interpretations of the extra parameters are listed in Table 5.1 and can also be found in Jonker et al. (Jonker et al., 2004; 2005).

Table 5.1 Interpretation of parameters (*a* and *b*) that define the interaction patterns in MIXTOX model (adapted from Jonker et al. 2005)

Pattern	Parameter	Value	Interpretation
Synergy or antagonism(S/A)	<i>a</i>	<0	Synergy
		>0	Antagonism
Dose-ratio dependence(DR)	<i>a</i>	<0	Synergy, except for those mixture ratios where significant positive b_i indicate antagonism
		>0	Antagonism, except for those mixture ratios where significant negative b_i indicate synergy
	b_i	>0	Antagonism where the toxicity of the mixture is caused mainly by toxicant <i>i</i>
		<0	Synergy where the toxicity of the mixture is caused mainly by toxicant <i>i</i>
Dose-level dependence(DL)	<i>a</i>	<0	Synergy at low dose level and antagonism at high
		>0	Antagonism at low dose level and Synergy at low
	b_{DL}	>1	Change at lower dose level than the EC50
		=1	Change at EC50 level
		0~1	Change at higher dose level than the EC50
<0	No change, but the magnitude of synergy or antagonism is dose level dependent		

5.3 Results

Soil and porewater properties

The organic carbon content of the soil used was 5.7 ± 0.2 %. All values are presented as arithmetic means of three replicates and standard deviation unless stated otherwise. Clay content accounted for 7.0 ± 0.6 % and CEC was 10.1 ± 0.3 cmol/kg. The pH of the porewater from the original soil sample was 6.67 ± 0.15 . The addition of Cd, or Zn, or a mixture of Cd and Zn had negligible effect (usually < 0.3 unit) on the porewater pH (Data not shown). In all metal-spiked soil samples, the porewater pH was 6.49 ± 0.28 , which was not statistically different ($p > 0.05$) from the pH prior to spiking. The DOC concentration in the porewater of the untreated soil was 171.4 ± 9.8 mg/L. Under the influence of metal addition, the concentration of DOC decreased to an average of 122 ± 13.1 mg/L after 28 d of exposure.

For both metals, the nominal total concentrations correlated linearly with the concentrations measured in total soil, porewater and CaCl₂-extracts (see Table 5.2). In this experiment, not all metal concentrations were measured. Instead, the missing values were estimated based on the nominal total concentration using the equations listed in Table 5.2. Measured total metal concentrations in soil were in agreement with the nominal total concentrations (usually <10 % deviation). The measured total Cd concentration showed a good linear relationship with the porewater Cd concentration and the amount of CaCl₂-extractable Cd. The measured total Zn concentration also was significantly correlated with the Zn concentration in the porewater ($p < 0.0001$) and the Zn concentration in CaCl₂ extracts

($p < 0.0001$). Addition of pH or DOC as explanatory variables did not significantly improve the regression equation presented in Table 5.2 (Data not shown).

Table 5.2 Linear regression relationships between nominal total concentrations (mg/kg), measured total concentrations (mg/kg), porewater concentrations ($\mu\text{g/L}$) and CaCl_2 -extractable concentrations (mg/kg) of metals

Equations	R^2 *	n	p	F
$\log(\text{measured total Cd})=0.93 \log(\text{nominal Cd})+0.21$	0.95	115	<0.0001	2300
$\log(\text{porewater Cd})=1.04 \log(\text{nominal Cd})+0.10$	0.81	95	<0.0001	395
$\log(\text{CaCl}_2\text{-extr. Cd})=1.03 \log(\text{nominal Cd})-0.57$	0.82	166	<0.0001	763
$\log(\text{porewater Cd})=1.17 \log(\text{measured total Cd})+0.65$	0.84	91	<0.0001	453
$\log(\text{CaCl}_2\text{-extr. Cd})=1.04 \log(\text{measured total Cd})-0.66$	0.67	95	<0.0001	185
$\log(\text{measured total Zn})=0.70 \log(\text{nominal Zn})+0.99$	0.92	116	<0.0001	1372
$\log(\text{porewater Zn})=0.82 \log(\text{nominal Zn})+1.58$	0.81	95	<0.0001	397
$\log(\text{CaCl}_2\text{-extr. Zn})=0.79 \log(\text{nominal Zn})-0.08$	0.77	171	<0.0001	567
$\log(\text{porewater Zn})=1.19 \log(\text{measured total Zn})+0.37$	0.83	91	<0.0001	440
$\log(\text{CaCl}_2\text{-extr. Zn})=1.08 \log(\text{measured total Zn})-1.03$	0.74	96	<0.0001	262

* R^2 is the coefficient of determination; n indicates the number of data points; p indicates the statistical significance level; F is the value of F test.

Partitioning of Cd and Zn mixtures

The mutual interactions of Cd and Zn at the exposure level were determined in a direct way, by comparison of partition coefficients of one metal in the presence and absence of the second metal. The partition coefficient is usually used to evaluate the partitioning of metal between the soil solid phase and solution phase. It can be defined as the ratio of the measured total concentration to the porewater concentration (K_d) (Sauvé et al., 2000), the ratio of the measured total concentration to the CaCl_2 -extractable concentration (K_d') (Luo et al., 2006), or the ratio of the CaCl_2 -extractable concentration to the porewater concentration (K_d'') (Goody et al., 1995). In the present study, all the above three forms of partition coefficients were adopted in order to provide more reliable evaluation of metal partitioning.

