Combination toxicity effects of heavy metals on terrestrial animal (Earthworm- *Eisenia andre)*

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ABSTRACT: Combination toxicity of heavy metals to earthworms has been studied for the first time. Three metals, namely Cadmium (Cd), Lead (Pb) and Zinc (Zn) were used as toxicants. The study was carried out in line with the OECD recommended procedure for ecotoxicology tests of chemical substances to soil. Hence, the OECD approved earthworm species Eisenia Andrei was used as biological test organism. OECD artificial soil was used as test substrate. The parameters of interest in the study were the effect of logarithmically increasing concentrations of single, and finally of combinations of metals, to reproduction and body growth of earthworms. These parameters were chosen for the study since sublethal effect on growth and reproduction are of more ecological relevance than an acute toxicity effect studying mortality. Growth was measured as the increased in body weight of the worms in time. Reproduction was estimated based on the number of cocoons produced per worm and the number of emerged juveniles produced per cocoon. The EC50 values of the effect of the toxicants to the organisms were estimated by an extended logistic model which is able to take into account the presence or absence of hormesis in calculating EC50 values. The EC50 values after three weeks of the three metals to growth and cocoon production were first determined singly. Cocoon production and juvenile numbers appeared to be more sensitive parameters than growth. A continuous increase in weight was observed in almost all tested concentrations of each metal except at the highest concentration of lead. Cd appeared to have the strongest adverse on cocoon production, followed by Zn, and then Pb. This is judged from the observed EC50 values of each of the metals on cocoon production. The observed EC50 values were 104.0mg/kg (1.076 mmol/kg), 418.3mg/kg (6.398 mmol/kg), 1570.3mg/kg (7.578 mmol/kg) for Cd, Zn and Pb respectively. Based on the observed EC50 values of the single toxicity tests, combination toxicity tests were carried out for Zn & Cd, Cd & Pb and Pb & Zn. Zn & Cd were found to be antagonistic (less than concentration additive) other tests where not interpretable. The ecological implication of the result of combination toxicity test of Cd & Zn is that single toxicity test alone may not indicate the fate of earthworms exposed to sites polluted with combinations of metals.

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INTRODUCTION

A literature inventory using a computer mainframe programme Biosis Data base has revealed that combination toxicity of metals has never been studied using earthworms or other terrestrial organisms, apart from bacteria. It is only in aquatic literature that such studies have been recorded. Pharmacological achievements and other cases of successful development of chemical mixtures for economic reasons, have been reported to originate from ancient knowledge of the advantages of mixtures of chemicals over chemicals applied singly (Marking, 1977). Some chemicals are reducing or increasing the toxic effects of the other toxicants for target organisms (Marking, 1977).

Environmental risk assessment of metals to the soil compartment has always been tested with single toxicants in the laboratory by using earthworms or other species as biological test organisms. Yet in nature earthworms, like other terrestrial organisms in contaminated ecosystems, are usually exposed to mixtures of toxicants. This study, which is the first of its kind, is aimed at determining the toxic effects of combinations of metals to earthworms of the species Eisenia andrei. The study should prove the accuracy of single toxicity tests in determining the toxic effect of metals to earthworms. Comparisons of the result of these experiments can be made with available findings will help ascertain whether or not combination toxicity of metals describes the toxicity metals to earthworms in a better way than when metals are tested singly. This will, of course throw more light on the approach of toxicity tests of metals to estimate risks for the polluted soil compartment.

The importance of earthworms has been long recognized. Darwin, as far back as 1881 has this to say about earthworms and 1 quote from Lee (1985): “It may be doubted whether there are many other animals which have played so important a part in the history of the world, as have these lowly organized creatures". Earthworms have been used as human food and to alleviate some illnesses, nut there scientific relevance originates from the fact that they have been known to be important soil fauna (Edwards & Lofty, 1977). They enhance soil fertility in many ways, for example by mixing of the soil, formation of water stable aggregates, aeration of the soil and improvement of its water holding capacity by their burrowing activities (Edwards & Lofty 1977). Therefore substances that effect the ecology of earthworms will also interfere with the productivity of the soil. Earthworms have specific metal binding proteins (Satchell, 1983). They have been reported by many authors to be able to take up and accumulate heavy metals in their tissues. These metals could be accumulated further in birds and animals that feed on earthworms and finally in man. This type of problem is expected to occur more in industrialized countries (Lee, 1985). Earthworms exist in large number in many soils. Some species can be easily grown in the laboratory. They can be readily collected and identified (Van Gestel, 1991). Thus they are cheap test organisms. It is because these profitable characteristics of earthworms that they have been selected as indicator organisms for testing ecological effects of industrial chemicals (OECD, 1984; EEC, 1985).

The toxicity of metals is concerned with some 80 elements and their compounds (Musch, 1992). Cd, Zn and Pb have been selected for this study because they occur commonly together in minerals and soils (Musch, 1992). Cd is usually a by-product of Zn refining, so it is the level of production of zinc and not the demand for Cadmium that determines the supply of Cd (IHE, Ecotoxicology Workshop, 1992). These three metals are among the suspected carcinogenic metals. Zinc and lead have been proven to be carcinogenic to experimental animals (Musch, 1992). Their tested concentrations are within the range they occur at polluted sites. They have been found to occur in concentration of up to 0.988mol/g, 445mol/g and 60.5mol/g respectively for Cadmium, zinc and lead at polluted sites in north – Western Europe (Posthuma, 1992). They have been selected, among other metals, for the validation project operating in Budel, a metal polluted site in the southern part of the Netherlands. The result of this research will provide part of the database for that project.

Soil characteristics can have great influence on the absorption and therefore on the bioavailability of chemicals in soil (Van Gestel, 1991). Therefore it is pertinent that earthworms toxicity test should be carried out with a standard substrate like OECD artificial soil. For reasons of standardization and reproducibility, OECD artificial soil was chosen as test substrate. This artificial soil was made specifically for these tests and has the same absorption capacity as a typical loam soil (Van Gestel, 1991).

A parameter like acute toxicity effect can only give information on the number of organisms lethally affected by the toxicant. Earthworms at polluted sites are exposed to toxicants all through their life time and acute toxicity tests alone cannot give information on what an organism suffered before death. A toxicant which does not lead to immediate death, but affects example the genetic constitution or reproductive capacity or growth of an organism, may disturb the ecological balance of that population. According to Spurgeon et al. (1993), reproduction and growth disturbances are far more likely to mediate population effects, and reproduction is likely to be of particular importance in ecotoxicological risk assessment because of its influence on population dynamics. From this view point, sublethal effects on reproduction (cocoon and juvenile production) and growth were chosen as test parameters.

2. MATERIALS AND METHODS

2.1. TYPES AND SOURCES OF TEST MATERIALS

2.1.1. Biological Material

Earthworm of the species *Eisenia andrei* were used as test organisms. All the worms used were adults of about 18 weeks of age with well developed clitellum. They were retrieved from RIVM earthworm mass culture, reared following a standard operation method (Van Gestel, 1991).

