Heavy metal accumulation in earthworms exposed to spatially variable soil contamination

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Stellingen

1. Ecotoxicologen houden onvoldoende rekening met omstandigheden van bodemverontreiniging *in situ*.
   dit proefschrift

2. Ook voor regenwormen blootgesteld aan ruimtelijk variabele bodem-verontreiniging, is het mogelijk de blootstelling te kwantificeren.
   dit proefschrift

3. Het gebruik van 'Internal Threshold Concentration' (Wensem et al., 1994) suggereert ten onrechte een onvoorwaardelijk verband tussen het optreden van stoornissen in groei, reproductie en/of overleving enerzijds en de interne concentratie van een bepaalde stof anderzijds.
   Wensem, J. van, J.J. Vegter, and N.M. van Straalen (1994) Soil quality criteria derived from critical body concentrations of metals in soil invertebrates. *Appl. Soil Ecology* 1: 185-191.

4. De bodem is geen bodemloze put.

5. "Biologische beschikbaarheid" is een verwarrend begrip.

6. Wie tijd spaart, verliest tijd.
   Marianne Cense in een persoonlijk gesprek op 21 september 1993

7. Structureel overwerken is een belangrijke oorzaak van de heersende werkloosheid.

8. De heersende concurrentie tussen wetenschappers, mede veroorzaakt door de strijd om financiering van het onderzoek, is moordend voor de wetenschap en de wetenschappers.

9. Mensen hebben het recht te kunnen kiezen voor euthanasie; leven is een recht en geen plicht.

10. Leven is een beetje sterven.
    Simone de Beauvoir in haar roman: "de Mandarijnen"

Stellingen behorend bij het proefschrift "Heavy metal accumulation in earthworms exposed to spatially variable soil contamination". Mari Marinussen, 21 februari 1997.

Aan ons pa en ons ma

Abstract

Ecotoxicity of contaminated soil is commonly tested in standard laboratory tests. Extrapolation of these data to the field scale is complicated due to considerable differences between conditions in laboratory tests and conditions *in situ* in contaminated soils. In this thesis, heavy metal accumulation in earthworms was studied under various laboratory conditions to identify and obtain knowledge that is needed to predict accumulation in earthworms exposed to *in situ* soil contamination. Both Cu accumulation-rate and Cu excretion-rate appeared large, which implies that Cu tissue concentrations in earthworms change rapidly in spatially variable contaminated soils. Soil moisture content did not influence the heavy metal accumulation. Soil pH did not influence the Cu availability for uptake, but significantly affected the toxicity of Cu contaminated soil. This indicates that there are two different exposure routes. Earthworms were also exposed to soil containing a mixture of Cd and Cu. A considerable tissue Cd accumulation, which could result in an increase in Cu binding capacity of earthworms, did not affect tissue Cu accumulation in these group of earthworms. Also the addition of Pb to Cu contaminated soil, which could result in an increase in Cu availability, did not affect tissue Cu accumulation. The results of the laboratory experiments imply that of factors studied only the total extractable soil Cu content controls tissue Cu accumulation, whereas mortality is controlled by Cu concentrations in soil solution. Predictions of tissue Cu accumulation in earthworms that were introduced in a heavy metal (Cu, Pb, Zn) contaminated site were based on these assumptions. Measurements of tissue Cu concentration appeared in excellent agreement with the observations in laboratory studies. Hence, laboratory studies with earthworms are very useful for estimation of the effects of soil contamination *in situ* on earthworms, provided that laboratory conditions are realistic with regard to field conditions.

Additional index words: soil contamination, spatial variability, heterogeneity, Cu, copper, field-studies, soil-chemical interactions, earthworms ecology.

Contents

Chapter *1*

General introduction

UTCH SOIL PROTECTION legislation intends to protect public health and environment against adverse effects of soil contamination on the functions of soil that are important for humans, flora or fauna (Ministerie van VROM 1994a). Much effort has been dedicated to scientific research into human-toxicological and ecotoxicological effects of soil contamination to provide a scientific basis for soil quality criteria. The study in this thesis has been performed within the scope of risk assessment of heavy metal contamination of soil ecosystems.

COMPLICATIONS IN CONTAMINATED SOIL RISK ASSESSMENT

REDICTIONS OF ECOTOXICOLOGICAL effects of soil contamination in contaminated sites are commonly based on data obtained by laboratory studies. Extrapolation of ecotoxicological data obtained by laboratory studies to the field scale is difficult (TCB 1991, Wensem *et al.* 1994, Straalen and Bergema 1995). Discrepancies between field observations and laboratory studies may to some extent result from differences between conditions in standard laboratory toxicity tests and conditions *in-situ* in contaminated soils. Below, I discuss briefly three of the differences that can be identified. I mention only these differences, because they were subject of my research.

1. *Soil spatial variability*
Soil properties show various degrees of spatial variation and correlation. The variation generally depends on land use and soil type. The spatial variability of soil contamination depends also on *e.g.* the source of contamination, and soil

physical and soil chemical interactions. Soil spatial variability complicates ecotoxicological risk assessment of contaminated soil in at least three ways:

(i)   The reliability of estimations of the degree and extent of soil contamination are limited or even small.

(ii)   Soil spatial variability of soil contamination leads to temporal variation in exposure of migrating soil dwelling organisms. It is unknown whether this variation has consequences for the sensitivity of individual organisms to soil contaminants. Recently Newman and Jagoe (1996) showed mathematically that "realistic time lags" in exposure lead to oscillations of concentrations in organisms. Oscillations in concentrations can also occur in organisms inhabiting contaminated soils. Since oscillations may have significant implications for *e.g.* food chain transfer models and the use of accumulation models in establishing environmental quality criteria (Newman and Jagoe 1996), it is important to test the mathematical estimations in field and laboratory experiments.

(iii)   Soil spatial variability results in differences in exposure of each (group of) individual(s) belonging to a population inhabiting a contaminated site. An example concerning a concentration gradient as a function of depth was provided by Lofs-Holmin (1980) who discussed that juvenile earthworms are exposed to much larger pesticide concentrations than adults, because juveniles mainly live in the upper layers of soil (Rundgren 1975) whereas adults inhabit both upper and lower layers. Lethal exposure of juveniles only may still lead to eradication of earthworms. Planar spatial variability may imply that adverse effect concentrations are exceeded at only a few spots in the upper layer of soil. A particular fraction of organisms moving through such a contaminated area will be exposed to contaminants. The number of organisms that will be exposed may depend on both the degree of variability and the mobility of soil animals. The latter is associated with the ecology of the particular organism and varies with time due to temporal variation in *e.g.* soil-temperature and soil-moisture.

2. *Soil properties*

The toxicity of contaminants in soil is associated with soil properties that affect the contaminant bioavailability such as pH, cation exchange capacity (CEC) and composition and concentration of soil organic matter (OM) (*e.g.* Straalen 1993). In soil ecosystems, these parameters vary as a function of time and space, and may be different from the soil that is used in laboratory experiments. Standardization of the soil type by using an artificial "soil" which is a mixture of 70% sand, 20% kaolinite and 10% peat (OECD 1984) may help in communicating differences between either contaminants or organisms, but does not enable a simple translation to natural soils. The reason is that this mixture differs very much from natural soils in at least three respects.

(i)   Soil is not a fresh mixture of its constituents, but rather a product of long-term mechanical, chemical and biological processes. Its constituents (mineral matter, organic matter, water solution and soil air) have interacted with each other and these interactions determine to a large extent the soil-chemical properties that affect the bioavailability.

(ii) In Dutch soils, the main clay mineral is illite (Jelgersma 1994). The specific surface area of kaolinite that is used in the OECD mixture is small (1-40 m^2 g^{-1}) compared to the specific surface area of illite (50-200 m^2 g^{-1}; Bolt *et al.* 1978), which implies that the CEC of illite is larger than the CEC of kaolinite. Hence, illite will much more affect the contaminant bioavailability than kaolinite.

(iii) Peat which is used as organic matter in the OECD mixture is a parent material of reactive humic substances in soil ecosystems. Due to chemical and biological weathering of raw organic matter, it becomes chemically reactive and is able to adsorb large amounts of heavy metals (*e.g.* Wit 1992). Peat may be a poor model constituent for the large variety of different organic substances that is present in soil ecosystems.

3. *Mixtures of contaminants*

Generally soil contamination consists of mixtures of contaminants. This may have

at least two ecotoxicologically important consequences. First, soil-chemical interactions with other pollutants may affect the bioavailability of each pollutant. Second, interaction with other pollutants accumulated in organisms may change the toxicity of each pollutant (Hansen and Lambert 1987). The mixture toxicity may be less (antagonism) or greater (synergism) than the toxicity of the most toxic component, or it may be equal. As the number of possible contaminant combinations may be unlimited, it is too expensive to study all possible interaction effects in laboratory studies.