Partition coefficients of Cd and Zn presented singly and in mixtures are shown in Figure 5.2. In single metal exposures, the effects of metal concentrations on metal partitioning were negligible as the slopes were close to 0 (linear regression equations: $K_d(\text{Cd}) = -0.009 \text{ total Cd} + 83.0$ ($p > 0.05$, $n = 11$), $K_d(\text{Zn}) = -0.009 \text{ total Zn} + 115.1$ ($p > 0.05$, $n = 9$)). In the mixtures, partitioning of Zn was not significantly different from that in the treatments of Zn alone ($p > 0.05$). Partitioning of Cd was similar. The K_d values for Cd and Zn were on average $91 \pm 36 \text{ L/mg}$ ($n=91$) and $141 \pm 57 \text{ L/mg}$ ($n=91$) respectively. Nearly all values of K_d were within 50 to 125 L/kg for Cd and 50 to 200 L/kg for Zn (Figure 5.2, A, B). Values of K_d' (Figure 5.2, C, D) and K_d'' (Figure 5.2, E, F) for both metals mostly fell within a relatively narrow range. For all three forms of partition coefficients, the highest values were around 3 to 4 times higher than that of the lowest. Data points were evenly distributed between the highest and the lowest values. Metal partitioning into porewater, into CaCl_2 extracts and between porewater and CaCl_2 extracts was affected neither by their concentration nor presence of the other metal.

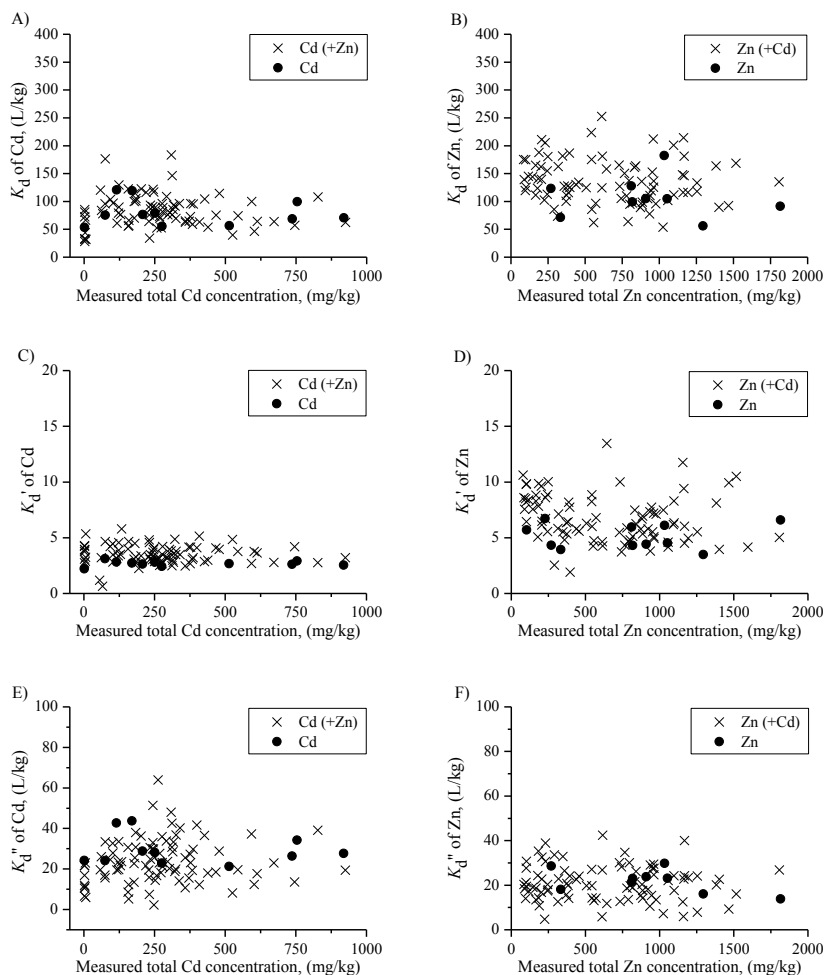


Figure 5.2 Partition coefficients of Cd and Zn presented singly (•) or in mixtures (×). “Cd (+Zn)” represents Cd partitioning in the presence of Zn of the whole concentration range studied, while “Zn (+Cd)” represents Zn partitioning in the presence of Cd of the whole concentration range studied. Partition coefficients were calculated as follows: the ratio of the measured total concentration to the porewater concentration (K_d , L/kg) (A, B), the ratio of the measured total concentration to the CaCl_2 -extractable concentration (K_d' , dimensionless) (C, D), and the ratio of the CaCl_2 -extractable concentration to the porewater concentration (K_d'' , L/kg) (E, F).

Single metal toxicity

Earthworm mortality increased with increasing total concentrations of each metal tested (Table S5.2, Supporting Information). Similar trends were also found when porewater concentration or CaCl_2 -extractable concentration was assumed to be the exposure pathway.

Values of LC50 of Cd alone and Zn alone for *A. caliginosa* on the basis of measured total, porewater and CaCl₂-extractable concentration are shown in Table 5.3. Cadmium caused higher mortality than Zn in *A. caliginosa* when present at the same concentration. Here, the relative toxicity of Cd was approximately 7 times higher than Zn on a molar basis (by dividing measured total concentration to molecular weight).

Table 5.3 Median lethal concentrations (LC50) of Cd, Zn for *Aporrectodea caliginosa* in single metal toxicity tests.

Expressions of exposure	LC50 (95% confidence interval)	
	Cd	Zn
Measured total (mg/kg)	344 (317-375)	1499 (1318-1592)
Porewater (µg/L)	4890 (4155-5416)	13588 (12850-14001)
CaCl ₂ -extractable (mg/kg)	112 (93-137)	317 (279-353)

Impact of Cd on Zn toxicity

Changes in 28 d LC50 values of Zn to *A. caliginosa* at various Cd levels are presented in Figure 5.3. Values of LC50 of Zn, expressed on the basis of measured total, porewater and CaCl₂-extractable concentrations, were plotted against added Cd concentration of the three corresponding Cd fractions. Generally, an elevation in Cd content coincided with a reduction in LC50 of Zn, the more Cd was added, the lower was the LC50 value of Zn.

