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Effects of metals and metal mixtures on survival and cocoon production of the earthworm *Aporrectodea caliginosa*

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Summary. The capacity to reproduce is an essential factor for the long-term survival of populations at the basis of the food web. Ecological effects of soil pollutants may be assessed in terms of exposure levels that reduce reproduction, relative to survival. In this study, the earthworm *Aporrectodea caliginosa* (Annelida, Oligochaeta) was exposed to cadmium, copper and zinc in a natural soil. Each metal was mixed into the soil, as a sulphate salt, in a different series of concentrations. Mortality and cocoon production were measured over 56 days to determine LC_{50}, EC_{50} and EC_{10} values for each metal separately. LC_{50} decreased with exposure time and it approached a constant value for each metal. EC_{50} and EC_{10} values were determined following a loglogistic curve fitting of cocoon production versus exposure concentration. The ratio between the LC_{50} and the EC_{10} for effects on reproduction (sublethal sensitivity index, SSI), was different for each metal. The ratio was greatest for cadmium (a factor of 300) and smallest for zinc and copper (a factor of 6 to 10). Based on the single metal EC_{50}s a mixture of the three metals was prepared and effects on cocoon production were assessed. The EC_{50} of the mixture was 1.4 toxic units and this value differed significantly from 1. It may be concluded that 1) SSI is highly metal-specific, and 2) the three metals, cadmium, copper and zinc, in the concentration levels tested, have an antagonistic action on each other in their effects on cocoon production of *Aporrectodea caliginosa*.

Key words: Earthworms, *Aporrectodea caliginosa*, survival, reproduction, metal toxicity, soil, mixtures, cadmium, copper, zinc

Introduction

Metal pollution may disturb soil ecosystems by affecting how soil invertebrate populations function. Successful protection of communities demands a knowledge of the ecophysiology of metals in invertebrates and their susceptibility to metal intoxication (Dallinger & Rainbow 1993; Hopkin 1989). Among the various effects that soil pollutants may have, inhibition of invertebrate reproduction seems to be most relevant, because the long-term survival of populations that form the basis of a food-web depends on their capacity to reproduce (Kooijman & Metz 1984; Van Straalen et al. 1989).
Responses to stress resulting from toxicants may be expressed as effects on survival, growth or reproduction. Since the energy intake of the organism is distributed over these activities, differences in energy allocation can be expected under toxicant stress. Crommentuijn et al. (1995) introduced the Sublethal Sensitivity Index (SSI) as an ecotoxicity parameter that

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can be used to indicate energy allocation under toxicant stress. SSI was defined as the ratio between the median lethal concentration (LC_{50}), relative to the threshold concentration for effects on reproduction (EC_{10} or NOEC). Crommentuijn et al. (1995) studied this parameter with respect to the effects of cadmium on the growth and reproduction of four soil arthropods. In our study we aimed to determine if this parameter (SSI) is metal-specific or not, by comparing the sublethal effects (reproduction) related to the lethal effects of three heavy metals.

The second aim of this study was to determine the effect of mixtures. Although metals are often present in soils in a mixture of three to five different contaminants according to the contamination source (Spurgeon et al. 1994), few studies have been done on the effect of mixtures in soil invertebrates (Medici & Taylor 1967 and Van Gestel & Hensbergen (submitted). For aquatic invertebrates, Kraak et al. (1994) analysed the effects of mixtures according to the toxic unit model. In this model, the mixture concentrations are expressed as fractions of the EC_{50}s of the individual toxicants and the data are tested against the null hypothesis of relative concentration additivity, which states that the effects of the mixture can be predicted from the sum of the toxic units of the individual compounds. In our study an attempt was made to estimate the effects of metal mixtures on the earthworm *Aporrectodea caliginosa*.

Materials and Methods

Earthworms

A culture of the earthworm *Aporrectodea caliginosa* was established from animals collected from Al-Qanater Al-Khahiriya city, Egypt. The culture was maintained in the laboratory of the Faculty of Science, Tanta University, at 25 ± 3 °C. Adult worms from the culture were used for experiments.

Chemical and soil characteristics

Solutions of the sulphate salts of cadmium, copper and zinc were used (purity 90%, Merck). Soils were collected from the same area where the animals were collected and were analysed for some main constituents; organic matter content was 21.6%, pH and conductivity were measured in an aqueous extract (5 ml water per 1 g of soil); pH was 7.05, conductivity was 750 µS. For more details on the physico-chemical properties of the soils used, see Khalil et al. (1996).