2.1.2. Food

Cow dung was used as food for the worms. The dung was obtained from a local farm, oven-dried over night and ground to a diameter of 0.5

2.1.3. Substrate

OECD artificial soil was used as test substrate. It was prepared in line with the OECD guideline for testing chemicals 207, entitled "Earthworms Acute Toxicity Test", adopted 4th April, 1984 , as updated by Van Gestel (1991). The different percentages of substances below used for the artificial soil were weighed into a container (big enough to make 12.50 kg of soil at a time) and mixed together with a cement mixer. The humidity and pH of the soil before being used for tests were aimed to be 55% and a pH $5.0^{+}_{-}0.5$, respectively, which was checked in random samples from the test series.

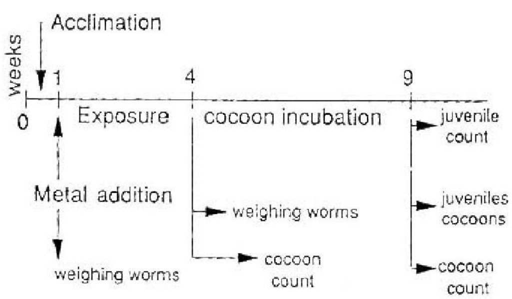
2.1.4. Metals

The metals were added to the artificial soil in the form of nitrate salts; that is cadmium nitrate (Cd $(NO_3)_2.4H_20$, Zine Nitrate ($Zn(NO_3)_2.6H_2O$ and Lead Nitrate ($Pb(NO_3)_2$) respectively.

2.2. PROCEDURE FOR SINGLE AND COMBINATION TOXICITY TEST EXPERIMENTS

The experiments were carried out in two consecutive parts. The first part consisted of the single toxicity effect tests of the individual metals. This part is necessary to provide the necessary data needed for the combination toxicity tests. The second part consisted the combination toxicity effect tests of the different combinations of the metals used for the combination toxicity test. All the worms used for both the single and combination toxicity test passed through three major phases as shown below namely:

a. The Acclimation Phase
b. Metal Exposure Phase
c. Cocoon Incubation Phase



2.2.1. Single Toxicity Tests

Acclimation. The test worms were passed through an acclimation phase in clean artificial soil. This is necessary for the worms to get used to living in a surrogate environment in the laboratory. For this purpose, 24 test jars were labeled for the five different tested concentrations of the metal and a control (each concentration, including the control had 4 replicates).

The moisture contents of the artificial soil to be used was raised to 20%. This was done by adding an equivalent of 20% of the dry weight of the soil in the form of demineralised water. The soil and the water were then mixed together with a kitchen mixer. The 20% humid soil was left to stand for a minimum of 5 days and a minimum of 7 days for a microbial population to develop before being used for the experiment.

At the end of this period, the moisture content of the was raised to 55% by adding the percentage difference of 35% of demineralised waster.

Two different samples of ca. 20g of the soil were then taken pH and humidity measurements respectively. An amount of 625g of 55% humidity soil was added into each jar. Holes were made in the centre of the soul content of the jars and 6.3g of the 55% wet weight of dung was put in the holes. The holes where up by slightly shaking each jar with hand.

Adult worms were hand-picked from the mass culture and put in a big petridish with moistened filter paper. The worms were washed with tap water in small plastic containers in batches of 10 and surface dried by keeping them on humid filter paper for some seconds before weighing. Surface attached water was lost by this process. The worms were weighed in batches of 10, but individual weight was determined for one of the replicates of the 6 concentrations. Each set of 10 worms was put in one of the test jars which already had soil and dung.

Jars were covered with glass Petri-dishes to allow enough air to reach the worms, minimizing evaporation, and preventing the worms from crawling out. The average wet weight of the worms was 0.360+_ g. each set of 10 worms were put in the test jars which already had soil and dung.

The weight of each test jar with soil, worms, dung and lid were taken. The jars were then put in a climate-room (temperature control-room) at a temperature of 20^{o}c+_2 and an illumination of 400-800 lux. Minimum-maximum thermometers were also put in the cabinets were jars were kept, together with humidity chart. This is for constant observation of the temperature and humidity respectively. Appendix 19 shows one of such charts.

This phase lasted for a week. The worms were then ready for the text phase, that is the metal exposure phase after washing, drying and weighing. Samples of the soil were taken to determine if there was change in pH of the soil after the acclimation phase.

Cocoons produced during this acclimation phase were washed from the soil and the numbers of cocoons produced per jar/per worm were noted. This observation was made to assure that all the worms used had reached maturity actually of age of reproduction.

Metal exposure. The different nominal concentrations of metals tested were (in mg/kg):
Cadmium: 0, 3.2, 10, 100, 320
Lead: (First experiment): 0, 10, 32, 100, 320, 1000
Lead: (Second experiment): 0, 50, 158.1, 500, 1581.1, 5000
Zinc: 0, 10, 32, 100, 320, 1000.

Two experiments were run for lead. The first one showed only minor effects on cocoon production, i.e. EC50 was not reached. Therefore, the second test was run with higher lead exposure. From here on, only the results for the interpretable experiment for lead (the second) are shown. Original data for both experiments are summarized in the Appendix section (Appendix 2 and 4).

Below is a typical approach for the exposure of worms to metal “X” where “X” could be Cd or Zn or Pb. A number of 24 test jars where labeled (4 replicates per concentration). The moisture content of the artificial soil was raised to 20% with demineralised water and the pH was taken. The soil was left to stand for a minimum of 5 days and a maximum of 7 days for the establishment of a microbial population.

The stock solution of each metal was made from their Nitrate salts. Equal amounts of Nitrate were maintained each tested concentration of the three metals. This was achieved by calculating the concentration at which the highest amount of Nitrate was obtained; this was at the highest concentration minus the amount already added through the nitrate salt in that particular concentration. The difference was made up with the addition of sodium nitrate. Thus in the control, the highest difference was made up with the addition of sodium nitrate. Thus in the control, the highest amount of sodium nitrate was added while none was added to the highest tested concentration range of the metals.

The exact amount of stock of the metal needed in 1 kg dry soil was pipetted into a 250 ml flask. This was prepared in duplo. Twelve 250 ml flask with 6 different concentrations (in duplicates) of each tested were prepared by adding calculated amount of stocks. Each flask content was then made up to 250 ml fluid liquid content with demineralised water.

The 20% humidity soil was weighed out in batches of 1200g. Each batch was put into a bowl of a kitchen mixer placed under a laminal flow hood.