Linear regression demonstrated that LC50 values of Zn decreased significantly upon increasing total Cd concentration ($p < 0.0001$) (Figure 5.3). The linear relationship became more apparent when LC50 values of Zn were expressed on the basis of porewater concentration of Cd or CaCl₂-extractable Cd concentration. All the LC50 values of Zn in mixtures were lower than that in the treatments of Zn alone. An increase in the measured total concentration of Cd from 2 mg/kg to 734 mg/kg resulted in a 2.5-fold decrease (1499 to 600 mg/kg of Zn) of the LC50 of Zn for *A. caliginosa*. Up to 3-fold reduction in LC50 values of porewater Zn was observed when the porewater concentration of Cd increased from 31 to 8194 µg/L. An almost 4-fold decrease in LC50 values of CaCl₂-extractable Zn was found when the concentration of CaCl₂-extractable Cd increased from 1 to 211 mg/kg. These results are consistent with the null hypothesis that Zn toxicity increases with the increasing Cd content.

The joint effects (TU_{mix}) of Cd-Zn were estimated using Equation 5-2 and presented in Figure 5.4. Detailed methods of calculation are shown in Tables S5.3 through S5.5 (Supporting Information). When the measured total concentration was used to express exposure, all values of TU_{mix} of Cd-Zn combinations were above 1. Cadmium addition at any ratio resulted in an antagonistic effect regarding Zn toxicity. TU_{mix} increased from 1.10 to 2.54 with increasing measured total Cd concentration, indicating that more Cd in the mixture resulted in the enhancement of the magnitude of antagonism. TU_{mix} values over the full concentration range decreased to some extent but were still above 1 when the exposures were normalized to porewater or CaCl₂-extractable metal concentrations instead of measured total concentrations. There was a tendency towards nearly concentration-addition (TU_{mix} close to 1)

when the concentrations of Cd in the porewater and the CaCl_2 -extractable Cd were less than $600 \mu\text{g/L}$ and 20 mg/kg respectively.

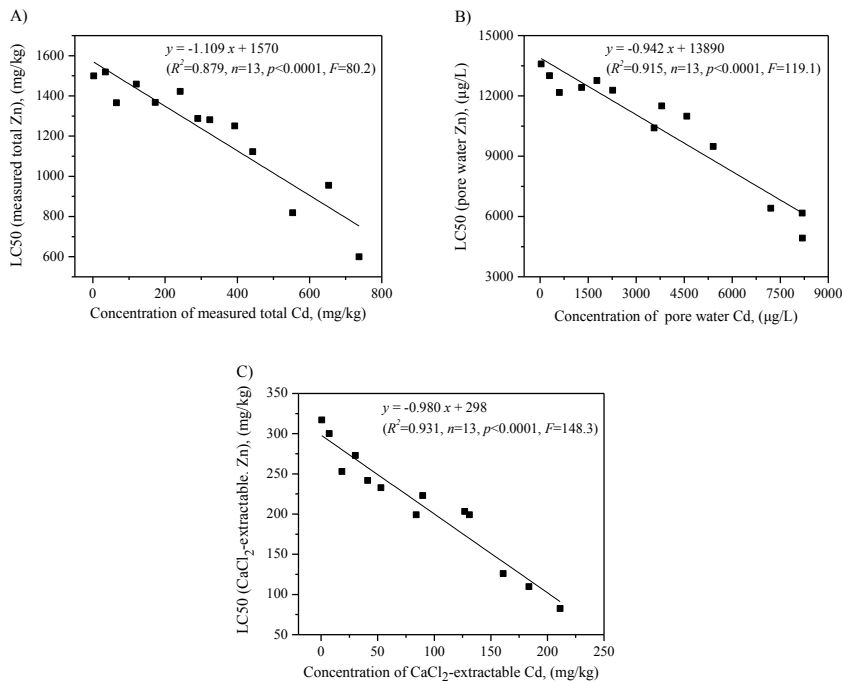


Figure 5.3 Median lethal concentrations (LC50) of Zn for *Aporrectodea caliginosa* under the influence of Cd after 28 d of exposures related to measured total (A), porewater (B) and CaCl_2 -extractable (C) concentrations. The solid line is the linear fit.

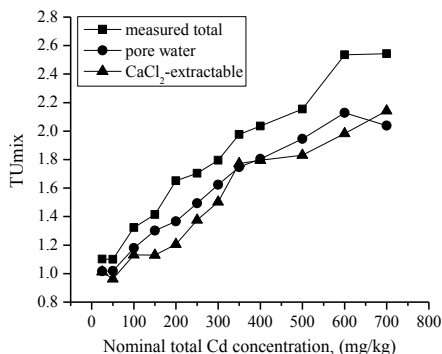


Figure 5.4 Toxic units (TU_{mix}) of Cd-Zn combinations (data points in Figure 5.3) causing 50% mortality of *Aporrectodea caliginosa*. Calculations of TU_{mix} were related to three different exposure metrics (measured total, porewater and CaCl_2 -extractable concentrations) using equation 5-2.

Impact of Zn on Cd toxicity

A typical observation when establishing dose-response curves was that Zn displayed a protective effect at Cd levels exceeding the LC50 of Cd alone. For instance at a nominal Cd-level of 500 mg/kg, 100% mortality was observed. Subsequent addition of a small amount of Zn alleviated toxicity and induced survival of the earthworms at the same Cd level. Higher concentrations of Zn (nominal total Zn > 600 mg/kg in this example) resulted in mortality again.

Variations in 28 d LC50 values of Cd to *A. caliginosa* at different Zn levels are plotted in Figure 5.5. The LC50 values of Cd varied over a range of Zn concentrations. Firstly, a substantial reduction of Cd toxicity under Zn addition was observed for all three expressions of metal concentrations. When measured total Zn was varied from 100 to 1000 mg/kg, LC50 values of Cd increased from 344 to 680 mg/kg. At these levels, Zn thus exerted a protective effect on Cd toxicity. Secondly, beyond a critical Zn concentration of around 1000 mg/kg for total Zn, 9000 µg/L for porewater Zn or 150 mg/kg for CaCl₂-extractable Zn, LC50 values of Cd decreased dramatically with increasing Zn concentration.