Survival experiments

The soil was artificially contaminated by mixing dry soil with solutions containing different concentrations of metals to obtain the following series: Cd: 300, 400, 500, 600, 700 and 800 µg/g dry soil; Cu: 500, 600, 700, 800, 900 and 1000 µg/g and Zn: 2500, 3000, 3500, 4000, 4500, and 5000 µg/g. The toxicants were mixed in homogeneously with the soil. The final water content of the soil was 40%. Control soil received water only. A series of metal treated soils were placed in plastic containers, 400 g of moist soil to a container. Each concentration of the metals, including the control, was represented by five replicates and ten adult earthworms were added to each container. Every week each container was checked, dead worms were removed and the surviving worms were counted, and returned to the soil. The duration of the test was 8 weeks at room temperature and natural daylength (c. 12 h).

Reproduction toxicity experiments

For the reproduction toxicity tests the soils were contaminated with concentrations of Cd: 10, 50, 100 and 300 µg/g dry soil; Cu: 50, 100, 200 and 400 µg/g; Zn: 300, 600, 1000 and 1600 µg/g. These concentrations were chosen in such a way that, based on the survival experiments, no mortality was expected at the highest exposure level. Test metals, as sulphate salts, were mixed with the soil to achieve a homogeneous concentration. Ten adult earthworms, with well developed clitellum, were introduced

in test boxes (surface area 300 cm^2), with 1 kg of dry weight natural soil. Ground cattle manure (0.5 g per worm per week) was added as a food source. The soil moisture content was 45%. The duration of the test was 8 weeks. Each treatment was replicated five times. Cocoons were collected and counted each week by hand-sorting the soil of each replicate. Every week survival of the adults was also checked and then the adults were returned to the soil for further incubation.
For the mixture toxicity experiments, mixtures of one third each of the above mentioned concentrations of the three metals were prepared; each mixture and the control were represented by five replicates. Ten adults earthworms were added to each replicate, as mentioned above.

Data analysis

Survival data were analysed using the toxicokinetics-based survival model of Kooijman (1981). Maximum likelihood estimates for LC_{50} were obtained for each exposure time. Reproduction data (cumulative cocoon production) were analysed as concentration response relationships, and EC_{50} and EC_{10} values were estimated from the data using the non-linear least squares fitting routine in the SYSTAT® software package, using a loglogistic model (Haanstra et al. 1985). The model was written so that EC_{50} and EC_{10} were the parameters to estimate, while the response in the control was given a fixed value, the observed average cocoon production (see Khalil et al., 1996). The same model was also applied to data from the mixture toxicity experiment after the concentrations in the mixture were expressed as fractions of the individual EC_{50} (toxic units) and the sum of the toxic units had been evaluated for each mixture.
A generalised likelihood ratio test (Sokal and Rohlf 1996) was applied to test for significance of the difference between EC_{50} values for the single metals and the mixture. To do this the concentrations of cadmium, copper and zinc in the mixture and in the single series were expressed in toxic units (on an EC_{50} basis) and a model was fitted that assumed a different EC_{50} and slope for each metal and for the mixture. The results were then compared with a model that assumed a single EC_{50}, and the likelihood ratio statistic was estimated from the least squares of both models. This allowed a test of the hypothesis of whether the metals acted according to additivity of toxic units (Van Gestel & Hensbergen, submitted). All calculations were done using the SYSTAT® programme.

Results

Survival experiment

In the survival experiment no mortality in the control was observed. The changes in LC_{50} over eight weeks for Cd, Cu and Zn are demonstrated in Fig. 1. For all three metals LC_{50} decreased with exposure time until it approached a constant value. The changes conform to the toxicokinetics-based model, which explains the decrease of LC_{50} with exposure time from the gradual accumulation of metals in the worms, until an equilibrium between uptake and elimination is reached. The fit of the model was best for the Cd data, for Cu and Zn there was a tendency for continued decrease of the LC_{50} with exposure time. Estimates for LC_{50} after 8 weeks of exposure are shown in Table 1. It is obvious that Cd is the most toxic metal, followed by Cu and Zn.