The content of the 250 ml flask was then added each 1200g weight of 20% humidity soil starting with the control. After emptying, each flask was rinsed with 100 ml of demineralised water and this was added to the soil content making the total moisture content of each initially 20% humidity soil to be 55%. The final weight of the soil sample in the bowl then became 1550 g of 55% moisture content. This 1550g of soil was mixed for about 1 minute with the barters of the mixer. A amounts of 625 g of the 55% humidity soil were weighed out in 2 different jars labeled A and B. the remaining soil from the 1550 g soil was put in another flask for chemical analysis.

The other replicates of the same concentration were also treated as above and 2 different 625 g weight of soil were weighed out and put in jars labeled C and D. a similar procedure was followed to prepare all the different concentrations and the controls. Hence, each tested concentration and the control had four replicates of soil samples in the jar.

All the 24 jars were put in the climate room for 1 week to allow for metal absorption and desorption to and from the soil. This started at the same day at which the acclimation phase of the worms was started. At the end of each week 6.3g wet weight of 55% humidity dung was added to each jar of the polluted soil as at phase A. the phase A jars were emptied in a flat plastic container one after the other, and the worms were hand picked, washed with tap-water, dried on a filter-paper, weighed and put in the exposure jars. Lids were put on the jars. Then the weight of the jars, including worms dung, soil and lids, were taken. The jars were taken then put in the climate room.

The exposure phase lasted for three weeks. Every week, the water content of the jars was determined by taking the weight of the jars. The weight of the content for a particular week minus the weight of the previous week gave an insight into the amount of moisture lost. Moisture loss was corrected by adding an amount of water equivalent to the loss amount of demineralised water every week.

At the end of the third week exposure the temperature of the cabinet pH, and the humidity of a sample of the test jars were taken randomly from different test jars. The worms were retrieved by washing off the soil through a 2 mm sieve. This 2 mm sieve had another 1 mm sieve underneath to collect small sized cocoons that might pass from the former. The number of cocoons produced were counted by eye observation with the aid of tweezers. The weight of each set of worms in the jars were taken after washing and drying.

Cocoon Incubation. The cocoons produced per jar were taken from the sieves, and were put in petridishes containing 65g of 35% wet weight of unpolluted soil mixed up with 1g of dung. The cocoons were again covered up with another 65g of 35% wet weight of soil mixed up with 1g of dung.

The Petri dishes were put in the climate room for 5 weeks for incubation. Each week the moisture loss was made up as in case of the worms. At the end of the 5th week the juveniles from hatched cocoons were calculated after washing out the soul through a 0.5 mm sieve.

2.2.2. Combination Toxicity Tests

At the of the single toxicity test part, the EC50 of the three different metals on growth, cocoon production and juvenile production were estimated statistically. This was done by using a statistical model, written in Genstat 5, which performs curve-fitting to estimate toxicity parameters in dose-effect research. The model is a standard logistic model which is able to take into account the possibility of subtoxic stimulus (hormesis) in calculating the EC50 (Van Ewijk & Hoekstra, 1992).

The toxic unit approach for the combination toxicity test was applied to toxicity data for cocoon production rather than body weight. This is because, the single toxicity tests showed consistent and severe effects of each of the three metals on cocoon production against insignificant effects on growth (see Result section).

The combination studied for their effect on cocoon production were:

Cd & Zn
Cd & Pb
Pb & Zn

The basic data for the combination toxicity test in this study were the EC50 values of the metals tested singly on cocoon production. The EC50 of each metal on cocoon production also refers to 1 TU of the metal based on the definition of 1 TU for a singe. To obtain the theoretical 1 TU for two metals, ½ of the EC50 (1 TU) of the individual metals in the mixture were added together. This theoretical TU was usually made to be at concentration 4. the upper and lower concentration ranges were obtained by dividing or multiplying the concentration of the theoretical 1 TU with 6 root 10, 4 root 10, or square root 10, increments or decrements of 4 root 10 were chosen to have more narrow adjacent concentrations around EC50 expected. Four replicates of jars per concentration were used. For the combination toxicity test of Cd & Pb and Pb & Zn two replicates per concentration were used, since twelve different concentrations were applied rather than six, as usual in single toxicity tests. The same test substances, method of addition of metal and exposure method of the worms as in single toxicity test phase were applied.

2.2.3. Chemical Analysis of Soil Samples

Chemical analysis was carried out to determine the amount of metals that the worms were actually exposed to after absorption and desorption of metals to and from soil. The background concentrations of the metals in the unpolluted soil samples were also determined. For single toxicity tests Cd, Zn and Pb the metal concentration at the beginning and end of the experiments were determined, but since result of the analysis showed no difference between the amount extracted at the beginning and the amount extracted at the end, samples for chemical analysis were only taken at the end of the experiment for Pb and the combination toxicity tests.

Known amounts of soil samples were randomly taken from each replicate of the different tested concentrations of the metal. The samples were extracted with 0.1M $CaCl^2$ and 0.43 N HNO_3 separately, and shaken for 24 hours. The metal ion concentrations in the solutions were measured by Atomic absorption Spectrophotometer (flame-AAS). Measurements of total metal contents through completely dissolving the samples in heated strong acid, and measuring metal ions in solution by AAS were also done. All chemical analysis were downe by the FCT (RIVM – department of physical and Chemical Techniques).

2.2.4. Statistical Analysis

The data were analyzed with a logistic model as described by Van Ewijk and Hoekstra (1993). The logistic model describes a situation without hormesis. An extension of the model is able to incorporate hormesis, and to assess its significance. In the non-hormesis model, the dose-response curve is given by three parameters.

B = Slope parameter, indicating steepness of slope at the EC50

M = Estimate of the in (EC50), from this, EC50 itself can be calculated

C = Estimate of performance when toxicity is absent.

From the estimate of M, the EC50 was calculated. From the accuracy of the fit, the range of the 95% confidence interval of the EC50 estimate was calculated. The hormesis model is determined by an additional parameter, namely:

F=Value to express hormesis, if significant

The models were fitted by least-square estimation, and results are presented for the non-hormetic model if hormesis was not significant ($P>0.05$), or if the hormetic model showed a worse fit than the non-hormetic model. In the latter case, both results will be shown. The accuracy of the fit is given by the models through the percentage of variation accounted for; a high value indicates an accurate fit.

3. RESULT

3.1. ABIOTIC FACTORS

3.1.1. Total and Extractable Amounts of Metals

Nominal, extractable and actual concentrations of metals in the single toxicity test of Cd, Zn, Pb and in the combination toxicity test of Zn & Cd, Pb, Cd and Pb & Zn, respectively, were measured during the experiments. There is no difference between the amounts of metals extracted at the beginning and at the end of each experiment except for Zn, for which a peculiar difference exists between the amount of metals extracted at the beginning and the end. Since 16.4 mg Zn/kg soil was extracted at the beginning, the low concentration recorded at the end of the experiment might be probably attributed to an analytical error. It can be conclude from all measured metal concentrations, that the worms were exposed to the same concentrations throughout the exposure periods. The detailed data are summarized in the Appendix section (see Appendices 6-18).