The patterns of Zn-Cd interaction reflected by the values of TU_{mix} are demonstrated in Figure 5.6. Calculation procedures are shown in Tables S5.6 through S5.8 (Supporting Information). It was found that TU_{mix} increased from 1.56 to 2.70 upon measured total Zn addition of 183 to 1087 mg/kg, and then reduced slowly to nearly 1 at higher concentrations of measured total Zn. The trends were similar for all three exposure metrics. Nearly all Zn-Cd mixtures showed an antagonistic relationship (TU_{mix} > 1.10) except when 1200 mg/kg of nominal total Zn was added. At this exception, the low LC50 value of Cd indicated that Zn is the dominant cause of mortality in the mixture.

Mixture toxicity modelling

The mixture data of all three exposure metrics were all analyzed. The parameters (*a* and *b*) and significance test results ($p(\chi^2)$) obtained from fitting the nested MIXTOX model are shown in Table 5.4. Generally, the interaction patterns reflected by the modelling were consistent with the results obtained by the calculations of TU_{mix}. In the present study, both the TU approach and MIXTOX modelling showed that the interaction of the metals studied was mainly antagonistic, and the magnitude was both dose ratio- and dose level-dependent.

Fitting of the concentration addition model to the data explained 47% of the variation in the data. Inclusion of parameter *a* in Equation 5-3 to describe synergy or antagonism decreased the sum of the square residuals significantly ($p(\chi^2) = 3.9 \times 10^{-12}$) and explained 57% of the variation in the data. Parameter *a* being positive was an indication of an antagonistic relationship between Cd and Zn. Extending the concentration addition model with a second parameter *b*_{Zn} to describe a dose ratio-dependent deviation provided the best description of the data and 61% of the variation in the data was explained. Estimated values of the deviation parameters were *a* = 3.94 and *b*_{Zn} = -3.99. It can be concluded from these values that the deviation from additivity was mainly antagonistic. An increased joint effect was connected to a high proportion of Zn in the mixture and a decreased joint effect was related to a high proportion of Cd in the mixture. Similar analysis demonstrated that including a second parameter *b*_{DL} to describe a dose level dependence also improved the model fit significantly. In this case, parameter *a* being positive and *b*_{DL} being negative

revealed that the interaction pattern was antagonism and the magnitude of antagonism was dose level dependent.

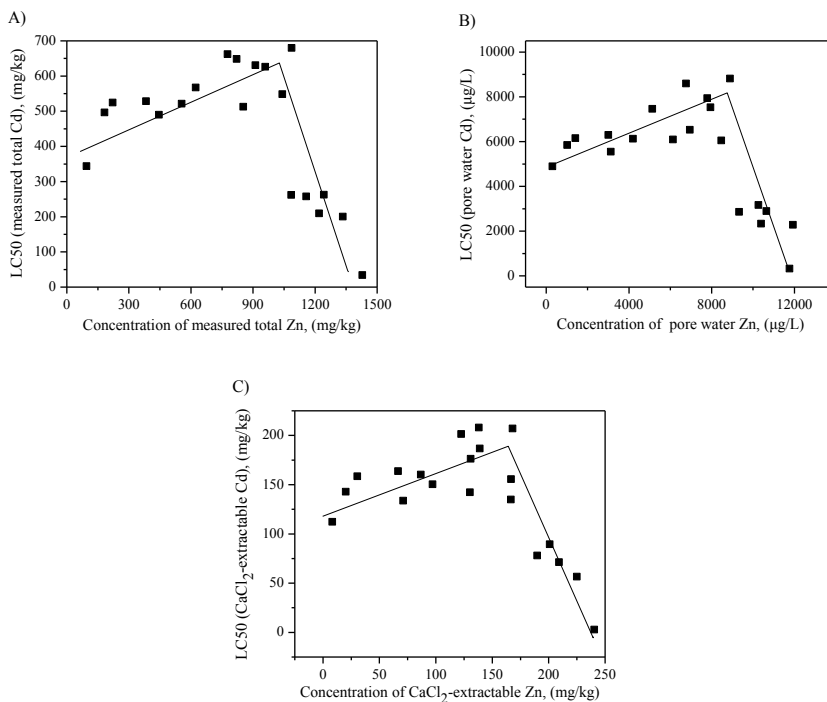


Figure 5.5 Median lethal concentrations (LC50) of Cd for *Aporrectodea caliginosa* under the influence of Zn after 28 d of exposures related to measured total (A), porewater (B) and CaCl₂-extractable (C) concentrations. The solid line is freehand and was added to schematically depict trends in mixture toxicity.

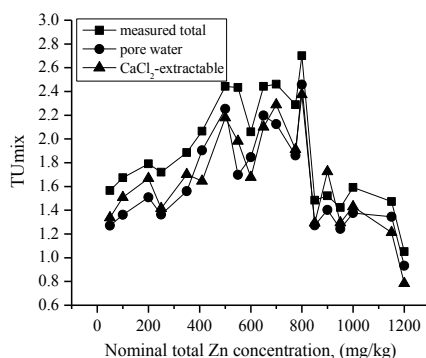


Figure 5.6 Toxic units (TU_{mix}) of Zn-Cd combinations (data points in Figure 5.5) causing 50% mortality of *Aporrectodea caliginosa*. Calculations of TU_{mix} were related to three different exposure metrics (measured total, porewater and CaCl₂-extractable concentrations) using equation 5-2.

When relating toxicity to the porewater concentrations or the CaCl₂-extractable concentrations, the identified interaction patterns were similar to those of the measured total concentrations.