Table 1.1 *Heavy metal concentration factors (ratio heavy metal concentration to soil heavy metal concentration) for cadmium, copper, lead and zinc at contaminated sites (Ireland 1983)*

Site	Species	Cd	Cu	Pb	Zn	reference
Acid mine spoil	*Dendrobaena rubida*			2.4	0.3	Ireland (1975)
Near roadside	*Lumbricus terrestris* (degutted)	17	0.6	0.4	7.3	Czarnowska and Jopkiewicz (1978)
Lead mine complex	*L. terrestris*			0.03	0.02	Roberts and Johnson (1978)
Soil amended with sludge	*L. terrestris*	26		0.4		Andersen (1979)
	4 *Allolobophora* spp.	9-17		0.1-0.2		
Soil amended with sludge	*L. terrestris*	9		0.1		
	4 *Allolobophora* spp.	9-20		0.1-0.2		
Soil amended with sludge	*Aporrectodea tuberculata*	236			4.5	Helmke *et al.* (1979)
Lead mine complex	*Lumbricus rubellus*	7.5	0.7	2.7	5.4	Ireland (1979)
Soils amended with metals	*Eisenia foetida*	4.7			0.3	Mori and Kurihara (1979)
Sludge amended with metals	*E. foetida*	1.7	0.2	0.01	0.07	Hartenstein *et al.* (1980a)
	E. foetida	0.8	0.1	0.6	0.6	Hartenstein *et al.* (1980b)
Sludge near zinc smelter	5 *Allolobophora* spp.	4-6		0.4-0.6	1-2	Wright and Stringer (1980)
	L. terrestris	5.6		0.3	2.3	

AIM OF THE STUDY

THE MAIN OBJECTIVES of my study were: (i) to determine whether or not data obtained by laboratory experiments can be used for assessing actual ecotoxicological risks at the field scale (*in situ*); (ii) to identify which knowledge and experimental data are lacking for assessing actual ecotoxicological risks *in situ*; and (iii) to fill part of these gaps in knowledge.

To investigate how laboratory data should be handled to enable the assessment of ecotoxicological effects in the field, data regarding exposure and accumulation or toxic effects of a mobile soil-dwelling organism for a contaminant were needed from both laboratory and *in situ* field site conditions. In view of both other studies and the available facilities, the investigated contaminant was a heavy metal. Earthworms were chosen as the mobile soil- dwelling organism for practical reasons: mobile, but limited travel distance during the course of experiments which lasted typically days or weeks; ease of sampling and analysis; heavy metals accumulate in earthworms as is illustrated in Table 1.1. Tissue heavy metal concentrations in earthworms correlate well with soil heavy metal concentrations (*e.g.* Ireland 1979, Ash and Lee 1980, Morgan and Morgan 1993). Hence, the tissue heavy metal concentration may be an indicator of the degree of exposure of earthworms to soil heavy metal contamination. However, interactions with other contaminants may affect the heavy metal availability for uptake. This hypothesis was also tested in research described in this thesis.

OUTLINE OF THIS THESIS

THE CHAPTERS 2-6 of this thesis consist of papers that have been submitted to or published by international scientific journals. In Chapter 2, the effect of home-range on the exposure of organisms to spatially variable soil contamination is examined. Species may differ in home-range which affects the exposure time and exposure level. In a mathematical model, the

toxicokinetic one-compartment model was used to investigate whether there is a considerable variation in exposure within a group of individuals living in the same living area.

In Chapter 3, the tissue copper accumulation in earthworms *Lumbricus rubellus* was studied under both laboratory and field conditions. The field experiment has been performed at an arable field that was artificially contaminated in 1982. The spatial variability of soil copper content within the plough layer was not large and tissue copper accumulation under field conditions were expected to be in good agreement with tissue copper accumulation under laboratory conditions.

In Chapter 4, copper accumulation and excretion rates in earthworms *Dendrobaena veneta* were studied under laboratory conditions. Earthworms had been exposed in soils that were sampled at a contaminated site in The Netherlands. Both copper accumulation and excretion rate determine whether or not earthworms tissue copper concentrations change rapidly when earthworms move through a spatial variable soil copper contamination.

In Chapter 5, copper accumulation in earthworms *Dendrobaena veneta* exposed in soils containing a mixture of either copper and cadmium or copper and lead was studied under laboratory conditions. It was hypothesized that exposure to cadmium would induce the production of metallothioneins, *i.e.* heavy metal binding proteins. This induction may lead to an increased ability of copper accumulation. Lead was added to copper contaminated soil to affect the copper sorption equilibrium, which may lead to an increased tissue copper accumulation.

In Chapter 6, copper accumulation in earthworms *Dendrobaena veneta* that were introduced in a heavy metal contaminated site was studied and predictions on tissue copper accumulation based on laboratory observations were evaluated. It was expected that at high soil sampling density in the field site, it may be possible to accurately estimate tissue copper accumulation under field conditions.

In Chapter 7, computer simulations illustrate the implications of the work described in the previous chapters for tissue Cu accumulation and mortality under field conditions. A few suggestions for future research are provided and implications for soil protection policy are discussed.

Chapter 2

Conceptual approach to estimating the effect of home-range size on the exposure of organisms to spatially variable soil contamination

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ABSTRACT

Spatial variability of soil properties leads to uncertainties in the ecotoxicological risk assessment of polluted soil *in situ*. Mobility of organisms causes that they are not exposed chronically to pollutants in soil, which is in contrast with laboratory experiments. Moreover, only a fraction of the total amount of contaminants is available for organisms. Developing a conceptual model, we identify the information that is required for an ecotoxicologically based risk assessment. Field data of Cd polluted soil are used in a Monte Carlo simulation for the illustration of the concept. The data are analysed geostatistically and predictions at unsampled locations are made using Ordinary Block Kriging. The accumulation of the pollutant in fictitious organisms is estimated with the one-compartment toxicokinetic model. Both the home-range size of the organism and the spatial pattern of cadmium content affect the extent of the area where exposure to the pollution leads to exceeding of a specific Cd-concentration in the organisms. On average, larger home-range sizes lead to lower Cd-concentrations in organisms. However, larger home-range sizes lead to an increase of the probability that a specific exposure level is exceeded. Research in uptake- and assimilation coefficients, excretion activities, and the behaviour of organisms in a polluted area is needed.

INTRODUCTION

URING THE PAST decades, awareness of the hazards due to soil pollution has grown. This has resulted in legal constraints regarding disposal and emission of pollutants. Additionally, soil cleaning methodologies for already polluted soil have been developed.

Risks due to soil pollution need to be quantified to assess whether or not a soil is polluted at a level that warrants expensive clean-up programs. In The Netherlands and some other countries, total contents in soil are compared with references in the procedure of the risk assessment of polluted soil. Straalen and Denneman (1989) developed the RAPS-method (risk analysis of polluted soil) to estimate an ecotoxicologically based maximum allowable content of a specific pollutant in a soil-ecosystem. They assumed that the ecosystem itself will be protected by protecting 95% of the species in the system. The corresponding maximum allowable content of pollutant is based upon the NOEC (No Observed Effect Concentration) of at least five species. The NOEC is determined in laboratory experiments so it is of limited value. Extrapolations from laboratory to field scale result in large uncertainties in ecotoxicology (TCB 1991, Straalen *et al.* 1991).

Field conditions differ from that in the laboratory in at least two ways. First, polluted soil generally contains several pollutants, hence one has to deal with combinational toxicity (synergistic, antagonistic). The second difference is the temporal and spatial variability in exposure of organisms to the pollutant. Organisms are not exposed chronically to one specific degree of pollution due to spatial variability of soil physical and soil chemical parameters.

The aim of this paper is to show how the effect of soil spatial variability on the exposure of mobile organisms to soil contamination can be taken into account in risk assessment. In particular we considered fictitious organisms that differ only with respect to their home-range size. Hence they are equal in metabolism, age, weight, health, etcetera. We used the one-compartment toxicokinetic model and field data of a Cd polluted soil to estimate the Cd concentrations in organisms.

Comparison of the concentrations in the organisms with different home-range sizes resulted in conclusions about the effect of soil spatial variability on the accumulation of pollutants.

CONCEPTUAL MODEL

Limited availability of soil contaminants

FOR ECOTOXICOLOGICAL RISK assessment, consideration of soil quality should be based on concentrations that are available for the organisms. Assessment that only concerns the total contents is not satisfying. Organisms are exposed to contaminants by eating, by drinking and by absorption through respiratory organs and/or body surface. The amount of contaminant that is assimilated by animals is not well known in many cases (Hopkin 1989). In Figure 2.1, uptake routes are depicted schematically. It is obvious that the composition of the diet of a specific organism affects its daily uptake of pollutants.

Soil dwelling species are exposed to concentrations in the soil solution, as was shown for earthworms by Gestel (1988). The concentration in the soil solution is often in equilibrium with the amount adsorbed by the solid phase (clay minerals, soil organic matter). For heavy metals, this equilibrium can often be described with the Freundlich adsorption equation:

$$q = K_F C^n \qquad (2.1)$$

where q is the amount adsorbed [mg kg^{-1}], K_F is the Freundlich apparent affinity parameter [mg^{1-n} m^{3n} kg^{-1}], C is the concentration in the soil solution [mg m^{-3}] and n is the Freundlich power [-] (García-Miragaya and Page 1977,1978).
The total content Q [mg m^{-3}] equals

$$Q = \rho q + \theta C \qquad (2.2)$$

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where ρ is the dry soil bulk density [kg m^{-3}] and θ is the volumetric water fraction [m^3 m^{-3}].