Nitric acid prepared to have extracted a higher proportion of added metals than calcium chloride based on the percentages estractable amounts of metal by the two substances. Whereas $CaCl_2$ extracted on the average 15% of Cd, 28% of Zn, 0.8% of Pb, HNO_3, extracted 70% of Cd, 69% of Zn and 69% of Pb.

Also, in the combination toxicity experiment with Cd and Zn, $CaCl_2$ extracted 15% of Cd and 19.7% of Zn while HNO_3 extracted 40% of Cd and 60%.

The desired similarly of concentrations of nitrate in all the tested concentrations of the metals was almost completely achieved for Cd, Pb and Cd & Zn, and for Zn up to concentration 4 (for details: see Appendices 6-18). However, there were differences between the amounts of nitrate added among metals, depending on the amounts of nitrate added in the highest concentration. Therefore, the nitrate concentration in all Cadmium exposure groups was lowest, and increasing in the order Pb (first experiment), Zn, Cd & Zn and finally Pb (seconf experiment).

3.1.2. PH and Humidity Readings

Table 1 shows the Ph and humidity measurement of the different OECD soil samples using the experiments. Apart from the combination toxicity tests of Cd & Pb, and Zn & Pb which disrupted due to factors of which lack of optimum Ph is suspected to be one and Cd & Zn combination toxicity test in which the humidity measurement was 37% in all the other tests, the soil pH and humidity were on the average of 4.76-5.90 and 50.75-57.90% respectively.

Table 1 shows the Ph (at the beginning and end of experiment) and humidity (beginning of experiment) of the OECD soil used in each toxicity test of metal(s).

Table 1. Abiotic factors measured during the experiments

Added metal	**Average of pH Measurement**		**Average % Humidity**
	Begin	End	
Cd	4.82	n.d.	51.80
Pb(1st)exp	4.76	5.40	50.75
Zn	4.76	5.07	57.75
Pb(2nd)exp	5.30	5.60	55.50
Zn & Cd	5.48	5.90	37.06
Cd & Pb--	n.d.	6.60	n.d.
Pb & Zn--			

n.d. = not determined

Experiments ended as a result of lack of production of cocoons even in the control at t=21 days. This was attributed to high pH among other factors

3.2. EFFECT OF THE SINGLE METALS TO EISENIA ANDREI

First, data from the single toxicity test experiments are introduced (section 4.2.), because these data are necessary to interpret the combination toxicity studies, these are treated in section (4.3.).

3.2.1. Growth and Mortality

There was no pronounced dose effect relationship recorded for growth at different concentrations of the metals tested in all the single toxicity tests (figure 3 and appendix 1-5). Except for the fact that there was mortality of 2 worms, 6 worms and 5 worms at the highest concentration of Cd, Zn and Pb respectively. This implies that growth is not a sensitive parameter to estimate effects of these metals on Eisenia Andrei.

3.2.2. Cocoon Production

Figure 4, 5 and 6 summarises model fits of the dose effect relationships for cocoon production of Eisenia Andrei. The model parameters, and other information on model fits to these data, are summarized in Table 2.

The observed EC50 on cocoon production in the experiment with Cadmium was 103.9 mg/kg, at 102.4 – 105.5 95% confidence interval for this estimate. The percentage variance accounted for by the model was 84.4, which indicates an accurate fit of the model to the data.

In the experiment with Zn, when the data on cocoon production were fitted with the non-hormetic model, the EC50 observed was 279.21 mg/kg, and the percentage variance accounted for was 55.9. when fitted with hormetic model, the EC50 was 418.2 mg/kg, while the percentage variance accounted for was 85.2. moreover, hormesis was found to be significant ($P<0.001$). The presence of hormesis at low dose is indicative of the ability of the worms to be stimulated at the low doses of Zn to produce more cocoons than in the control.

Effects of Metals on Growth

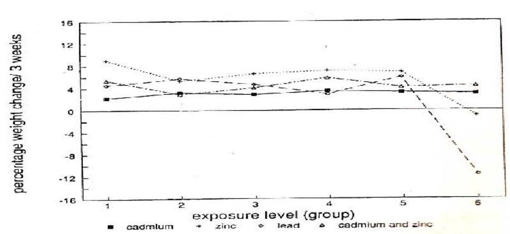


Figure 3. Dose effect relationship of Cadmium, Zinc, Lead and a mixture of Cadmium and Zinc on body growth of Eisenia andrei

With Pb, the EC50 could not be estimated by the model at the tested highest concentration of 1000 mg/kg dry soil in the first experiment. Hence, the experiment was repeated with higher concentration ranges and the EC50 was then estimated to be 1570mg/kg by the non-hormetic model. The percentage variance accounted for by this model was 68.5. This showed that the model gives an accurate description of the observations.

The number of cocoons per worm per week varied from 1.67 to 0.34 in the Cd experiment, 1.12 to 0.00 with Zn 0.80 to 0.00 with Pb (Appendix 1-4). For all metals, there was a clear effect of the dose of metals on the numbers of cocoons produced, since performance reduced by at least 50 percent in the exposed groups (Table 3); only with Zn and Pb there was no cocoon produced at the highest applied concentration. This means that the EC50 could be calculated by interpolation for all metals, which implies accurate estimation. Generally, it appeared that the accuracy of fitting of the model was higher for Cd, followed by Pb and then Zn.

The number of cocoons produced in the different experiments appeared to vary among experiments. This was demonstrated by the controls of each experiment. An average of 34 cocoons were produced in the control experiment with Cd, 33 in the control of Zn and 24 in the control of Pb (Appendix 1-4). Apparently, there is a factor which influences basic cocoon production, which might be either the physiological condition of the worms or the nitrate present. With additional information from the other experiments it is shown later (figure 12), that the number of cocoons decreased from the nitrate present in the control of experiment with Cd (upper left), via Pb (first experiment), Zn and Cd & Zn, towards Pb (second experiment, lower right). The order of decrement shows that the number of cocoons produced decreased with increasing nitrate concentration. Since the model is normally used to fit the data of a single experiment, it would be improper to fit the model to the data of five separate experiments, as indicated in the figure.