Table 5.4 Parameter values of the deviation function and significance

	R^{2*}	a	b_{Zn}/b_{DL}	$p(\chi^2)$
Measured total				
CA**	0.47	NA	NA	NA
S/A	0.57	1.62	NA	3.9×10^{-12}
DR	0.61	3.94	-3.99	1.2×10^{-15}
DL	0.59	0.04	-23.82	3.5×10^{-13}
Porewater				
CA	0.48	NA	NA	NA
S/A	0.54	1.86	NA	8.6×10^{-8}
DR	0.59	4.27	-4.68	6.5×10^{-12}
DL	0.55	0.05	-22.96	4.8×10^{-8}
CaCl ₂ -extractable				
CA	0.52	NA	NA	NA
S/A	0.54	1.02	NA	5.1×10^{-4}
DR	0.58	3.67	-4.79	9.1×10^{-8}
DL	0.55	0.02	-42.17	5.3×10^{-4}

*The value R^2 quantifies goodness of fit. Parameters (a , b_{Zn}/b_{DL}) define the deviation patterns from concentration additive model, and $p(\chi^2)$ indicates the statistical outcome of the Chi-squared test. **CA indicates concentration addition; S/A indicates synergy/antagonism; DR indicates dose ratio dependent; DL indicates dose level dependent. NA indicates not applicable.

5.4 Discussion

A number of studies have examined the toxicity of Cd and Zn presented singly for selected life-cycle traits in earthworms (Fitzpatrick et al., 1996; Khalil et al., 1996; Spurgeon et al., 2000). Although variations in soil types, exposure time and species sensitivity all contributed to the variety of metal toxicity reported, the LC50 values of individual Cd and Zn in this study were still comparable to data in some literature (Fitzpatrick et al., 1996; Spurgeon et al., 2000). The effect of a mixture of Cd and Zn on the mortality of *A. caliginosa* was mainly antagonistic regardless of how the exposure conditions were measured. Previous studies revealed that the most frequently observed pattern of interaction in Cd and Zn mixtures was antagonism, followed by additivity (Khalil et al., 1996; Van Gestel and Hensbergen, 1997; Weltje, 1998). Possible interactions between Cd and Zn limited the predictive capability of the simple concentration-addition model. Mixture toxicity was a result of the simultaneous action at all interaction levels. This study focused on the interaction at the exposure level and at the toxicological level. The relative contributions of interactions outside the organism and inside the organism to toxic effects were then distinguished.

Partition coefficients of each component in the mixtures were predictive for metal interactions occurring in the soil. The average K_d value of metals in the present study indicated that the order of sorption affinity was $Zn > Cd$. Empirically, the metal with higher K_d value affected partitioning of the metal with lower K_d (Jonker et al., 2004). Here, however, the partitioning of Cd was largely unaffected by the presence of Zn, and vice versa. Apparently, sufficient soil sorption sites are available to preclude competition of Cd and Zn for sorption to the solid phase, as evidenced by the fairly constant K_d values found even at highly elevated Cd or Zn concentrations. Hence, there was no interaction of Cd and Zn at the exposure level. In a study carried out by Van Gestel and Hensbergen (1997), Cd did not affect the water-soluble concentration of Zn in an artificial soil substrate, in agreement with our results. On the other hand, water extractability of Cd was significantly increased by Zn ($p = 0.003$). These authors attributed this to increased complexation of Cd with chloride, which was less important for Zn. There was no chloride complexation in the soil we studied as the metals were spiked as their acetate salts. The addition of Cd alone and Zn alone and as a mixture did not cause a dose-related decrease of pH, and only minor fluctuations in pH values. This indicated that after equilibration for two months, the acetate had been mineralized. Zidar et al. (2009) found that water extractability of Cd in artificially contaminated food for the isopod *Porcellio scaber* was not significantly affected by the presence of Zn at any concentration, and vice versa ($p > 0.05$). Since metals were added as nitrates, they concluded that the organic food source, high in organic matter, probably accounted for the non-interaction between Cd and Zn at the exposure level.

Unraveling the role of interactions in soil requires further analysis of the relationship between toxic effects and metal concentrations occurring in different phases and species (Jonker et al., 2004). It is generally agreed that exposure is governed mainly by the solution phase (porewater), and the toxicity of metals is dependent on the nature of the metal species present (Peijnenburg et al., 2007; Peijnenburg and Jager, 2003; Saxe et al., 2001). Porewater and $CaCl_2$ -extractable concentrations were often used to express exposure and were assumed to be available for uptake (Janssen et al., 1997; Kalis et al., 2008). When examining the combined toxicity of simple mixtures, Jonker et al. (2004) found that the Freundlich adsorption constant of one metal was influenced by the presence of another metal, suggesting interaction at the exposure level. As a result, both the composition of the mixture and the relative toxicity of each mixture component differed between total, water-soluble, and $CaCl_2$ -extractable concentrations. In this experiment, relative consistent modes of interaction were observed when LC50s were expressed as total, porewater and $CaCl_2$ -extractable metal concentrations. One comment should be made with regard to the different expressions of exposure yielding similar results. It may be due partially to our use of only one soil type. Total metal concentrations correlated well with the porewater and $CaCl_2$ -extractable concentrations. As the bioavailability of metals can vary over several orders of magnitude depending on the soil properties, it is probably critical to include more soil types to study the interaction patterns of metal mixtures.

Metal toxicity involves three steps: bioavailable metal causes exposure; exposure leads to uptake; and effects result from reaction with a biological target. In *A. caliginosa* Cd and Zn interacted at the effect level. Addition of Cd induced higher Zn toxicity. Already at a low

concentration (measured total Cd = 65 mg/kg), Cd antagonized Zn toxicity. When the primary constituent was Cd, addition of Zn reduced toxicity.

Because competitive displacement between Cd and Zn did not occur in the soil we studied, we examined whether interactions could be identified during uptake and at the effect level. Competition between Cd and Zn during the uptake processes has been reported in plants (Cataldo et al., 1983). It is likely that Cd and Zn share the same pathway of uptake since they both belong to the group IIB of the periodic table and have similar physicochemical properties. Nevertheless, Li et al. (2010) provided evidence that the uptake of Cd by *Eisenia fetida* proceeds via Ca channels whereas Zn uptake is carrier-mediated by proteins or other sulphhydryl-containing compounds, implying that the mechanisms of Cd and Zn uptake in *Eisenia fetida* are essentially different. Therefore, Cd and Zn may employ different mechanisms in influencing the uptake of each other.