Depending on pH, ionic strength and organic matter content, large total contents may correspond with either high and low concentrations in the soil solution. For cadmium Boekhold and Van der Zee (1992) showed that equation (2.1) can be written as:

$$q = K^* f_{oc} (H^+)^{-1/2} C^n \tag{2.3}$$

where K^* is the apparent affinity parameter [mg^{1-n} mol$^{1/2}$ m$^{3n-1/2}$ kg^{-1}], (H^+) is the proton activity in soil [mol m^{-3}], f_{OC} is the organic carbon content of soil [kg kg^{-1}].
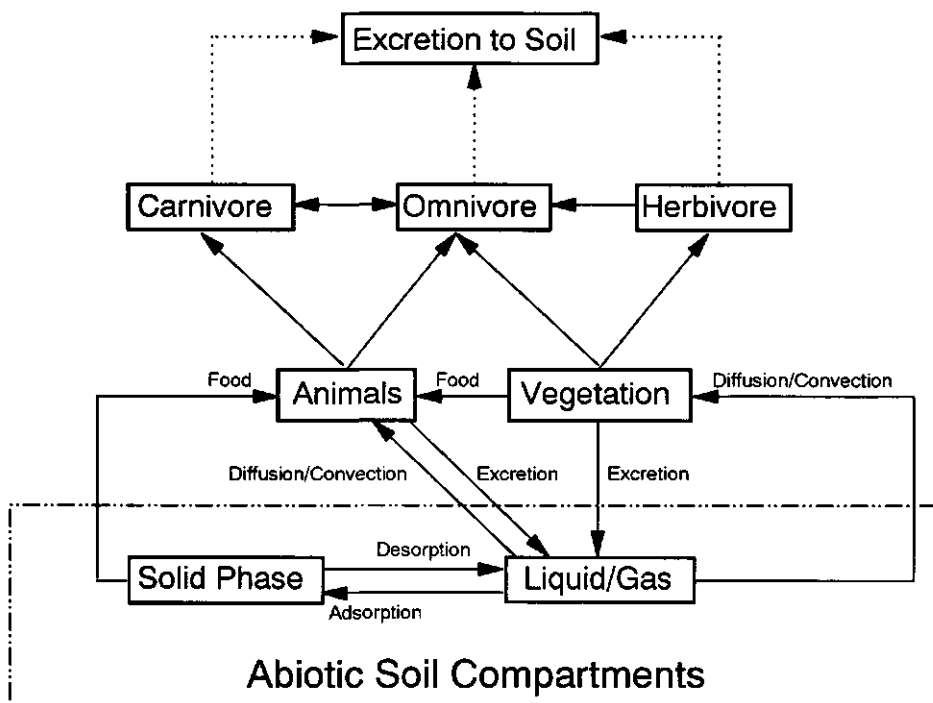

Figure 2.1 *Interactions between soil pollution and terrestrial organisms*

Assimilation and excretion

While an organism is exposed to a contaminated soil the pollutant concentration in the organism, P, changes as a function of time, which can be described by a one compartment toxicokinetic model:

$$\frac{dP(t)}{dt} = \frac{I(t)}{M(t)} - k(t)P(t) \tag{2.4a}$$

where $dP(t)/dt$ is the rate of change of the concentration per weight of the organism [mg day^{-1} kg^{-1}], $I(t)$ is the assimilation rate of the contaminant [mg day^{-1}], $M(t)$ is the weight of the organism [kg] and $k(t)$ is the excretion rate [day^{-1}]. The weight of the organism and the assimilation- and excretion rate vary with time, depending on several environmental parameters (physical, chemical and biological). We will not consider these relationships in detail because they do not affect the conceptual approach that we intend to illustrate. We replace $I(t)/M(t)$ by a constant I [mg kg^{-1} day^{-1}], and we assumed also a constant excretion rate, resulting in:

$$\frac{dP(t)}{dt} = I - kP(t) \tag{2.4b}$$

Both assimilation and absorption of contaminants depend on the available quantity in soil. This quantity depends on total content, soil pH, f_{OC} and K_F (equations (2.1), (2.2), and (2.3)). During digestion part of the ingested contaminants will be assimilated, depending on the assimilation efficiency of the organism. We assume that this efficiency is constant in time (Atkins 1969) resulting in:

$$I = \alpha Q_A \tag{2.5}$$

where α is the assimilation coefficient and Q_A is the biologically available concentration.

Substituting equation (2.5) into equation (2.4b), the analytical solution of equation (2.4b) is:

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$$P(T) = \frac{\alpha}{k} Q_A (1 - e^{-kT}) + P(0)e^{-kT} \tag{2.6}$$

where $P(T)$ is the concentration in a individual that has been exposed to a contamination level Q_A during a time period T, and $P(0)$ is the initial concentration in the organism.

Variability of exposure

Due to soil spatial variability a foraging organism is exposed to the pollution level at a specific location for its limited residence time (Δt) at this location. In our model, we assumed that the organism utilizes its living area in an optimal way, i.e. the averaged residence time at a particular location is uniform for the locations within the boundaries of the living area. It randomly moves through the living area without either avoiding or preferring any part of the living area. Organisms

TABLE 2.1 *Illustration of order of magnitude of the size of the living area for a few terrestrial organisms (from: Fuchs et al. 1985)*

Organisms	size [m²]
Vegetation	1
Earthworm	5-10
Mole	400
Shrew	200-800

have differently sized home-ranges (Table 2.1). The average residence time per square meter (Δt) can be calculated by dividing the total residence time in the living area (T) by the size of the living area (A):

$$\Delta t = \frac{T}{A} \tag{2.7}$$

Hence, as the home-range size increases, exposure time to a particular level of

pollution becomes smaller. We consider that the life expectancy of the organisms is not decreased by the contamination (i.e. there is no mortality risk). The concentration of $P(T)$ of a individual that is living in a specific living area is obtained by iterative computation of the concentration according to:

$$P(t=t+\Delta t)=\frac{\alpha}{k}Q_A(1-e^{-k\Delta t})+P(t)e^{-k\Delta t} \tag{2.8}$$

until t equals T. The estimation of $P(T)$ for a specific living area can be obtained by a Monte Carlo simulation. In our model, the organism randomly moves around in its living area, beginning in the centre and with $P(0)=0$. After calculating $P(T)$ for a great number of organisms for a specific living area the corresponding expectation of P is estimated by

$$\hat{E}\{P\}=P(T)_{avg}=\frac{1}{n_c}\sum_{i=1}^{n_c}P(T)_i \tag{2.9}$$

where $P(T)_i$ is the concentration in a individual as estimated at computation number i, and n_c the number of calculations in each living area. The advantage of the Monte Carlo procedure in comparison with averaging the soil pollution within a living area is the possibility of the assessment of a confidence interval of P. This interval reflects the uncertainty about the accumulated amount of pollutant, which is caused by soil spatial variability. The 95% confidence interval of P is estimated by

$$P(T)_{avg}-t_\alpha\sqrt{var\{P\}} \leq P \leq P(T)_{avg}+t_\alpha\sqrt{var\{P\}} \tag{2.10}$$

where t_α is the *Student's t*.

In the following example, we investigated the effects of soil spatial variability on the exposure of fictitious soil dwelling organisms to a heavy metal soil contamination. In our calculations we assumed that $\alpha=k=0.1$ for various organisms with differently sized home-ranges. We want to emphasize the effect of mobility of organisms on exposure to polluted soil. In practice α and k are not equal; they

differ for each species and are e.g. age dependent. Large α-values may correspond both with large and low values of k. This ratio is also species-dependent. Nevertheless, we assume α=k, without loss of generality for the conceptual approach.

MATERIALS AND METHODS

FOR ILLUSTRATION, we use data of a polluted arable field in the 'Kempen' region in the south of The Netherlands (Boekhold and Van der Zee 1992). Due to atmospheric deposition the soil received large amounts of zinc (Zn) and cadmium (Cd), that were emitted by zinc ore smelters during the past century. The source was diffuse so the initial spatial variability of the contamination is limited. Soil samples were taken at 166 spatially distributed points in approximately 0.5 ha. Total extractable cadmium content of the soil (Cd_T [mg kg^{-1}]) was determined using 0.43 M HNO_3 as an extractant, assuming this quantity reflects the amount of Cd that can potentially desorb (Houba *et al.* 1989). Additionally, soil was extracted with 0.01 M $CaCl_2$ to determine Cd_S. Eriksson (1990) has shown that Cd_S has a better correlation with the Cd-contents of wheat and oats than Cd_T. Hence, it is plausible that Cd_S reflects better the bioavailability of Cd than Cd_T. In our calculations we use Cd_S as the concentration that is available for the organisms. For illustration of the limitations of the use of Cd_T as a soil quality standard, we also present Cd_T as comparison with Cd_S.

Predictions of contents at unsampled locations were made by a geostatistical interpolation-method: Ordinary Block Kriging (Davis 1986). Using GeoEAS (Geostatistical Environmental Assessment Software) we estimated both Cd_S and Cd_T at every square meter of the field by block-kriging (Englund and Sparks 1988). Contour graphs are depicted in Figure 2.2. The highest levels of Cd_T (Figure 2.2A) were in the north of the field. High levels of Cd_S (Figure 2.2B and 2.2C) were found in the south of the field and a few square meters in the north of the field. Cd_S exceeds 0.5 mg kg^{-1} in about 25% of the total area (Figure 2.2B) whereas Cd_S
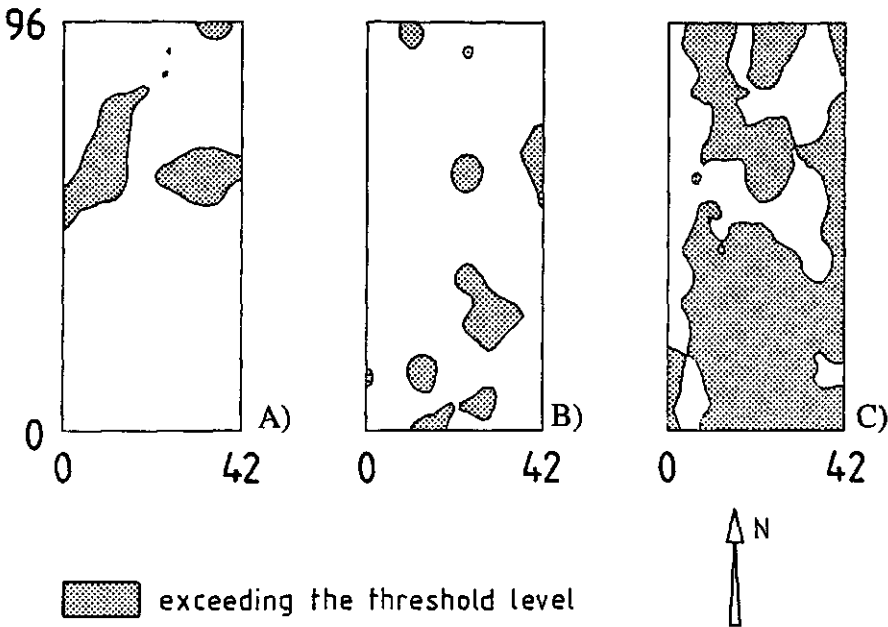
Figure 2.2 *Contour graphs of cadmium pollution in an arable field in the Kempen region: (A) Cd_T exceeds 5.0 mg kg^{-1}. (B) Cd_S exceeds 0.50 mg kg^{-1}. (C) Cd_S exceeds 0.35 mg kg^{-1}.*