Single metal experiments for reproduction

Even at the highest concentration of all three metals no mortality was observed. Fig. 2 shows the cumulative production of cocoons over the 8 weeks exposure period. The linear increase of cocoon numbers with time shows that there is a constant rate of cocoon production; also from Fig. 2 it can also be seen that for Cu, cocoon production was stimulated at the lowest test concentration; for Zn, 600 µg/g gives less effect than 300 µg/g. At the high concentrations production was affected by all metals, especially after 2 weeks, no difference was observed in the first 2 weeks. The highest exposure inhibited cocoon production completely. Based on the data given in Fig. 2, it was decided to cumulate

Table 1. LC_{50}, EC_{50} and EC_{10} values for the effect of cadmium, copper and zinc on cocoon production of *Aporrectodea caliginosa* in a natural soil. EC_{50} and EC_{10} were calculated for the cumulative cocoon production over exposure weeks 3–8

Parameter	Cd		Cu		Zn	
LC_{50} (µg/g)	540		640		3610	
95% conf. limits	510	560	610	670	3510	3710
EC_{50} (µg/g)	34.8		185.8		826	
95% conf. limits	19.1	63.4	69.0	500.0	539.5	1265
EC_{50} (µmol/g)	0.309		2.924		12.635	
95% conf. limits	0.169	0.564	1.085	7.869	8.253	19.34
EC_{10} (µg/g)	1.86		69.8		567.5	
95% conf. limits	0.30	11.4	11.7	415.9	217.3	1483
EC_{10} (µmol/g)	0.016		1.098		8.681	
95% conf. limits	0.003	0.101	0.184	6.542	3.324	22.685
SSI (LC_{50}/EC_{10})	290		9.2		6.4	

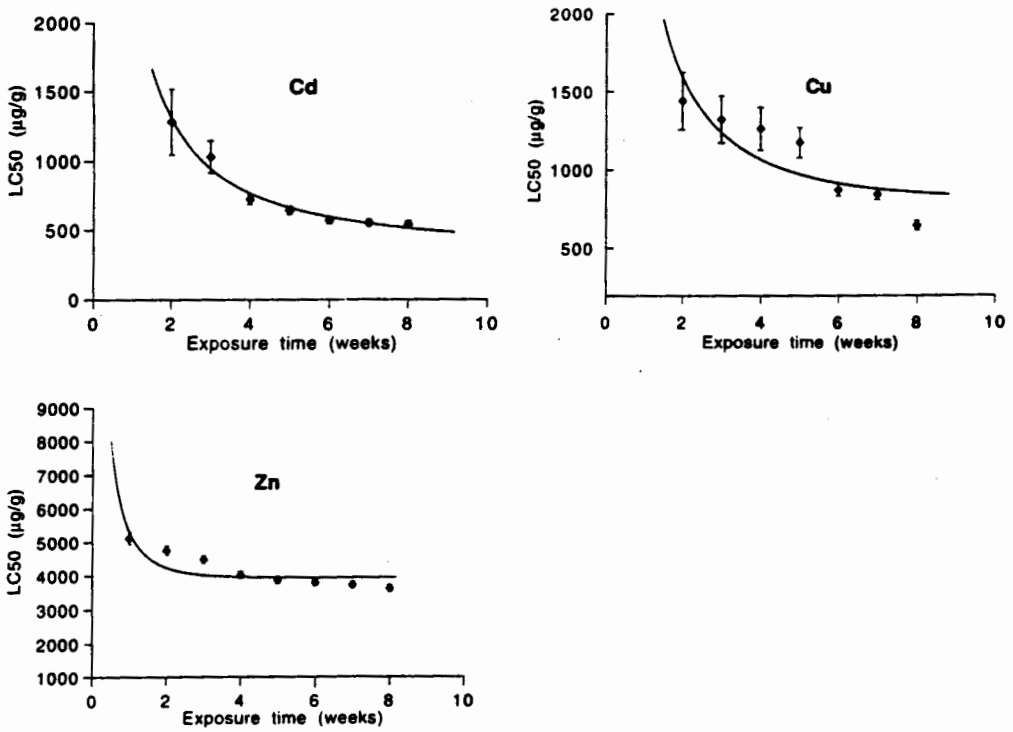

Fig. 1. Changes in LC_{50} over eight weeks for the effects of Cd, Cu and Zn on survival of the earthworm *Aporrectodea caliginosa*. The LC_{50} and its 95% confidence interval is given for each exposure time, estimated using the toxicokinetics-based survival model of Kooijman (1981). The solid line represents the model fitted to all data simultaneously