Effect of Cadmium on Cocoon production

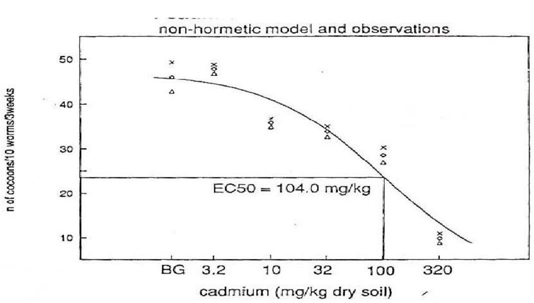


Figure 4. Dose-effect relationship of Cadmium exposure for cocoon in Eisenia andrei

Effect of Zinc on Cocoon Production

Hormetic model and observations

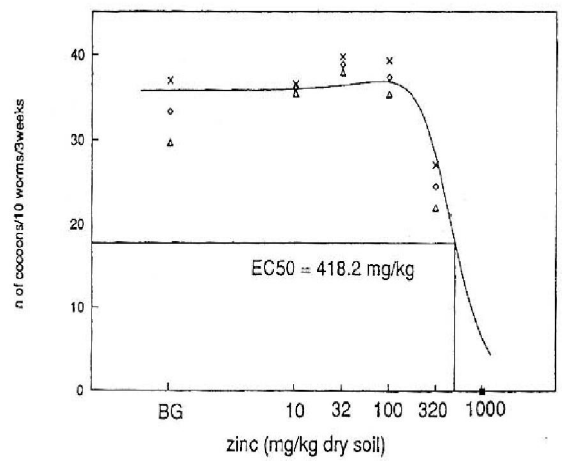


Figure 5. Dose-effect relationship of Zinc for cocoon production of Eisenia andrei

Effect of Lead on Cocoon Production

Non-Hormetic model and observations

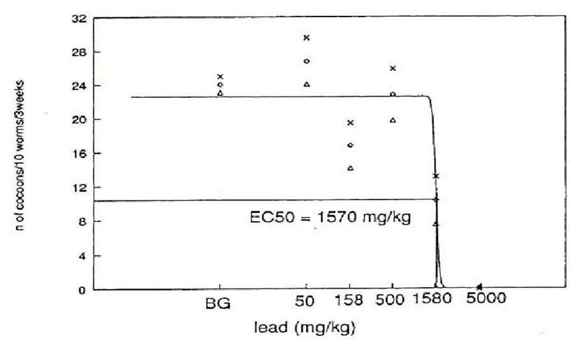


Figure 6. Dose-effect relationship for lead exposure for cocoon production in Eisenia andrei

3.2.3. Hatchability of Cocoons

The percentage for hatchability of cocoons were high in all tested concentrations of the metals, except for the highest concentration range of Zn and Pb, where no cocoons were produced and, therefore, no juveniles were found (Appendices 1-4). On the average, calculated over all concentrations within an experiment, the observed percentages hatchability were 92%, 95% and 93% for Cd, Zn and Pb, respectively. This indicates that, on the overall, the hatchability of the produced cocoons was not really affected by the metals.

3.2.4. **Effects on Juveniles Production**

There is slight difference between the number of juveniles produced per fertile cocoon in the control and the different tested concentrations of each metal in the experiments: the juvenile number per fertile cocoon decreased from 3.07 to 2.00 for Cd, 2.93 to 2.24 for Zn, 2.67 to 0.79 for Pb (Appendices 1-4). This implies that the metals do not interfere with the number of juveniles per cocoon.

Tables 2 and 3 summarize the model fits to data of juveniles production. The estimated EC50 of the effect of Cd on juveniles production was 28.51, at a 95% confidence interval of 28.51-29.82, by the non-hormetic model. The percentage variance accounted for was 89.4. apparently, juveniles numbers are more sensitive than cocoon numbers for Cd exposure (compare Fig. 4 and 7).

Table 2. Summary of results of fitting models to toxicity data. Model: "N" indicates the non-hormetic model, "H" indicates the hormetic model, "%" indicates the percentage of variation accounted for by the model (fit), "B" indicates the slope parameter of both models, 'M' is the estimate of the In(EC50), 'C' is the estimate of unstressed performance, 'F' indicates the hormetic parameter (if significant). The models were fitted both to the parameters C (Cocoon numbers) and J (juvenile numbers).

Substance and parameter	Model	%	B	M	C	F
Cd, C	N	86.6	-0.834	4.644	46.87	-
Cd, J	N	89.4	-0.659	3.350	160.90	-
Zn, C	N	55.9	-0.625	5.632	41.89	-
Zn, C	H1*	85.2	2.380	6.036	35.75	0.00064
Zn, C	H2*	18.1	1.091	13.82	1000.00	-
Zn, J	N	59.5	-0.669	5.270	114.40	-
Zn, J	H	-	-	-	-	-
Pb, C	N	68.5	-26.630	7.359	22.56	-
Pb, C	H	-	-	-	-	
Pb, J	N	71.2	-2.358	6.942	54.24	
Cd & Zn, C	N**	61.1	-11.832	0.946	27.13	
Cd & Zn, C	N***	76.7	-8.410	0.664	28.36	
Cd & Zn, J	N	65.9	-2.190	0.275	78.09	

Hormetic model: the estimate of b in the non-hormetic model is equivalent to the estimate –b of the hormetic model, and indicates the slope at inflection (for further details: see text).

Model ran with defective cocoons inclusive

There are relatively high numbers of defective numbers only the highest conc. Since this affect in a major way the EC50 estimate, the programme was ran both with and without these cocoons.

For Zn, the estimated EC50 of its effect on juvenile production was 194.58mg/kg and the percentage variance accounted for was 59.5 with the non-hormetic model (Fig.8). With the hormetic model, the EC50 tended to infinity while the confidence interval could not be estimated; no variance could be accounted for by the model, and thus indicates bad fit of data. Within the same organism, it appears to be possible to observe hormesis in one reproduction characteristic (cocoon production), while it is lacking in the other (juvenile production).

In the experiment with Pb, the EC50 was 1034.4mg/kg with the non-hormetic model and the percentage variance accounted for by the model was 71.2 (Fig.9, Tables 2 and 3). Again, similar to Cd and Zn, juvenile production was more sensitive to metal exposure than cocoon production (compare Fig. 6 and 9).

Table 3. Summary of EC50 estimates (calculated from parameter M of the models) from fitting models to toxicity data (legends as in former table).

Substance	Parameter	Model	EC50(mg)	95% Confidence interval
Cd	Cocoon	N	103.96	102.42-105.53
	Juveniles	N	28.51	27.25-29.82
Zn	Cocoon	N	279.21	-
		H1*	418.2	-
		H2	(infinity)	(infinity)
	Juveniles	N	194.58	-
		H	-	-
Pb	Cocoon	N	1570.25	-
		H	-	-
	Juveniles	N	1034.40	-
Zn & Cd	Cocoon	N**	2.57(TU)	-
		N***	1.94(TU)	1.93-1.96
	Juveniles	N	1.32(TU)	1.30-1.33

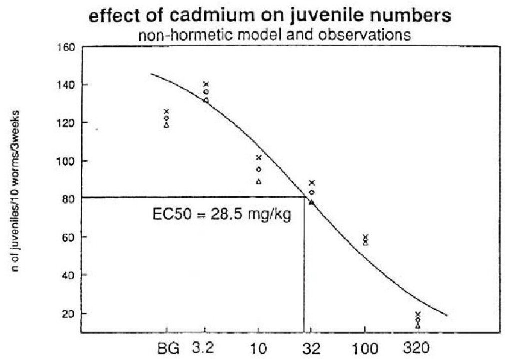
In this case, the hormetic model was run without (HI) and with (H2) defective cocoons. The model H2 did not show interpretable results.