exceeds 0.35 mg kg^{-1} in about 60% of the total area (Figure 2.2C). These threshold levels were chosen arbitrarily to show the degree of pollution and its spatial pattern in this field.

Using the geostatistical estimations, we estimated the concentration in an organism that is exposed to Cd_S for its residence time in its living area in this field, using equations (2.8) and (2.9). The residence time in the living areas was arbitrarily set to half a year. The living areas are supposed to be squares. We move this squares over the field surface as a moving window with intervals of 1 m. For each living area we did a Monte Carlo simulation with 250 realisations of $P(T)$. Organisms were randomly moving in the living areas with residence time Δt at every square meter (equation (2.7)). With equations (2.9) and (2.10) we calculated P and its 95% confidence interval (t_α=1.96) in the specific living area.

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RESULTS AND DISCUSSION

THE RESULTS OF the calculations are shown in the Figures 2.3 and 2.4. In Figure 2.3 we show the areas where $\hat{E}\{P\}$ exceeded 0.50 mg Cd kg^{-1}. Organisms with living areas up to 200 m^2 contained more than 0.50 mg Cd kg^{-1} in the south as well as in the north of the field. For animals with larger sized living areas (>200 m^2), the Cd concentration did not exceed 0.50 mg kg^{-1}. Enlarging the home-range size in this arable field lead to a decrease of the area in which $\hat{E}\{P\}$ exceeded 0.50 mg Cd kg^{-1}.

Ê [P] exceeded 0.50 mg/kg
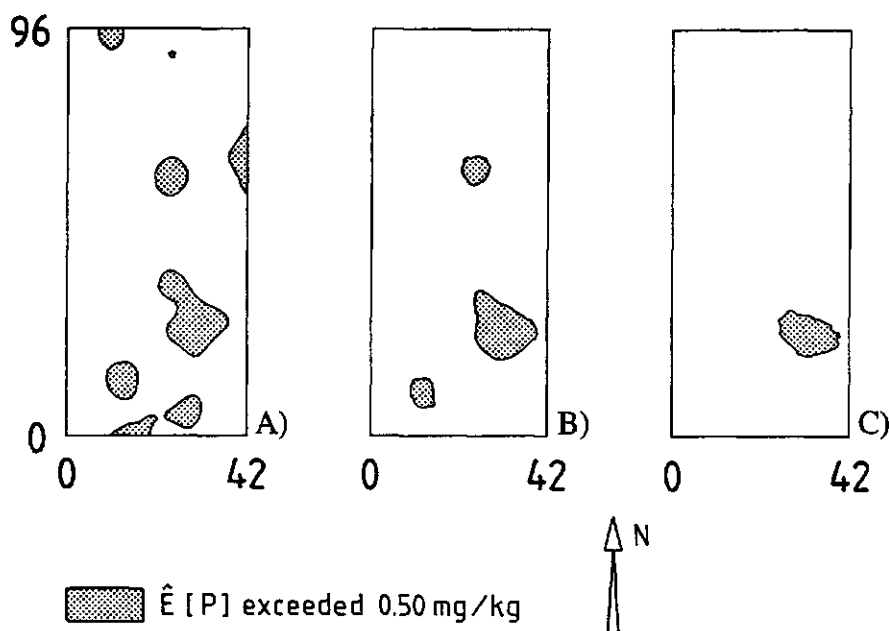
Figure 2.3 *Areas where $\hat{E}\{P\}$ exceeded 0.50 mg kg^{-1}.*
Living areas are: (A) 10 m^2, (B) 100 m^2, and (C) 200 m^2.

In Figure 2.4 we show the areas in which the lower limit or the upper limit of the 95% confidence interval of P exceeded 0.50 mg Cd kg^{-1}. When the home-range size
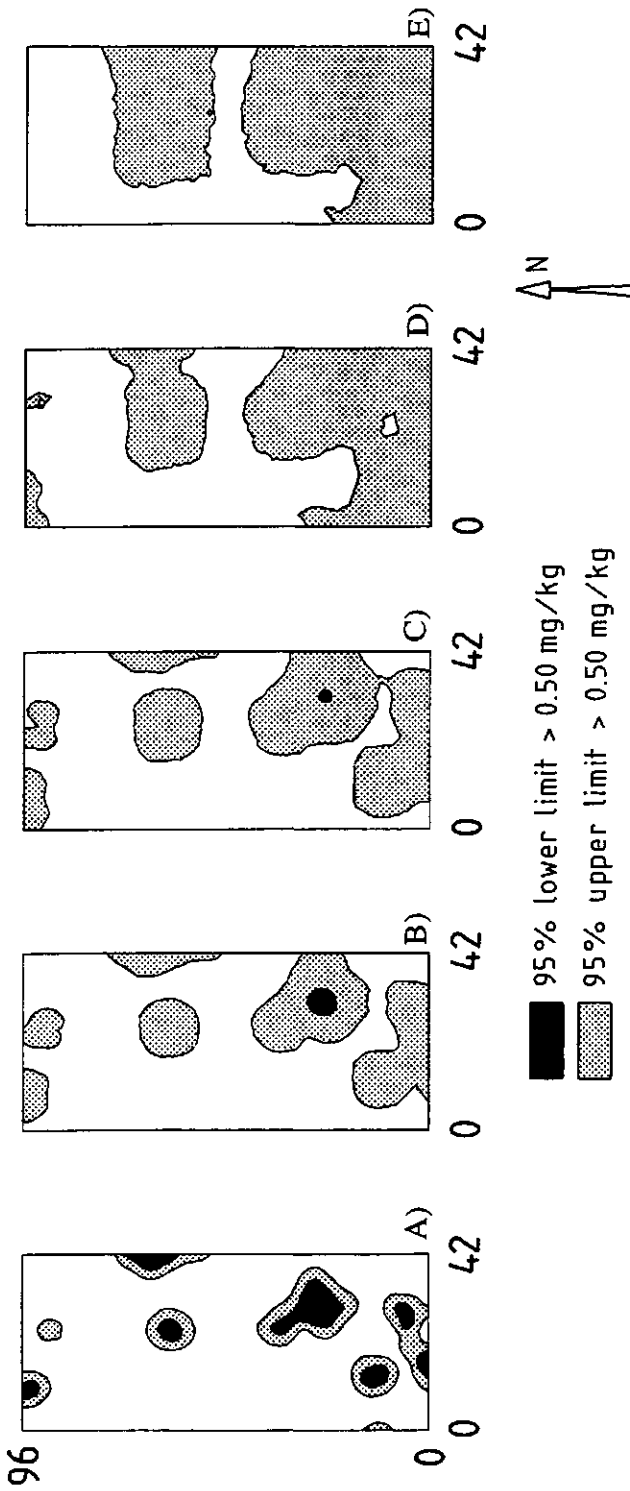
Figure 2.4 *Areas where the lower limit or the upper limit of the 95% confidence interval of P exceeded 0.50 mg Cd kg^{-1}. Living areas are: (A) 10 m^2, (B) 50 m^2, (C) 100 m^2, (D) 200 m^2, and (E) 400 m^2.*

95% lower limit > 0.50 mg/kg

95% upper limit > 0.50 mg/kg

is made larger, we observe different effects on the 95% lower and upper limit boundaries and the mean. Whereas the area where the lower limit is exceeded decreases, the opposite is the case for the area where the upper limit is exceeded. This indicates that the area within the confidence interval increases with increasing home-range size. This means that the uncertainty about P increases with increasing home-range size and this implies that the probability that an individual is exposed to a high level of contamination increases with increasing home-range size.

For organisms with small mobility we observe relatively small patches where the chosen threshold is exceeded (Figures 2.4A and 2.4B). The extent with which the threshold may be exceeded may be larger than for those organisms that have a large mobility. The latter smooth the boundaries but are also vulnerable to small 'hot spots' (compare Figures 2.2B and 2.4E). Hence, one small 'hot spot' may affect the exposure for mobile organisms over a large area.

These results imply that the resolution needed for mapping soil contamination depends on (i) the patchiness of contamination, (ii) the home-range size of the organism of interest, and (iii) the scale at which adverse effects are accepted. Thus, small patches and large home-range size imply a large density of soil samples even when (iii) is large. When, however, (iii) is small, a large sampling density is needed always.