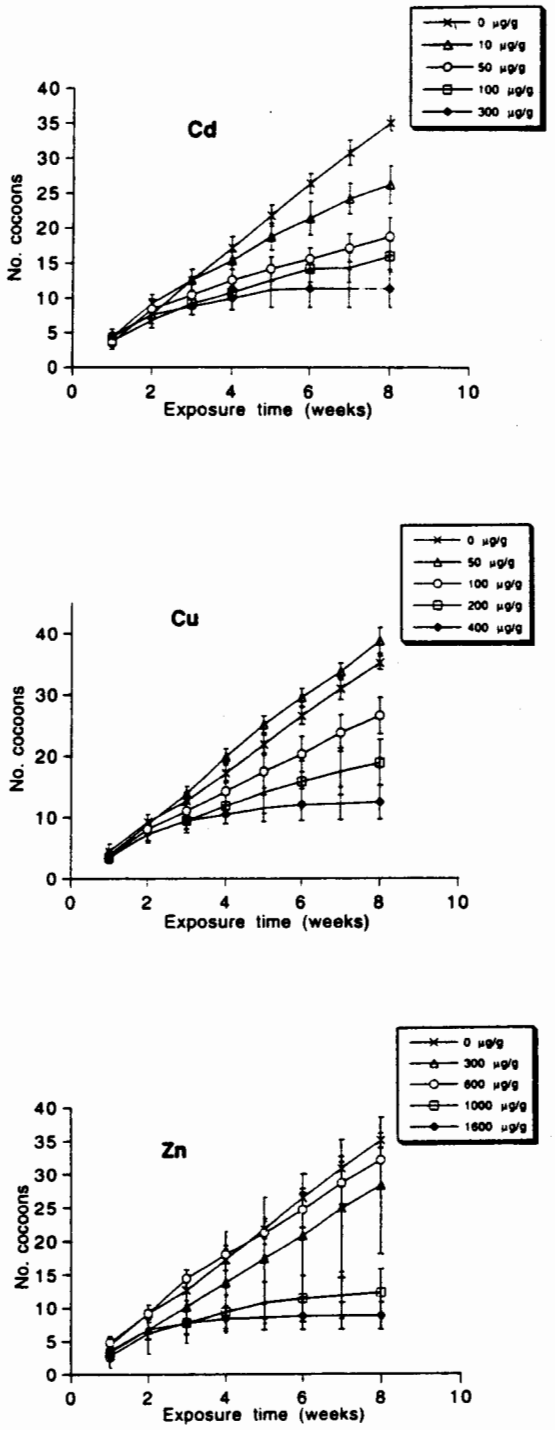

Fig. 2. Cumulative production of cocoons of the earthworm *Aporrectodea caliginosa* exposed to Cd, Cu and Zn over 8 weeks; data points show averages and standard deviations for five replicates per concentration

Table 2. Application of the toxic unit model to data for the effect of a mixture of three metals on cocoon production of *Aporrectodea caliginosa* in a natural soil including the cumulative cocoon production for the 3–8 week exposure interval

Cu		Cd		Zn		Cu+Cd+Zn
µg/g	TU	µg/g	TU	µg/g	TU	∑TU
16.9	0.090	3.3	0.094	100	0.121	0.306
33.3	0.179	16.9	0.485	200	0.242	0.907
66.6	0.358	33.0	0.948	330	0.399	1.706
133	0.715	100	2.873	533	0.645	4.234

Note: TU = toxic units (concentration relative to single metal EC_{50})

cocoon production over weeks 3–8 for each replicate, to obtain a measure of metal toxicity.

The concentration-response relationships of cocoon production (over weeks 3–8) are given in Fig. 3. The loglogistic model described the data reasonably well, the best fit was obtained for cadmium. Parameter estimates for the concentration response relationships for each metal are given in Table 1. Estimates for EC_{50} demonstrate that Cd is the most toxic metal followed by Cu and Zn; the relative toxicities are 1:10:42, on a molar basis.