-- Model run with defective cocoons observed particularly in the highest exposure concentration

--- Model run without the defective cocoons, which improved fit.

Based on the percentage variance accounted for by the models for different metals, the model fitted best data of the experiment with Cd, followed by that of Pb, and then Zn. Judged from the estimated EC50, Cd appeared to have a very strong effect on juvenile production, more than Zn and Pb, while Pb is least toxic to juvenile production.

Juvenile production appears to be a more sensitive parameter than cocoon production based on the estimated EC50 values of all the single toxicity experiments on both cocoon production and number of juveniles produced. The percentage variance accounted for by the model was always higher for effects on juvenile production than that of cocoon production except of Zn.



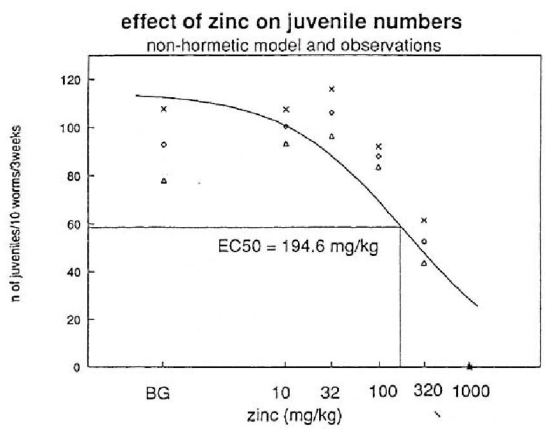


Figure 8. Dose-effect relationship of Zinc exposure for juvenile numbers in Eisenia andrei

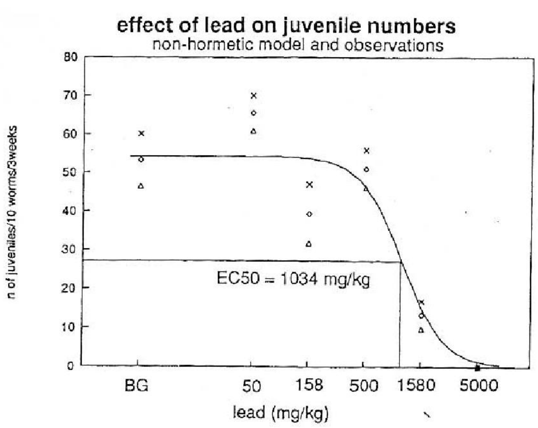


Figure 9. Dose-effect relationship of lead exposure for juvenile numbers in Eisenia andrei

3.3. COMBINATION TOXICITY EXPERIMENTS

3.3.1. Effect of a Cd & Zn Combination on Eisenia Andrei

Effects on Cocoon Production. Figure 10 summarises the dose-effect relationship of the effect of different concentrations of Zn & Cd combinations to cocoon production of E.andrei. at the highest two concentration ranges, an usually large numbers of defective cocoons was recorded. This may pose a problem to the interpretation of the combination toxicity test. To solve this, the model was run with one set of data with defective cocoons included and the other with defectibe cocoons excluded.

The EC50 observed with data including the defective cocoons was 2.57 (TU) with the percentage variance accounted for being 61.1 (Tables 2 and 3). Without defective cocoons the EC50 was 1.94 (TU) with a confidence interval of 1.93-1.96 and a percentage variance accounted for 76.7 (Tables 2 and 3). From these TU values, it is clear that addition of the defective cocoons affects in a major way the TU obtained. The percentage variance accounted for by the data without the defective cocoons is higher that that of the one with defective cocoons. Based on the accuracy of fit, it is concluded that interpreting the data without adding the defective cocoons is better than with defective cocoons included.

Both TU values indicate that toxicity of the mixture of Cd and Zn is less than concentration additive on cocoon production of Eisenia Andrei, both for the good fitting model and the probably imperfect model fit. For further details, see Appendix 5 which summarized the original data for cocoon production of Eisenia Andrei exposed to an equitoxic combination of Zn and Cd.

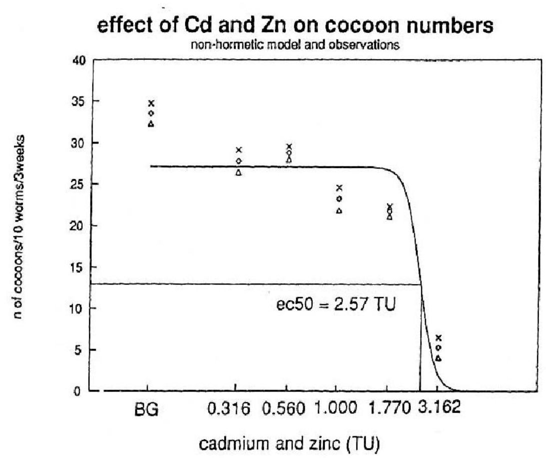


Figure 10. Dose-effect relationship of combined exposure to an equitoxic mixture of Cadmium and Zinc for Cocoon production in Eisenia Andrei

Effects on Hatchability of Cocoons. The percentage fertile cocoons for all the tested concentration ranges was on the average 94% (Appendix 5). This suggests that the hatchability of cocoons was not actually affected by the metal combinations, except for the recorded defective cocoons in concentration 6. the defective cocoons did not hatch, and were therefore, automatically, not influencing the model fits for juvenile numbers.

Effect on Juvenile Production. The mixture for combination toxicity test of Cd & Zn was prepared based on the EC50 of the single toxicity effect test of Cd and Zn on cocoon production of E.andrei, and not on their single toxicity tests results for juvenile production. This is because only one reproduction parameter can be studied in one combination toxicity test, and here major emphasis was put on the reproduction parameter cocoon production. Based on the EC50 values estimated for the single metals for juvenile production, TU were calculated as usual, but with the following principal difference for the interpretation. Since the equitoxicity was aimed to be valid for cocoon production alone, the principle of equitoxic concentrations was not applicable to the juvenile data. This means, that the mixture contained relatively more Zn than Cd. After calculation of the soil concentrations prepared, and estimating the TUs for juvenile numbers, it appeared that the ratio of concentrations of Cd and Zn in this mixture, expressed as toxic units, were 2.168, and not 1 as usual in an equitoxic mixture. The estimated EC50 from the non-hormetic model is 4.46 TU (Fig. 11, Table 2,3), this is equivalent to a mixture of Cd and Zn with concentrations of 87.0mg/kg of Cd and 274.0 mg/kg Zn. Apparently, much more Cd and Zn are needed than expected from the input data to obtain an EC50 effect. This indicates, again, an antagonistic effect between Cd and Zn.