CONCLUSIONS

I N OUR MODEL, we made many assumptions that were necessary to enable calculations. Much of the knowledge, needed to predict effects of polluted soil *in situ* on organisms in the field, is not available yet. Besides dose-effect relationships also soil-physical and soil-chemical processes, spatial variability of bioavailable quantities, assimilation rate, excretion rate, absorption rate, the daily uptake (food or soil ingestion) and exposure time (i.e. mobility of the organisms, avoidance behaviour) have to be taken into account in risk assessment. We

investigated the effect of both soil spatial variability and mobility of organisms on the exposure to soil contaminants of a population of fictitious organisms. The results reveal that the relationship between the soil contamination level and exposure of mobile organisms is complicated considerably by spatial variability. 'Hot spots' appear only to be a large risk-factor for the population of a species with small sized home-ranges, whereas they are a small risk-factor for a population of a species with large sized home-ranges This is due to the relatively low probability of getting exposed while foraging for food for species with large home-range size. Also when the home-range size is large in comparison with the polluted area, there will be always some individuals that will be exposed to the pollutant. Protection of an ecosystem can be realised without protecting 100% of the species in in the ecosystem. Which species are important for the sustainability of the ecosystem? The resolution with which soil contamination is to be mapped should preferably be related with the home-range size of these species. Ecotoxicologically based choices for the minimum patch size that is to be protected are needed in view of soil sampling costs.

Chapter 3

Cu accumulation in *Lumbricus rubellus* under laboratory conditions compared with accumulation under field conditions

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ABSTRACT

We performed experiments to determine Cu accumulation in earthworms under laboratory conditions using soil from a Cu contaminated site, followed by field experiments in this contaminated site. The aim of the laboratory experiments was a) to determine Cu accumulation rate, b) to determine the effect of soil Cu content on the steady state concentration, and c) to evaluate the effect of soil moisture on accumulation. The field experiments were performed to evaluate the use of accumulation data obtained from laboratory experiments for prediction of accumulation under field conditions. In the laboratory experiment, earthworms (*Lumbricus rubellus*) were introduced into four homogeneously mixed Cu contaminated soils and a reference soil. The total extractable Cu content in the soil (Cu_T) varied from 10 to 130 mg kg^{-1}; soil pH varied from 4.0 to 5.0; soil moisture content was set to approximately 25%, 35%, and 45% of the dry weight for each treatment. The tissue Cu concentration (Cu_w) was determined by sampling earthworms after 1, 7, 14, 28 and 56 days. In the field experiment, 500 earthworms were introduced at four differently Cu contaminated locations at a contaminated arable field. After 14, 28, and 70 days, earthworms were sampled to determine Cu_w. In both experiments, soil Cu contents significantly affected Cu_w. Soil moisture only significantly affected Cu accumulation for the wettest soil. Under laboratory conditions, a steady state did not seem to be achieved after 56 days; the Cu accumulation can be described by the toxicokinetic one-compartment model. The field experiment was considerably affected by variation in soil temperature resulting in significant fluctuations in tissue Cu concentrations. The tissue Cu accumulation was significantly correlated

to the Cu_T, which is in agreement with the results from the laboratory experiments. Variance of Cu_W at day 14 in earthworms from the field experiments were significantly larger than in the worms from the laboratory experiment. At day 28, the differences were not significant.

INTRODUCTION

STRAALEN AND DENNEMAN (1989) developed a model that claims to estimate ecotoxicologically based threshold level of contaminants in soil. The model uses NOEC's (No Observed Effect Concentrations) of at least five soil dwelling species. The NOEC's, however, are determined in laboratory experiments, hence they are applicable to only a limited set of conditions. Complications regarding extrapolation from laboratory to field scale result in large uncertainties in ecotoxicology (Straalen *et al.* 1991). Both soil heterogeneity and migration of soil dwelling species result in a time dependent exposure of the organism to contaminants. Marinussen and Van der Zee (1994) showed that the effect of soil heterogeneity on exposure to soil contaminants depends upon both the home-range of organisms and the pattern of soil contamination. In the studies of the present paper, Cu accumulation in *Lumbricus rubellus* under laboratory conditions was compared with Cu accumulation in these worms under field conditions. The aim was to evaluate the use of data obtained from laboratory experiments for prediction of accumulation under field conditions, and explain the differences, if observed.

The accumulation of heavy metals by earthworms is governed by the heavy metal content of soil, soil pH, organic matter content (*OM*) and Cation Exchange Capacity (*CEC*) (Ma 1982, Ma *et al.* 1983, Beyer *et al.* 1987, Morgan and Morgan 1988). Generally, sewage sludge is added to contaminate the soil (Hartenstein *et al* 1980, Ma 1988) or soil is artificially contaminated shortly before earthworms are introduced (Ma 1984, Streit 1984, Spurgeon *et al.* 1994). In the latter case, sorption kinetics may not be at equilibrium at the time of the experiments (Spurgeon *et al.* 1994). Our experiments, however, were performed using soils that were artificially

contaminated more than a decade ago, so that the sorption kinetics were at equilibrium at the time of the experiments.

The prediction of Cu accumulation by earthworms in contaminated sites has been shown to be difficult, several authors having found different relationships between total Cu concentration in soil samples and Cu accumulation in earthworms (Ireland 1979, Ash and Lee 1980, Ma _et al._ 1983, Beyer and Cromartie 1987, Beyer _et al._ 1987, Morgan and Morgan 1988, Morgan and Morgan 1991). Eijsackers (1987) showed avoidance of Cu contaminated soil by earthworms, which illustrates the uncertainty of exposure of earthworms in a contaminated site. As far as we know, in all surveys, soil spatial variability has been neglected by mixing the (randomly) taken soil samples. This may be one of the reasons that only part of the variation in the tissue Cu concentrations could be explained by total Cu in soil (and soil pH, OM, CEC). To evaluate soil spatial variability, the contaminated site of our field experiment has been sampled very intensively (sampling density: 17,500 samples ha^{-1}), and each soil sample chemically analysed.

To investigate whether temporal variation of soil moisture should be taken into account, we also examined the effect of soil moisture on the accumulation of Cu by _L.rubellus_ under laboratory conditions.

MATERIALS AND METHODS

Sampling schemes

THE EXPERIMENTS WERE performed using soils from a artificially contaminated agricultural field near Wageningen, The Netherlands. The soil is a slightly loamy fine sand and is low in organic matter content (loss-on-ignition: 3.5%). The soil was classified as a _Fimic A Horizon_ (FAO-UNESCO 1988) or a _Plaggen Epipedon_ according to the USDA Soil Taxonomy (U.S. Soil Conservation Service 1975). The agricultural field consists of 128 plots (each 6mx11m, Fig. 3.1). Four Cu levels were introduced in the autumn of 1982 by applying $CuSO_4$ at quantities of 0, 250, 500 and 750 kg Cu ha^{-1}. Four pH levels
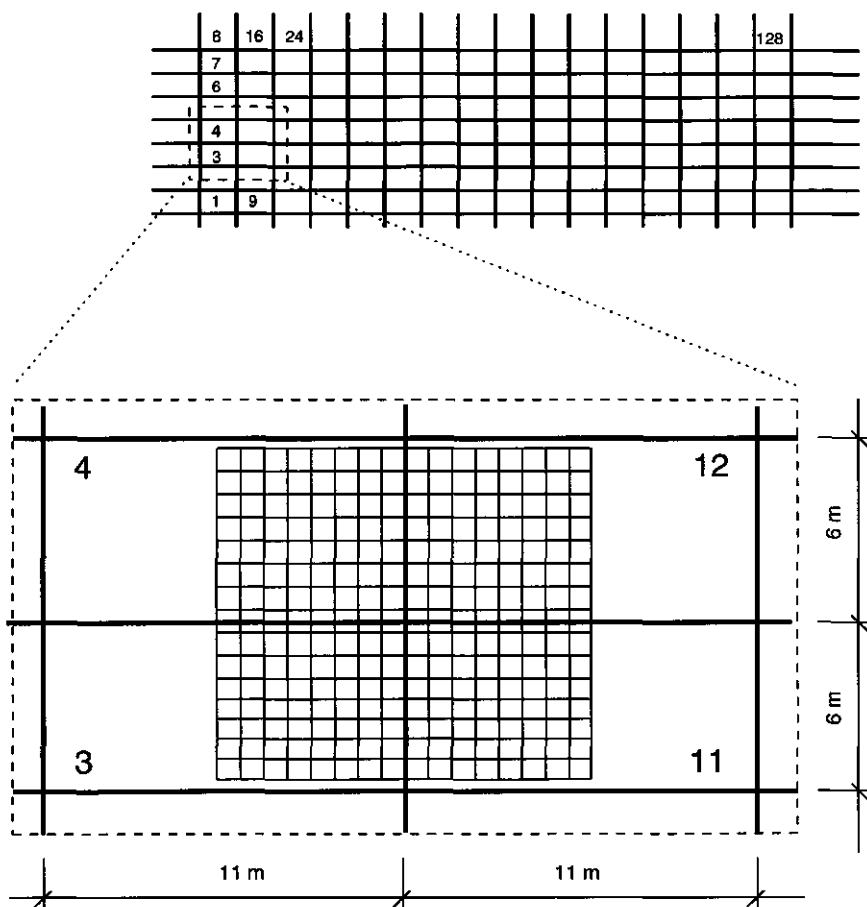
Figure 3.1 *Sampling scheme of the plots*

were established by either adding sulphur powder to lower the pH_{KCl} from 4.4 to 4.0, or by adding certain amounts of pulverized $CaCO_3$ to raise the pH_{KCl} from 4.4 to 4.7, 5.4, or 6.1. The methods of application have been described by Lexmond (1980). The pH levels are readjusted every 6 years.