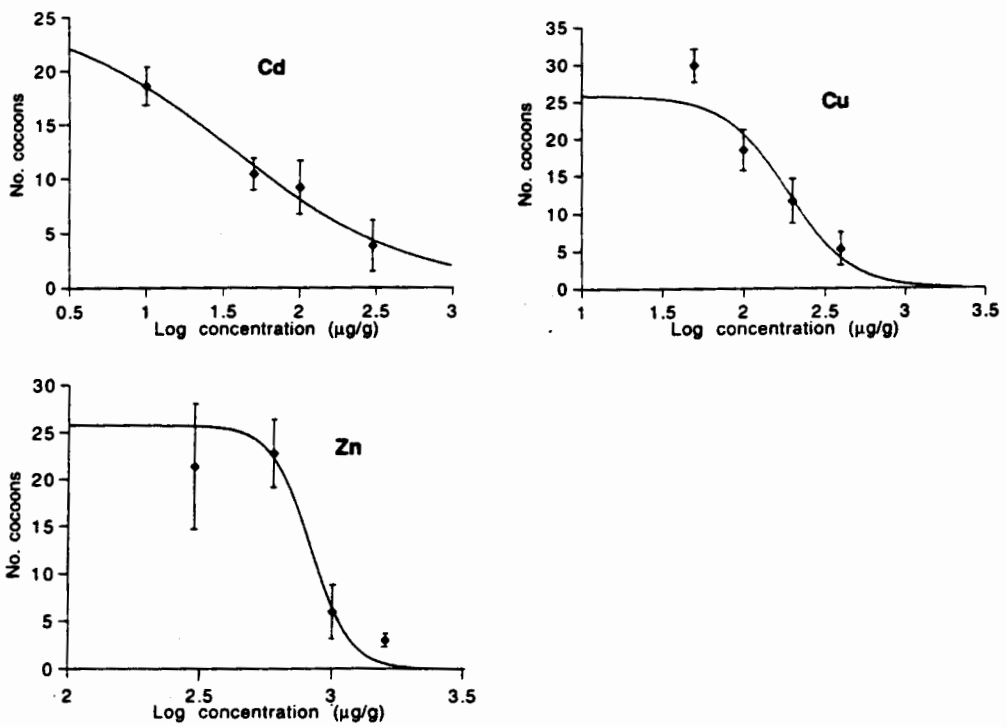

Fig. 3. Concentration-response relationship for the effects of Cd, Cu and Zn on cocoon production of the earthworm *Aporrectodea caliginosa* in soils treated with various metal concentrations. The average cocoon production over 6 weeks is plotted against the logarithm of concentration. Observed reproduction in the control was 25.8 ± 0.8 which was used as a constant in the curve fitting. The solid lines show a least square curve fit to the data, using the loglogistic model. Data points show averages and standard deviations for 5 replicates per concentration

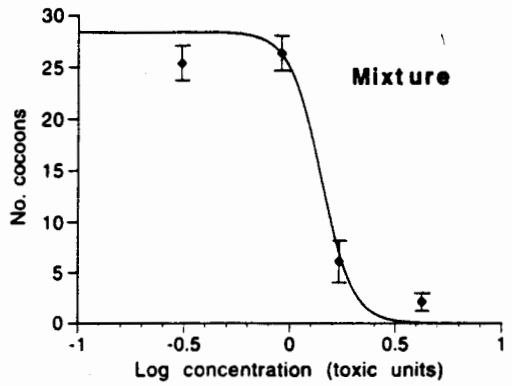

Fig. 4. Concentration-response relationship for the effect of three metals Cd, Cu and Zn, on the average cocoon production over 6 weeks of the earthworm (*Aporrectodea caliginosa*) in soils treated with various concentrations of a mixture of metals. The concentration axis is expressed in (logarithmic) toxic units. The solid line is a least squares curve fit of the loglogistic model. Observed reproduction in the control was 28.4 ± 2.58 which was used as a constant in the curve fitting. Data points show averages and standard deviations for five replicates per concentration

Table 1 also shows the values for SSI (= LC_{50}/EC_{10}). The effects of cadmium on the reproduction of earthworms begin at concentrations which are a factor of nearly 300 below the LC_{50}, while for Cu and Zn this factor is only 6 to 10.