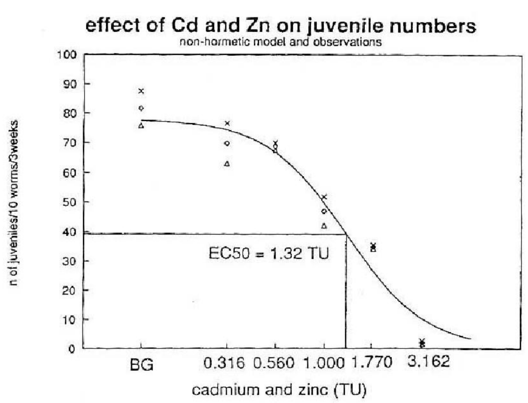


Figure 11. Dose-effect relationship of exposure to a mixture of Cadmium and Zinc for juvenile numbers in Eisenia Andrei. For further explanation: see text.

When judged by the EC50 of the single metals, the relative toxicities of Cd and Zn on juvenile production can be compared. Cd appeared to be 11 times more toxic than Zn for juvenile production.

3.3.2. Effects of Cd & Pb and Zn & Pb Combinations on Eisenia Andrei

With Pb & Zn and Pb & Cd, no scientifically reasonable data of the effect of the metal production to E. Andrei could be gathered. This is because at the end of 3-week exposure period of the worms to the metal combination of Pb & Cd, no cocoons were observed in the control of the experiment and only 1 cocoon was recorded for concentration number 3. hence, the result of the experiment was disregarded as far as this combination toxicity research is concerned. The test on Pb & Zn was also prematurely stopped after checking each replicate of the 6 concentration at the second-week of the duration of the experiment. This was due to the alarm raised by the result of the former combination test. In both of the experiments, the number of cocoons produced in the acclimation phase were on the average 7 versus an average of 13 recorded in previous tests.

4. DISCUSSION

4.1. SINGLE TOXICITY TEST

Cd had the most adverse effect on cocoon production, followed by Zn and Pb. Similar results have been recorded on the effects of Cd and Zn on this parameter. Both Van GEstel (1991) and Spurgeon et al. (1993) observed a similar order of toxicity of Cd, Zn and Pb on cocoon production of Eisenia Andrei (Savigny). So, the present results for single toxicants are in good agreements with previous work by other authors. Therefore, the above order of relative toxicity should be taken to be scientifically reliable.

The numbers of juveniles produced were generally more affected by the individual metals than the numbers of cocoons produced. This is judged from the EC50 of the effect of the metals on production. This experiment also supports the observation of Spurgeon et al. (1993) that juvenile number is a more important parameter for toxicity test of metals, than cocoon production.

Growth was not affected by the metals at all concentrations tested, except for the highest concentration of Pb. There was a slight increase in weight of the worms except for the highest concentration of Zn, and in the experiment with Pb Growth appeared to be an insensitive parameter for toxicity tests Cadmium, Zinc and Lead in earthworms. Therefore, growth may not be an important parameter for calculation of data for combination toxicity tests of Cd, Zn and Pb in earthworms tests.

The largest numbers of cocoons were recorded for the control of Cd, then Zn, then Pb. This could potentially be attributed to the biological differences of batches of worms, and to the effects of nitrate. Since figure 12 shows that there is dose effect relationship of nitrate concentration to cocoon production, I conclude that nitrate influenced the number of cocoons produced. In future, equal concentrations of nitrate should be maintained in all the tested metals as a whole, and not just within the different concentrations of each metal. This is already applied by Weltje (RIVM, 1993) in further experiments with earthworms exposed to heavy metals (pers, comm.., Lenmnart Weltje).

4.2. COMBINATION TOXICITY TESTS

The present study forms a landmark in the combination toxicity studies of heavy metals using earthworms. However, information is generally lacking on the combination toxicity effects of heavy metals to terrestrial organisms. It is only in aquatic literature that extensive studies have been recorded on this subject. First, therefore, some comments will be made on the results from aquatic literature (4.2.1.), followed by an evaluation of the present results (4.3.2.).

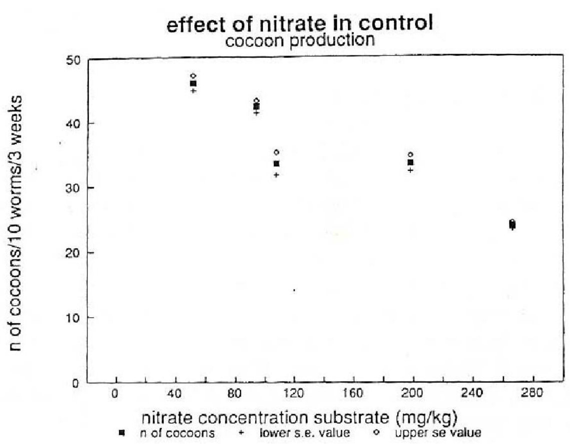


Figure 12. Relationship between amounts of nitrate in controls of (left to right) exp. With Cd, Pb(1), Zn, Cd & Zn, Pb(2) and cocoon production in Eisenia Andrei

4.2.1. Information From Aquatic Literature

The EIFAC (1987) presented a literature review on combined effects on freshwater fish and other aquatic life forms of mixtures of toxicants in water. Based on the report, the action of Cu and Zn appeared to be several-fold more than additive on the percentage survival of mummichog in synthetic sea water (20% salinity). The presentation reported conflicting result of combination of Cu and Cd to mummichog, baed on different parameters. Whereas Cu and Cd appeared to be less than concentration additive on their effect on the lateral lines of mummichog, the two metals have also been reported to be more than additive on both a response and concentration basis to survival of mummichog in synthetic sea water. Lethal effects of Cu and Cd in mixtures were found to be almost 2-fold more than additive to zebra fish in 96-hour acute tests.

Kraak (1992) evaluated the effects of equitoxic mixtures of Cu, Zn and Cd on the filtration rate of the freshwater mussel (Dreissena polymorpha) using the toxic unit concept, and he found that the effect of a mixture could not be predicted from the effects of the single metals. Cu and Cd contributed to the toxicity of a mixture of Cu, Zd and Cu at or below the NOEC for these metals, determined in single metal toxicity test, indicating concentration addition and potentiation, respectively.

Kraak (1992) also studied the chronic ecotoxicity of mixtures of cu, Zn amd Cd to the zebra mussel Dreissena polymorpha. In short-term experiments, Cu + Cd were strongly more than additive, indicating a loss of potential for additivity during prolonged exposure. It was concluded that the chronic effects of mixtures could neither be predicted from their short-term effects, nor from the chronic effects of the metals tested individual.

Enserink et al. (1991) did an ecological evaluation of reports on combined effects of metals. In their work, As, Cd, Cu, Hg, Pb, Ni and Zn were tested singly and in equitoxic mixtures based on the LC50 (individual Daphnia magna) or EC50 (population) of single metals. The result of their work showed nearly concentration addition of the metal mixture to individual survival as well as population growth of Daphnia magna in most cases.