The laboratory experiment was performed using soils that were collected from four adjacent plots (3, 4, 11, and 12, Fig. 3.1). Moreover, untreated reference soil

was collected from the agricultural field. About 120 kg soil from each plot was homogenized and split up into six 20 kg portions and put into plastic containers (24 L). Three water contents were established in duplicate by adding distilled water up to a water content of approximately 25%, 35%, and 45% of the dry weight. To get a sorption equilibrium, the containers were kept in a cold storage room for two weeks. Subsequently they were put into a phytotron, adjusted to 12 hrs daylight per day, room temperature at 15°C, and relative air humidity of 85%. Earthworms were kept in the untreated soil for one week before introducing 40 earthworms into each container. Dried alder leaves were added as a food source, and the containers were closed by paraffine film to prevent evaporation of water. Earthworms were sampled after five different exposure times (1, 7, 14, 28, or 56 days) to determine Cu accumulation; 25 earthworms were analysed to determine the initial tissue Cu concentration. The worms were rinsed with distilled water and kept in petri dishes on moist paper towels. After three days, the earthworms were killed by immersion in liquid nitrogen and individually put in 50 ml volumetric flasks for chemical analysis. The dry weight of each worm was determined after drying for one night in an oven at 105°C.

The field experiment was performed in the same plots (3, 4, 11, and 12). Soil samples were taken in the late summer of 1992. The schemes were based on a 7 by 7 grid in each plot with 0.75 m node distance (Fig. 3.1). The soil samples were air dried and sieved to remove aggregates larger than 2 mm before chemical analysis. In the middle of the sampled area of each plot, about 500 earthworms (*L. rubellus*) that were obtained from a local vermiculturist were introduced in the autumn of 1993 (there were no native earthworms). In each plot, approximately 30 earthworms were sampled three times: after 2, 4 and 10 weeks. Worms were moved to the soil surface by vibrating the soil at several locations using a dungfork. The advantage of this method is that the whole area can be sampled without adding toxic chemicals like formalin (Raw 1959, Ash and Lee 1980) that would drive away all the earthworms from the experimental site. The sampled worms were treated as described above.

Soil chemical analysis

Total extractable Cu contents of the soil (Cu_T [mg kg^{-1}]) were determined by using HNO_3 as the extractant assuming that this quantity reflects the amount of Cu that can potentially desorb (Houba *et al.* 1989). Three grams of air dried soil were equilibrated with 30 ml 0.43 M HNO_3 solution for 20 h in an end-over-end shaker. After filtering, the liquid phase was analysed for Cu concentrations on a flame atomic absorption spectrophotometer (Instrumental Laboratory AA/AE spectrophotometer S11). Soil pH was determined in a suspension with 0.01 M $CaCl_2$ as background solution. Three grams of air dried soil were equilibrated with 30 ml 0.01 M $CaCl_2$ solution for 20 h in an end-over-end shaker. The pH of the suspension was measured with a glass-calomel electrode. In soil fertility and contamination studies involving plant uptake it has been shown that the $CaCl_2$ extractable contents in soil are a better indicator of the bio-available fraction than the HNO_3 extractable fraction (Eriksson 1990, Novozamsky *et al.* 1993). Therefore, we also determined the amount of Cu in the 0.01 M $CaCl_2$ solution (Cu_S [mg L^{-1}]). The organic matter content of the soil (*OM*) was determined by loss-on-ignition.

Earthworm chemical analysis

The earthworms were individually put in 50 ml volumetric flasks and dried in an oven at 105°C. The earthworms were digested in 5 ml 65% HNO_3 heated to 130°C for one hour. Four ml of 20% H_2O_2 was added in aliquots of 0.5 ml. After cooling, the flasks were filled with distilled water up to the mark. The clear, colourless solutions were filtered through an ash-free filtering paper into plastic test-tubes. The filtrates were analysed for Cu concentrations on a furnace atomic absorption spectrophotometer (Varian SpectrAA 300/400 with Zeeman background correction). The Cu in the solutions is partly extracted from the residual soil in the earthworms. For this reason, we measured the amount of soil in each earthworm. The flasks were rinsed and soil particles were collected in the filter paper. The papers were folded and put in crucibles which were heated gradually in a furnace up to 550°C. The amount of residual soil in each worm was determined by weighing the ignition residue. The amount of Cu in this soil was estimated by

analyzing the Cu concentrations in the excrements of the worms in the petri dishes. The air-dry excrements were weighed and equilibrated with 5 ml HNO_3 in a head-over-end shaker. After filtering, the liquid phase was analysed for Cu concentrations on the flame atomic absorption spectrophotometer.

Statistics
The effect of soil moisture on Cu accumulation under laboratory conditions was tested by Analysis Of Variance (ANOVA) with soil moisture and time as factors. Pearson correlation coefficients for tissue Cu concentrations (Cu_W) and Cu in soil or soil pH were determined. The significance of differences in the mean tissue Cu concentrations was tested by the Student's t-test. Differences in variances in Cu accumulation in earthworms from the laboratory experiment (s^2_{LAB}) and earthworms from the field experiment (s^2_{FIELD}) were tested by the F-test. The 5% level of significance was used in all tests.

RESULTS

Soil analyses
MEAN VALUES OF Cu_T, Cu_S, and pH for the soils from both the laboratory experiment and the field experiment are presented in Table 3.1. In the field (Fig. 3.2A), Cu_T varied from 65 mg kg^{-1} (in plot 4) up to 204 mg kg^{-1} (in plot 3). The contourgraphs show that there is profound spatial variability of Cu_T. The laboratory soils 4 and 11 do not differ very much in Cu_T (67.7 mg kg^{-1} (soil 4) vs 79.9 mg kg^{-1} (soil 11)), they only differ in soil pH (4.0 (soil 4) vs 5.0 (soil 11)) and Cu_S. The same holds for the laboratory soils 3 and 12 where Cu_T was 132 mg kg^{-1} (soil 3) vs 112 mg kg^{-1} (soil 12) and pH was 4.7 (soil 3) vs 4.1 (soil 12). Cu_S in the soils from the laboratory varied from 0.005 mg L^{-1} up to 0.770 mg L^{-1} (Table 3.1). For the field samples, the maximum value measured for Cu_S was 1.20 mg L^{-1} (plot 12). In the field, pH is in the range of 3.9 (plot 12) to 5.7 (plot 11). The contourgraphs from Cu_S and pH (Figs 3.2B and 3.2C) show that
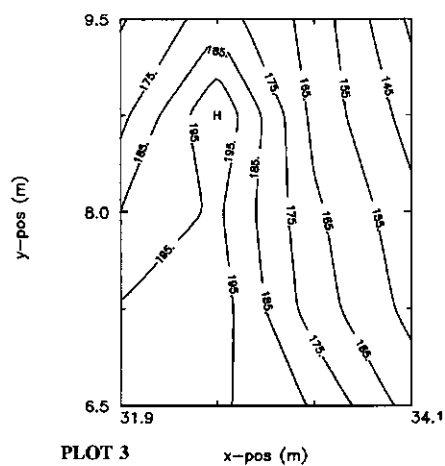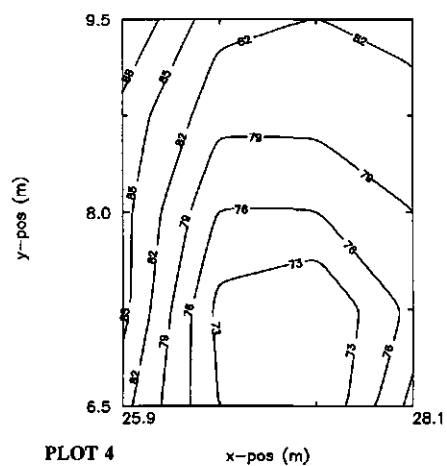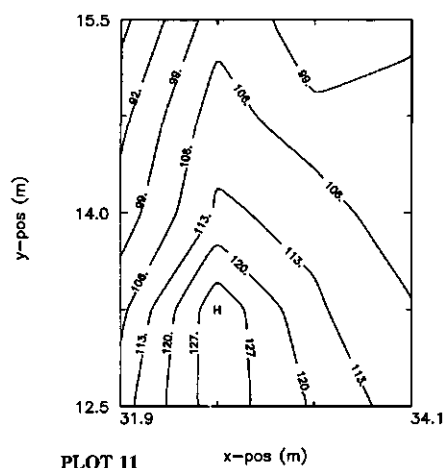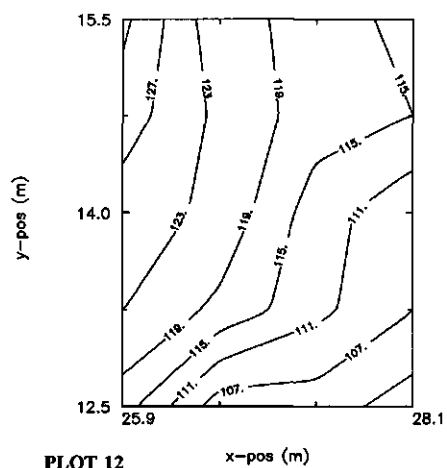
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Figure 3.2A *Contourgraphs of the experimental field as far as used in the experiment: Cu [mg kg⁻¹], extracted in 0.43 M HNO₃*

TABLE 3.1 *soil chemical analyses*

SOIL	Cu_T±s.d. [mg kg^{-1}]		Cu_S±s.d. [mg L^{-1}]		pH-CaCl$_2$	
	FIELD [A]	LAB [B]	FIELD [A]	LAB [B]	FIELD [A]	LAB [B]
reference [C]	14.4±14.0	10.1±0.68	0.014±0.007	0.005±0.003	4.6±0.34	4.5±0.14
3	154±25.8	132±1.68	0.27±0.14	0.27±0.007	4.7±0.25	4.7±0.01
4	87.5±12.6	67.7±1.56	0.47±0.12	0.43±0.014	4.1±0.14	4.0±0.02
11	112±20.3	79.9±1.05	0.088±0.04	0.076±0.003	5.2±0.23	5.0±0.03
12	113±12.1	112±1.82	0.73±0.25	0.77±0.025	4.1±0.16	4.1±0.01

[A] mean values for 64 samples in each plot, except in the reference soil

[B] mean values for 6 replicates

[C] field data of the reference soil are averages for 10 at random samples

there is also spatial variability in Cu_S and in pH. Comparison of the standard deviations show that the soil in the field experiment was considerably more heterogeneous than the soil in the laboratory experiment (Table 3.1).