Metal interaction experiment

Based on the single metal experiment, concentrations of metals in the mixtures were expressed as toxic units (fraction of their single EC_{50}), as shown in Table 2. The observed cumulative number of cocoons over weeks 3 – 8 were fitted to the sum of the toxic units. The concentration-response relationship of the mixture of copper, cadmium and zinc is given in Fig. 4. The loglogistic model provided a reasonable fit to the data, estimates of parameters for EC_{50} and EC_{10} and their 95% confidence intervals, represented in toxic units, were 1.40 (0.91 – 2.17) and 0.96 (0.35 – 2.65) respectively. The EC_{50} value for the mixture was significantly different from 1 TU according to a generalized likelihood ratio test ($p < 0.05$); this indicates an antagonistic effect for the mixture of Cd, Cu and Zn on cocoon production of the earthworm *Aporrectodea caliginosa.*

Discussion

In our study, the 8 weeks LC_{50} values recorded for copper and cadmium were more or less the same as those found by Spurgeon et al. (1994), for zinc it was somewhat higher but was comparable with the data found by Spurgeon & Hopkin (1996). Several studies have been carried out on earthworms to determine LC_{50} values for heavy metals. Van Gestel & Van Dis (1988) recorded a 14 day LC_{50} for cadmium of > 1000 µg/g in artificial soil. Neuhauser et al. (1985) estimated a 14 day LC_{50} of 1843 µg/g, our results seem to correspond with these values. Van Gestel et al. (1991) determined a 12 week LC_{50} for cadmium of 253 µg/g. For zinc Neuhauser et al. (1985) determined 14 day LC_{50} of 662 µg/g in artificial soils, for copper the 14 day LC_{50} was 643 µg/g which is much lower than our LC_{50}; this may be because the soil we used in our esperiment was fairly rich in organic matter, which may have reduced bioavailability, expecially for Cu.

In the cocoon production experiment, cadmium was the most toxic metal followed by copper and zinc. This order of toxicity has been found in other studies on the effect of

metals on reproduction in earthworms (Ma 1983; Bengtsson et al. 1986; Van Gestel et al. 1991). Cocoon production was significantly reduced at 560 μg Zn/g and 10 μg Cd/g (Van Gestel et al. 1993). NOEC values for the effect of copper on reproduction for *Dendrobaena rubida*, *Lumbricus rubellus* and *Aporrectodea caliginosa* were 122 μg/g, 30 μg/g and 50 μg/g respectively (Bengtsson et al. 1986; Ma 1982).

Crommentuijn et al. (1995) propose measuring the differences between lethal effects and sublethal effects using the "sublethal sensitivity index", SSI, and argue that this index indicates the priorities that an animal puts on the maintenance of reproduction when faced with toxic stress. A low value for SSI is a characteristic of "r-selected" life-history strategies, that rely on a high reproductive output. Crommentuijn et al. (1995) also indicate that SSI could be substance-specific, and this impression is confirmed by the present study. In the literature, differences between NOEC values for the effect of chemicals on earthworm reproduction and LC_{50} values (SSI) show a great variation. Ma (1983) gives a factor of 15 – 50 for the ratio between 6 weeks LC_{50} and NOEC values for four heavy metals and *Lumbricus rubellus*; this result can be compared with our results for copper and zinc. Van Gestel et al. (1992) give values for the effect of different chemicals on earthworm growth and reproduction; the ratio was lowest for benomyl, fentin and pentachlorophenol, a factor of 5 – 6, and highest for cadmium (a factor of >100); in our study the factor was even higher for cadmium (300). In the study of Ma (1983) difference in SSI is a factor of 5 to 20 for three pesticides. It can be concluded from data of Adema et al. (1981), Slooff & Canton (1983) and Slooff et al. (1986) that also for aquatic organisms differences between LC_{50} or EC_{50} values and NOECs for sublethal parameters are not constant. For eight chemicals tested on three fish species, Adema et al. (1981) found ratios LC_{50}/NOEC (SSI) of between 1.7 and 450 (cf. Van Gestel et al. 1992). Slooff & Canton (1983) concluded that for most chemicals differences between acute and chronic toxicity values were less than a factor of 100, but for some chemicals differences may be as high as a factor of 3200.

In the metal interaction experiment EC_{50} was estimated as 1.4 toxic units. This result confirms that the combination of these three metals has a tendency towards antagonism. Khalil et al. (1996) have observed a similar tendency for antagonism between Cd, Cu and Zn in earthworm growth experiments, although the antagonistic effect was not significant in this case. Van Gestel & Hensbergen (submitted) concluded that the combined effects of cadmium and zinc for the collembolan *Folsomia candida* are mainly antagonistic when considering growth as the endpoint, but mainly additive when reproduction is considered.

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