In addition, interactions at the internal pathways may happen, such as the synthesis of specific metal-binding proteins induced by another metal. Metallothionein (Mt), a metal-binding protein, plays an important role in preventing toxicity from heavy metals (e.g., Cd) by formation of metal-Mt compounds (Brzóska and Moniuszko-Jakoniuk, 2001). Metallothionein induced by Zn pretreatment or co-administration can immobilize Cd within the earthworms, which at least in part accounts for the protective action of Zn supply against Cd toxicity. Barata et al. (2006) mentioned that since both Cd and Zn can activate Mt synthesis, there is a high probability of competition with Mt compounds and that for this reason their combined effects may be expected to be antagonistic.

Different from traditional viewpoints, Wang et al. (2011b) argued that the actual toxic effect may not be due to interactions of the metals at binding sites. Their study showed that rather than site-specific competition, electrostatic interactions at the cell membrane surface of organisms may account for the alleviation of toxicity. It should be noted that all of the above assumptions of interaction at various uptake pathways or bindings sites only serve as examples of possible mechanisms in which interaction of the metals of interest could occur. More attention should be paid to the characterization of toxicokinetics and toxicodynamic of metals and target site interactions in future studies in order to obtain reliable interaction mechanisms and provide a robust biological basis for the observed differences.

After rescaling the concentrations in terms of toxic units, interactions between metals were obtained. When focusing on the impact of Cd on Zn toxicity, antagonism was noticed in nearly all cases. Since the addition of Cd linearly increased Zn toxicity, it was expected that the interaction type is synergy. On a molar basis, Cd is about 7 times more toxic than Zn. Therefore, the replacement of Zn by Cd in action sites may produce a larger adverse effect than Zn-only treatments. It is difficult to explain the reason why synergy was not observed. When focusing on the impact of Zn on Cd toxicity, it was found that Zn exerted a beneficial action on Cd toxicity when it was administrated at a measured total concentration in between 0 and 1000 mg/kg. Calculations of TU_{mix} revealed that the antagonistic effect of Cd and Zn coincided with the protective effect of Zn. The decreasing effects of Cd with increasing concentrations of Zn were described previously for the house cricket *Acheta domesticus* and the beetle *Tenebrio molitor* (Migula et al., 1989). The amount of Zn added is critical in determining whether a protective or harmful effect is exerted by Zn. When the total Zn

concentration was beyond 1000 mg/kg in this study, the LC50 of Cd rapidly decreases. Excess Zn thus induced a high toxicity and masked the toxicity of Cd.

In addition to toxic unit approach, the MIXTOX model approach revealed more complex deviation patterns. It is not easy to compare our results with those of others studies, because of the scarcity of exactly similar species and binary metal combinations. Jonker et al. (2005) analyzed the effect of Cd and Zn on reproduction of the springtail *F. candida*, a dose ratio-dependent deviation from concentration addition was found. Interaction pattern shifted from antagonism to synergy when Zn concentration was 13 times higher than Cd in the mixtures. In the present study, the magnitude of antagonism decreased with increasing concentration ratios of Zn to Cd in the mixtures. Besides, a concentration-dependent joint effect was also found. Lock and Janssen (2002) studied mixture toxicity of Cd and Zn to the potworm *Enchytraeus albidus* and found that the effect predictions based on the concentration addition model were always higher than those obtained with the independent action model. Therefore, it is reasonable to base results of this study on concentration addition model, which represents a worst-case scenario for risk assessment of mixture toxicity.

The interaction pattern can also vary when using different endpoints (Van Gestel and Hensbergen, 1997) and different expressions of exposure (Weltje, 1998). The combined effect of Cd and Zn on the growth of the springtail *F. candida* was antagonistic (EC50 significantly higher than a TU of 1.0), while the effect on reproduction was additive (EC50 not significantly different from a TU of 1.0) (Van Gestel and Hensbergen, 1997). Weltje (1998) compiled data on sublethal toxicity and tissue concentrations of Cd, Cu, Pb and Zn mixtures in earthworms. Mixture toxicity shifted from mainly antagonism towards nearly concentration-addition when the endpoints were based on extractable metal concentrations instead of total soil concentrations. Zidar et al. (2009) reported that effects of Cd and Zn on food consumption by the isopod *Porcellio scaber* were additive when based on total and water-soluble concentration but antagonistic when related to tissue concentrations. In this study, the toxic effect was estimated only based on mortality, so no conclusion on the impact of different endpoints could be drawn. There was also no observed shift in response types when expressing exposure using bioavailable concentrations instead of total concentrations.

5.5 Conclusions

This study provided insight into the interactive effect of binary Cd and Zn mixtures on mortality of *A. caliginosa* at different interaction levels in one soil type. Toxicity of Cd and Zn mixtures could not be predicted on the basis of the individual metals. Simultaneous exposure to Cd and Zn in soil jointly affected earthworm mortality, but did not influence their partitioning between solid and solution phases. Interactions of both Cd-Zn and Zn-Cd were mainly antagonistic as shown by the values of TU_{mix} . Using the MIXTOX model, deviation from additivity was statistically quantified to be mainly antagonistic, and the magnitude of antagonism depended on both relative concentrations and concentration magnitudes for the whole ranges of concentrations tested. Metal-metal interactions most likely occurred at the organism level.

Supporting Information

Table S5.1 Selected physicochemical properties of the tested soil.

Soil properties	Values
pH	6.1 ± 0.3*
Texture	sandy loam
Clay content (%)	7.0 ± 0.6
Organic matter (%)	5.7 ± 0.2
CEC (cmol/kg)	10.1 ± 0.3
Metal content	
Total Zn (mg/kg, dw)	104 ± 10.2
Total Cd (mg/kg, dw)	1.43 ± 0.37

*All values are presented as mean ± standard deviation for three replicates.

Table S5.2 Single metal (Cd, Zn) toxicity for *Aporrectodea caliginosa* after 28 d of exposure related to nominal, measured total, porewater and CaCl₂-extractable concentrations.