Earthworms from the laboratory experiment

Only in soil 11 and in the reference soil, earthworms survived the experiment (56 days) for all treatments. In soils 3, 4, and 12, however, there were problems to find living earthworms on day 28 and afterwards. At day 28, we did not find earthworms in either the driest replicates for soil 3, or the wettest replicates for soil 4 and found earthworms in only the wettest replicates for soil 12. At day 56, we did not find any earthworm in both soils 4 and 12, whereas we found earthworms in only the wettest replicates for soil 3.

The mean initial tissue Cu concentration (n=25) was 15.41±2.50 mg kg^{-1}. During the experiment, tissue Cu concentration (Cu_W^L) in soils 3, 4, 11, and 12 increased significantly, whereas there was no significant accumulation in worms in the reference soil (Table 3.2). Soil moisture significantly affected Cu accumulation in soils 4 and 12, whereas in soil 3, 11 and in the reference soil the effect of soil
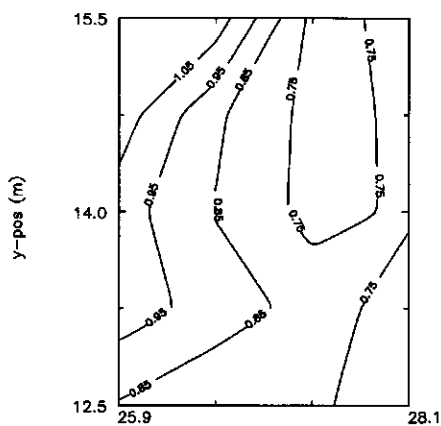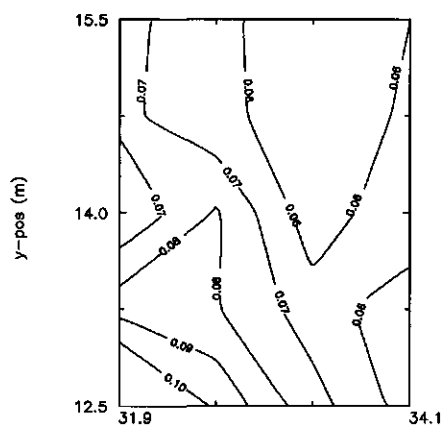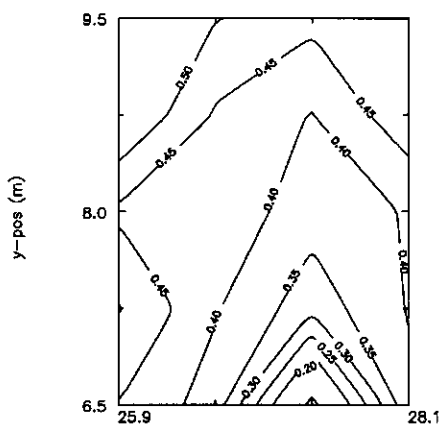
Figure 3.2B *Contourgraphs of the experimental field as far as used in the experiment: Cu [mg L⁻¹], in a 0.01 M CaCl₂ solution.*

Figure 3.2C: *Contourgraphs of the experimental field as far as used in the experiment: pH, measured in a 0.01 M CaCl₂ suspension.*

TABLE 3.2 *Earthworm analyses laboratory experiment*

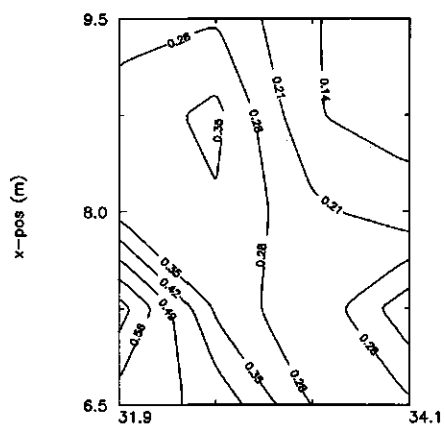| TIME [A] [days]	REF. SOIL $Cu_w \pm s.d.$ [mg/kg d.m.]	SOIL 3 $Cu_w \pm s.d.$ [mg/kg d.m.]	SOIL 4 $Cu_w \pm s.d.$ [mg/kg d.m.]	SOIL 11 $Cu_w \pm s.d.$ [mg/kg d.m.]	SOIL 12 $Cu_w \pm s.d.$ [mg/kg d.m.]
0	15.4±2.50	15.4±2.50	15.4±2.50	15.4±2.50	15.4±2.50
1(25)	11.9±0.74	28.6±5.36	20.7±2.52	20.0±0.53	29.2±5.36
1(35)	12.3±0.49	41.5±6.71	22.7±3.57	25.9±2.52	33.5±3.16
1(45)	11.6±0.02	30.1±17.8	13.6±1.26	27.8±0.98	15.6±2.06
7(25)	13.2±0.41	39.3±4.96	24.8±3.31	21.5±3.44	37.5±6.83
7(35)	14.1±2.35	43.0±3.40	25.3±2.19	28.8±4.25	44.4±11.1
7(45)	14.3±2.70	30.1±12.9	21.4±0.05	24.7±3.20	29.3±6.11
14(25)	12.8±0.85	44.1±9.16	31.8±6.45	25.7±3.95	44.1±4.09
14(35)	13.6±2.23	62.1±0.96	30.1±0.80	27.9±1.90	45.4±4.56
14(45)	12.8±1.49	52.0±13.5	22.2±3.99	29.8±3.08	40.2±9.85
28(25)	15.9±3.59	- [B]	54.4 [C]	37.4±5.11	- [B]
28(35)	15.6±4.38	108 ±8.77	53.2±7.49	38.9±3.92	- [B]
28(45)	15.7±2.09	83.3 [C]	- [B]	42.4±9.36	57.6 [C]
56(25)	18.3±9.77	- [B]	- [B]	44.0±1.92	- [B]
56(35)	20.1±0.08	- [B]	- [B]	45.8±9.30	- [B]
56(45)	14.3±2.76	144 [C]	- [B]	47.3±7.42	- [B]

[A] soil moisture between brackets (% dry weight)

[B] no data available, because no earthworms were found in both replicates

[C] no standard deviation, because earthworms were found in only one replicate

moisture on Cu accumulation was not significant. The moisture effect is particularly due to the relatively low Cu accumulation in the treatments with the largest soil moisture. Since earthworms avoid locations with larger soil moisture (Edwards and Lofty 1977), we repeated the statistical test without the data from
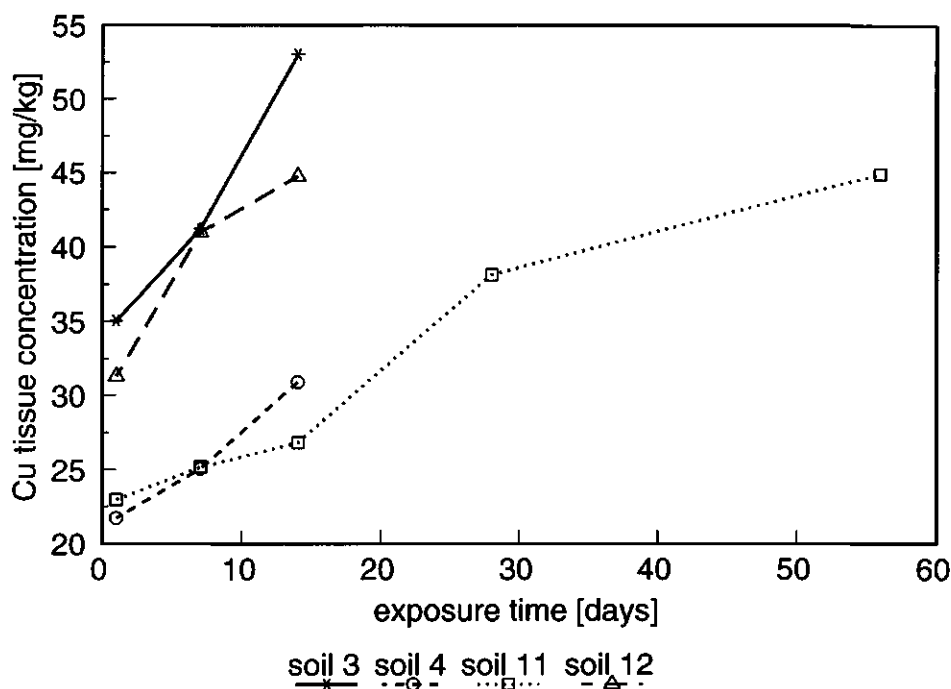
Figure 3.3 *Accumulation of Cu by L.rubellus under laboratory conditions*