Spehar and Fiandt (1986) worked on acute and chronic effects of water quality criteria-based metal mixtures on three aquatic species. In their work, As, Cd, Cr, Cu, Hg and Pb combined at criterium concentrations caused nearly 100% mortality in rainbow trout and daphnids (Ceriodaphnia dubia) during acute exposure. Fat-head minnows were not adversely affected at this or at two times this concentration, although a mixture of four to eight times the maximum values caused 15 to 60% mortality. This shows that effects of different metal combination differ from one organism to the other. Metals combined at the criterion average concentrations significantly reduced production of daphnid young and growth of fathead minnows after 7 and 32 days, respectively. In their further work, acute tests with metals at multiples of LC50 concentrations indicated that the joint action of the metals was more than additive for daphnids, based on toxic units calculated from the individual components of the mixture. Chronic tests showed that the joint action was less than additive for fathead minnows but nearly strictly additive for dapnids, indicating that long-term metal interationc may be different in fish compared to lower invertebrates. This result clearly pointed out that single chemical derived water quality criteria may not sufficiently protect some species when other toxicants are also present.

To summarise, experiment results available on combination toxicity in aquatic ecotoxicology show some contradicting results at first sight. Toxicity effects resulting from different combinations of metals differ with different parameters and different experimental conditions and duration of experiment. Despite this snag, the fact that combination toxicity experiments reflects the actual pollution of aquatic ecosystems in a more realistic way than experiments in which toxicants are tested individually, has been emphasized by many authors.

4.2.2. **Information From Experiments With Terrestrial Organisms**

Focusing on metal combination in soil, Bewley and Stotky (1983) investigated the effects of combinations of Cd and Zn on microbial activity in soil and the influence of clay minerals when metals were added simultaneously. They found that the effect on the lag period for population growth of the two metals together was concentration additive.

The EC50 for cocoon production in earthworms exposed to an equitoxic mixture of Cd and Zn was estimated to be 1.94 TU. Based on the concept of the Toxic Unit model, this result can be interpreted to mean that the metals are less than additive (antagonistic) (Sprague, 1970). Since the results of the combination toxicity experiment of Zn & Pb and Pb & Cd could not be obtained in this research, the effect of different mixture on cocoon production and juvenile production can not be compared favourably with that of their single toxicity.

The lack of observations for cocoon production was attributed to some possible factors:

1. The high pH value, as the pH read at the end of the experiment were 6.6 for Pb & Cd and 6.5 for Pb & Zn, versus the highest value of 5.0 recorded in previous experiments.

2. The weight of the works were on the average of 0.295 and 0.283 as against 0.35 and 0.37 recorded on average in previous tests.

3. May be that there was an unidentified adverse environmental factor that interfered with the experiment.

No previous or further information is available in terrestrial literature on effects of metal combinations to earthworms. If it should be taken that the two metals are antagonistic, it can be explained by the fact that the ions may complete for target molecules. In the presence of a high amount of Zn (a high number of molecules), Cd may not easily reach the target, so that toxicity decreases, in that case, Cd, which has been proved to be more toxic than Zn in single toxicity test, is rendered less toxic when acting in equitoxic concentration with Zn. It should be reminded that Zn is an essential element (micronutrient), whereas Cd is completely non-essential to any form of life.

4.3. **ECOLOGICAL TRANSLATION TO NATURAL CONDITIONS**

In the aquatic environment, discrepancies found between additive action and more than additive action of metals in organisms may be due to water quality characteristic, such as water hardness, that may alter metallic forms (EIFAC, 1997). In natural soils, similarly, different soil characteristics may also influence the toxicity of mixtures of chemicals to soil organisms. There is, however, no information available in literature to support this point. Mixtures of two substances have been found to be less than concentration additive in most cases, whereas mixtures of up to six substances are usually concentration additive (Spehar et al. 1970). In polluted sites, there would exist the probability of a Cd & Zn mixture being concentration additive in the presence of other metals.

It was observed that the metals did not interfere with the hatchability of the cocoons or with the number of juveniles per fertile cocoon. It could be possible that such an adverse effects of metals on juveniles could be observed in natural conditions, where the juveniles are exposed from hatching, in contrast to the laboratory situation. There may exist a difference in the survival and fitness of the juvenile if tested in a complete life cycle test.

Since E.andrei still acts like their aquatic ancestors (Edward & Lofty, 1977) it is recommended to carry out toxicity tests for the soil compartment with other important and completely terrestrial soil organisms in addition, like springtails from the upper litter layer of the soil, isopods, centipedes or millipedes, in order to obtain more reliable information on combination toxicity effects to terrestrial organisms in the soil compartment.

More experiments is need in this area for a favourable comparison of available information in aquatic literature on combination toxicity with results of similar studies with terrestrial organism.

4.4. CONCLUSION AND RECOMMENDATIONS

The model of combination toxicity of mixtures has been shown by this research to be applicable to E.andrei exposed to equitoxic mixtures of Zn & Cd. The toxicity of Cd to the reproduction of E. Andrei appeared to have been reduced by the presence of equitoxic concentration of Zn, or the other way round.

The result of the present study suggests that the toxicity of combination of metals might not be predicted from the EC50 of the single metals to the same parameter. Hence, single toxicity tests alone may not determine the fate of earthworms in sites polluted with combinations of chemicals.

This study demonstrates that the effect of Cd, Zn and Pb to cocoon production in the single toxicity test are in agreement with that of other authors like Van Gestel (1991) and Spurgeon et al. (1993).

For all the single toxicity test experiments, cocoon production and number of juveniles emerging from the produced cocoons appeared to be important basic data for calculation of mixtures for combination toxicity test.

The use of EC50 of 'number of juveniles' produced is recommended as basic data for calculation of mixture concentrations for combination toxicity tests when effect of metals on the life cycle of E.andrei are to be carried out. This will give a very good indication of the effect of the metals to the survival and fitness of the resultant juveniles. This will in turn give a better indication of long term effect of toxicants like metals to earthworms.

More experiments are needed in the area of terrestrial ecotoxicology on the mixture effects of metals, to enable a favourable comparison of the obtained results with available information in aquatic literature.

In future, abiotic factor, such as pH and humidity of OECD medium should be checked before starting of toxicity experiments. This is contrasting to the presently applied approach of measuring these abiotic factors after exposure of worms. Similar batches of different components of artificial soil substrates should be used throughout a research. These additional precautions will help to exclude possibilities of variation of environmental conditions in a particular research phase.

Equal amounts of nitrate should always be maintained in all the concentrations of metals tested, instead of within each series of concentrations for each tested metal separately. This is meant to adjust for the influence of nitrate on dose-effect relationships of metal salts to cocoon production.

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