	Nominal concentration (mg/kg)	Measure total concentration (mg/kg)	Porewater concentration (mg/kg)	CaCl ₂ -extractable concentration (mg/kg)	Mortality (%)
Cd	0	0	0	0	0
	50	75	993	24	0
	100	115	950	41	0
	150	170	1425	62	0
	200	207	2715	78	0
	250	251	3175	89	0
	300	301	3830	97	0
	350	348	4970	114	75
	500	514	9085	193	100
	650	737	10700	282	100
	800	754	7574	322	100
	1000	919	13050	361	100
Zn	0	0	0	0	0
	50	228	585	34	0
	100	270	2188	63	0
	200	333	4678	85	0
	410	812	6360	133	0
	600	818	7022	170	25
	650	902	7495	180	0
	775	909	8650	205	0

850	1053	10065	219	0
950	1033	10209	239	0
1200	1295	12350	288	0
1300	1461	13183	307	25
1350	1500	13594	317	50
1400	1538	14003	327	75
1500	1815	14813	347	100
2000	2252	18728	395	100

Table S5.3 Toxic units of binary mixtures of Cd and Zn, and the identified interaction types (Cd-Zn combinations used here are corresponding to data points of Figure 5.3A).

Nominal Cd (mg/kg)	Cd-Zn combinations causing 50% mortality of earthworms		TU _{Cd} *	TU _{Zn}	TU _{mix} (TU _{Cd} +TU _{Zn})	Interaction type
	Measured total Cd, (mg/kg)	LC50 (measured total Zn), (mg/kg)				
0	2	1499	-	-	-	-
25	35	1500	0.10	1.00	1.10	Antagonistic
50	65	1366	0.19	0.91	1.10	Antagonistic
100	120	1459	0.35	0.97	1.32	Antagonistic
150	173	1368	0.50	0.91	1.41	Antagonistic
200	242	1422	0.70	0.95	1.65	Antagonistic
250	291	1288	0.84	0.86	1.70	Antagonistic
300	324	1281	0.94	0.85	1.79	Antagonistic
350	393	1250	1.14	0.83	1.97	Antagonistic
400	443	1122	1.29	0.75	2.04	Antagonistic
500	553	818	1.61	0.55	2.16	Antagonistic
600	653	955	1.90	0.64	2.54	Antagonistic
700	737	600	2.14	0.40	2.54	Antagonistic

*Toxic unit of Cd (TU_{Cd}) was calculated by dividing measured total Cd concentration in mixture to median lethal concentration (LC50) of Cd presented singly. Mixture toxic units (TU_{mix}) are the sum of toxic units of Cd (TU_{Cd}) and Zn (TU_{Zn}).

Table S5.4 Toxic units of binary mixtures of Cd and Zn, and the identified interaction types (Cd-Zn combinations used here are corresponding to data points of Figure 5.3B).

Nominal Cd (mg/kg)	Cd-Zn combinations causing 50% mortality of earthworms		TU _{Cd} *	TU _{Zn}	TU _{mix} (TU _{Cd} +TU _{Zn})	Interaction type
	Porewater Cd, (µg/L)	LC50 (porewater Zn), (µg/L)				
0	31	13588	-	-	-	-
25	295	13000	0.06	0.96	1.02	Additive
50	601	12163	0.12	0.90	1.02	Additive
100	1299	12417	0.27	0.91	1.18	Antagonistic
150	1774	12764	0.36	0.94	1.30	Antagonistic
200	2269	12274	0.46	0.90	1.36	Antagonistic
250	3562	10399	0.73	0.77	1.50	Antagonistic
300	3803	11503	0.78	0.85	1.63	Antagonistic
350	4586	10990	0.94	0.81	1.75	Antagonistic
400	5408	9484	1.11	0.70	1.81	Antagonistic
500	7209	6412	1.47	0.47	1.94	Antagonistic
600	8186	6166	1.67	0.45	2.12	Antagonistic
700	8194	4920	1.68	0.36	2.04	Antagonistic

*Toxic unit of Cd (TU_{Cd}) was calculated by dividing porewater Cd concentration in mixture to median lethal concentration (LC50) of Cd presented singly. Mixture toxic units (TU_{mix}) are the sum of toxic units of Cd (TU_{Cd}) and Zn (TU_{Zn}).

Table S5.5 Toxic units of binary mixtures of Cd and Zn, and the identified interaction types (Cd-Zn combinations used here are corresponding to data points of Figure 5.3C).

Nominal Cd (mg/kg)	Cd-Zn combinations causing 50% mortality of earthworms		TU _{Cd} *	TU _{Zn}	TU _{mix} (TU _{Cd} +TU _{Zn})	Interaction type
	CaCl ₂ -extr. Cd, (mg/kg)	LC50 (CaCl ₂ -extr. Zn), (mg/kg)				
0	1	317	-	-	-	-
25	7	300	0.06	0.95	1.01	Additive
50	18	253	0.16	0.80	0.96	Additive
100	30	273	0.27	0.86	1.13	Antagonistic
150	41	242	0.37	0.76	1.13	Antagonistic
200	53	233	0.47	0.73	1.20	Antagonistic
250	84	199	0.75	0.63	1.38	Antagonistic
300	90	223	0.80	0.70	1.50	Antagonistic
350	127	203	1.13	0.64	1.77	Antagonistic
400	131	199	1.17	0.63	1.80	Antagonistic
500	161	126	1.43	0.40	1.83	Antagonistic
600	184	110	1.64	0.35	1.99	Antagonistic
700	211	83	1.88	0.26	2.14	Antagonistic

*Toxic unit of Cd (TU_{Cd}) was calculated by dividing CaCl₂-extractable Cd concentration in mixture to median lethal concentration (LC50) of Cd presented singly. Mixture toxic units (TU_{mix}) are the sum of toxic units of Cd (TU_{Cd}) and Zn (TU_{Zn}).

Table S5.6 Toxic units of binary mixtures of Cd and Zn, and the identified interaction types (Zn-Cd combinations used here are corresponding to data points of Figure 5.5A).

Nominal Zn (mg/kg)	Zn-Cd combinations causing 50% mortality of earthworms		TU _{Cd} *	TU _{Zn}	TU _{mix} (TU _{Cd} +TU _{Zn})	Interaction type
	LC50 (measured total Cd), (mg/kg)	Measured total Zn, (mg/kg)				
0	344	95	-	-	-	-
50	497	183	1.44	0.12	1.56	Antagonistic
100	525	222	1.53	0.15	1.68	Antagonistic
200	528	383	1.54	0.26	1.80	Antagonistic
250	490	445	1.42	0.30	1.72	Antagonistic
350	521	555	1.52	0.37	1.89	Antagonistic
410	568	623	1.65	0.42	2.07	Antagonistic
500	662	777	1.93	0.52	2.45	Antagonistic
550	649	821	1.89	0.55	2.44	Antagonistic
600	513	853	1.49	0.57	2.06	Antagonistic
650	631	911	1.83	0.61	2.44	Antagonistic
700	627	959	1.82	0.64	2.46	Antagonistic
775	548	1042	1.59	0.69	2.28	Antagonistic
800	680	1087	1.98	0.72	2.70	Antagonistic
850	262	1085	0.76	0.72	1.48	Antagonistic
900	258	1157	0.75	0.77	1.52	Antagonistic
950	209	1220	0.61	0.81	1.42	Antagonistic
1000	262	1243	0.76	0.83	1.59	Antagonistic
1150	200	1334	0.58	0.89	1.47	Antagonistic
1200	34	1429	0.10	0.95	1.05	Additive

*Toxic unit of Cd (TU_{Cd}) was calculated by dividing measured total Cd concentration in mixture to median lethal concentration (LC50) of Cd presented singly. Mixture toxic units (TU_{mix}) are the sum of toxic units of Cd (TU_{Cd}) and Zn (TU_{Zn}).

Table S5.7 Toxic units of binary mixtures of Cd and Zn, and the identified interaction types (Zn-Cd combinations used here are corresponding to data points of Figure 5.5B).

Nominal Zn (mg/kg)	Zn-Cd combinations causing 50% mortality of earthworms		TU _{Cd} *	TU _{Zn}	TUMix (TU _{Cd} +TU _{Zn})	Interaction type
	LC50 (porewater Cd), (µg/L)	Porewater Zn, (µg/L)				
0	4890	303	-	-	-	-
50	5848	1020	1.20	0.08	1.28	Antagonistic
100	6152	1406	1.26	0.10	1.36	Antagonistic
200	6295	3001	1.29	0.22	1.51	Antagonistic
250	5546	3120	1.13	0.23	1.36	Antagonistic
350	6124	4198	1.25	0.31	1.56	Antagonistic
410	7464	5133	1.53	0.38	1.91	Antagonistic
500	8590	6760	1.76	0.50	2.26	Antagonistic
550	6095	6129	1.25	0.45	1.70	Antagonistic
600	6531	6946	1.34	0.51	1.85	Antagonistic
650	7943	7786	1.62	0.57	2.19	Antagonistic
700	7534	7945	1.54	0.58	2.12	Antagonistic
775	6053	8469	1.24	0.62	1.86	Antagonistic
800	8819	8890	1.80	0.65	2.45	Antagonistic
850	2864	9325	0.59	0.69	1.28	Antagonistic
900	3169	10261	0.65	0.76	1.41	Antagonistic
950	2333	10390	0.48	0.76	1.24	Antagonistic
1000	2897	10645	0.59	0.78	1.37	Antagonistic
1150	2286	11929	0.47	0.88	1.35	Antagonistic
1200	325	11765	0.07	0.87	0.94	Additive

*Toxic unit of Cd (TU_{Cd}) was calculated by dividing porewater Cd concentration in mixture to median lethal concentration (LC50) of Cd presented singly. Mixture toxic units (TU_{mix}) are the sum of toxic units of Cd (TU_{Cd}) and Zn (TU_{Zn}).

Table S5.8 Toxic units of binary mixtures of Cd and Zn, and the identified interaction types (Zn-Cd combinations used here are corresponding to data points of Figure 5.5C).

Nominal Zn (mg/kg)	Zn-Cd combinations causing 50% mortality of earthworms		TU _{Cd} *	TU _{Zn}	TU _{mix} (TU _{Cd} +TU _{Zn})	Interaction type
	LC50 (CaCl ₂ -extr. Cd), (mg/kg)	CaCl ₂ -extr. Zn, (mg/kg)				
0	112	8	-	-	-	-
50	143	20	1.27	0.06	1.33	Antagonistic
100	158	31	1.41	0.10	1.51	Antagonistic
200	164	67	1.46	0.21	1.67	Antagonistic
250	134	71	1.19	0.22	1.41	Antagonistic
350	160	87	1.43	0.27	1.70	Antagonistic
410	150	97	1.34	0.31	1.65	Antagonistic
500	201	122	1.79	0.39	2.18	Antagonistic
550	176	131	1.57	0.41	1.98	Antagonistic
600	142	130	1.27	0.41	1.68	Antagonistic
650	187	139	1.66	0.44	2.10	Antagonistic
700	208	138	1.85	0.44	2.29	Antagonistic
775	156	167	1.39	0.53	1.92	Antagonistic
800	207	168	1.84	0.53	2.37	Antagonistic
850	78	190	0.69	0.60	1.29	Antagonistic
900	135	167	1.20	0.52	1.73	Antagonistic
950	71	209	0.64	0.66	1.30	Antagonistic
1000	90	201	0.80	0.63	1.43	Antagonistic
1150	57	225	0.50	0.71	1.21	Antagonistic
1200	3	240	0.03	0.76	0.79	Synergistic

*Toxic unit of Cd (TU_{Cd}) was calculated by dividing CaCl₂-extractable Cd concentration in mixture to median lethal concentration (LC50) of Cd presented singly. Mixture toxic units (TU_{mix}) are the sum of toxic units of Cd (TU_{Cd}) and Zn (TU_{Zn}).