the wettest soil. For that case the effect of soil moisture on Cu accumulation appeared to be not significant. Tissue Cu accumulation was significantly correlated with Cu_T (correlation coefficient $\rho=0.9$), whereas neither the correlation between accumulation and soil pH, nor between accumulation and Cu_S was significant. A steady state has not been achieved yet (Fig. 3.3). A fast accumulation at the beginning of the experiment seems to be followed by a slow accumulation. This is in agreement with Streit (1984) who concluded that at the beginning of the exposure the accumulation mainly takes place by absorption or diffusion through the skin and later on accumulation probably also takes place by uptake through the gut-wall. The accumulation could be described well by a one-compartment model (Atkins 1969):

$$Cu_w^L = C_0 + \alpha Cu_T(1 - \exp(-kt)) \tag{3.1}$$

with C_0= initial tissue Cu concentration [mg kg^{-1}] (15.41 mg kg^{-1}), α=coefficient, and k=excretion rate [day^{-1}]. Regression of the pooled data resulted in model parameters that were not significant. Therefore, we estimated the model parameters for every single soil. The estimated α-values varied between 0.2 (soils 3, 4 and 12) and 0.4 (soil 11). The estimated excretion rates differed considerably being 0.04 day^{-1} in soil 11, 0.59 day^{-1} in soil 4, 0.86 day^{-1} in soil 12 and 0.95 day^{-1} in soil 3. As shown in Figure 3.4, the model describes the data rather well.
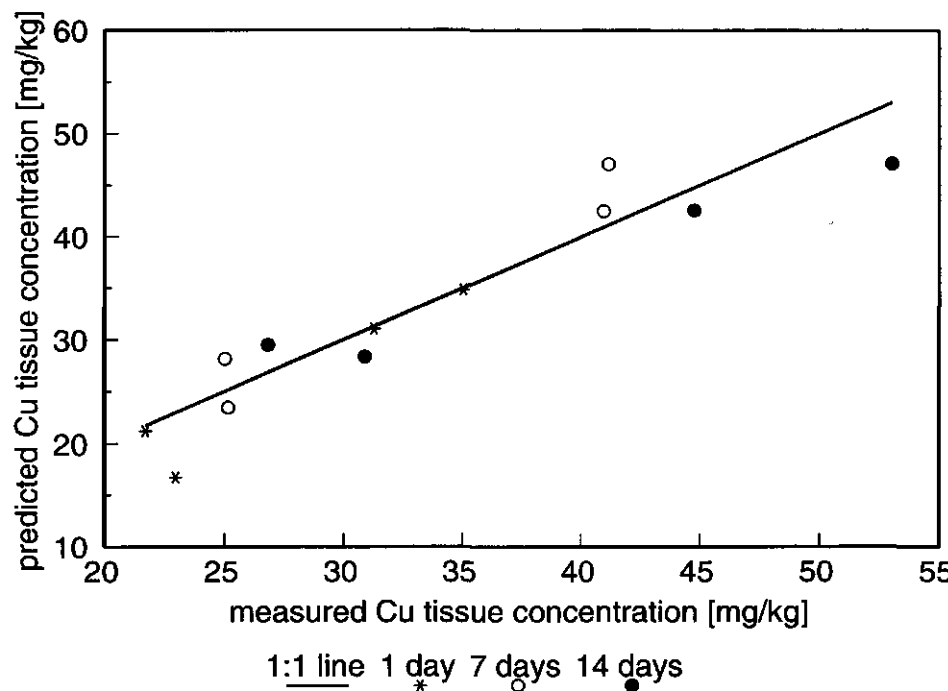
Figure 3.4 *One-compartment model of tissue Cu accumulation under laboratory conditions*

Earthworms from the field experiment

After 10 weeks, only a few living earthworms were found in plots 4 ($n=7$) and 12 ($n=2$). Upon excavation of the introduction points, only dead worms were found in these plots. In plots 3 and 11, many earthworms survived the experiment, which enabled us to sample about 30 earthworms at every sampling time. The observed mortality in plots 4 and 12 is in agreement with the observations in soils 4 and 12 for the laboratory experiment. Hence, it seems that the toxicity of Cu contaminated soil under field conditions is comparable to toxicity in laboratory experiments.

Fourteen days after the introduction, the tissue Cu concentration of the field worms (Cu_W^F) had increased significantly compared to the initial tissue Cu

TABLE 3.3 *Earthworm analyses field experiment*

TIME [days]	FIELD 3 $Cu_W \pm$ s.d. [mg/kg d.m.]	FIELD 4 $Cu_W \pm$ s.d. [mg/kg d.m.]	FIELD 11 $Cu_W \pm$ s.d. [mg/kg d.m.]	FIELD 12 $Cu_W \pm$ s.d. [mg/kg d.m.]
0	15.4±2.50 $n=25$	15.4±2.50 $n=25$	15.4±2.50 $n=25$	15.4±2.50 $n=25$
14	84.1±55.0 $n=29$	46.6±23.8 $n=24$	47.5±22.9 $n=27$	51.3±24.5 $n=21$
28	67.7±20.4 $n=28$	31.7±9.4 $n=24$	34.4±11.3 $n=32$	29.2±7.1 $n=22$
70	62.0±9.1 $n=25$	41.5±9.2 $n=7$	45.3±17.2 $n=27$	57.7±8.5 $n=2$

concentration (Table 3.3). At day 28, however, Cu_W^F had decreased significantly compared to Cu_W^F at day 14 in plots 4, 11 and 12 (Fig. 3.5). In both plot 4 and plot 11, Cu_W^F at day 70 was significantly larger than Cu_W^F at day 28. In plot 3, Cu_W^F at
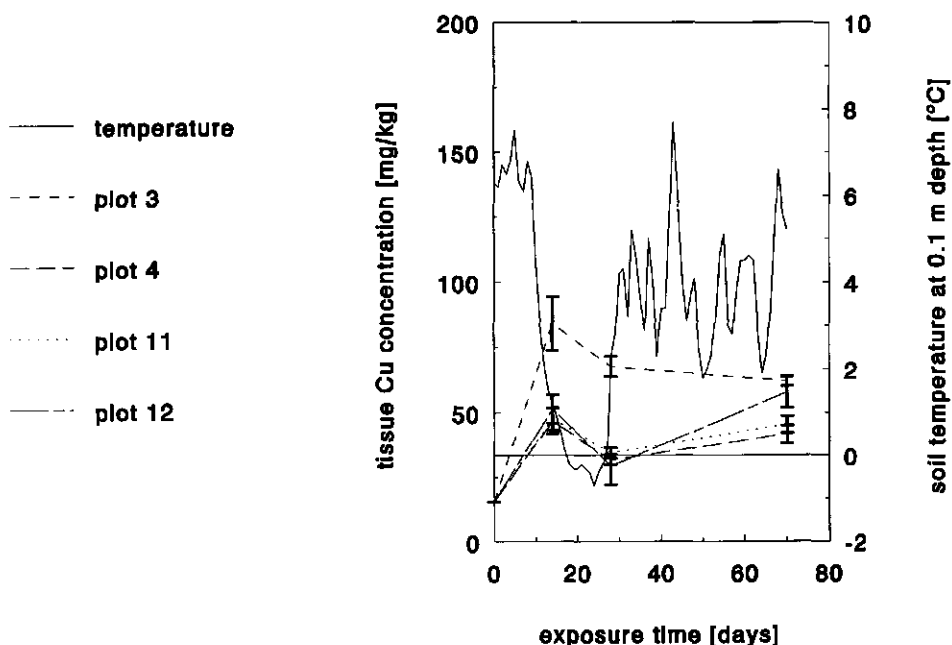
Figure 3.5 *Accumulation of Cu by L.rubellus under field conditions*

day 28 and Cu_W^F at day 70 did not differ significantly from Cu_W^F at day 14. At every sample time, Cu_W^F in plot 3 was significantly larger than Cu_W^F in the other plots. Cu_W^F in plots 4, 11 and 12 did not differ significantly from each other. Here, it should be noted that all worms were found within 1 m from the introduction point. Hence, the small differences in Cu_W^F in worms in plots 4, 11 and 12 can be explained by the relatively small differences in Cu_T at the introduction points in these plots (80, 110 and 120 mg kg^{-1} respectively), whereas Cu_T in plot 3 was much larger: 180 mg kg^{-1} (Fig. 3.2A). Cu_W^F was significantly correlated with these values of Cu_T ($\rho=0.791$), whereas it was neither correlated with soil pH nor correlated with Cu_S.

The significant decline in Cu_W^F during the period of two weeks after the first sampling coincided with relatively low soil temperatures (Fig. 3.5). The significant

increase in Cu_W^F in plots 4 and 11 during the period of five weeks after the second sampling coincided with an increase in soil temperature. The time dependent concentration factor (CF_t=the ratio of Cu_W^F at a sampling time t to Cu_T in the specific plot) was significantly correlated with soil temperature (ρ=0.643), hence tissue Cu accumulation in the field experiment could not be described by Eq.(3.1). At day 14, the variation in Cu_W^F (s^2_{FIELD}) was significantly larger than s^2_{LAB} for the corresponding soils (Tables 3.3 and 3.2 respectively). At day 28, the differences were not significant. At day 56, s^2_{LAB} in soil 11 did not differ significantly from s^2_{FIELD} in plot 11 at day 70. For the other soils, there were too few data on long-term accumulation to perform the F-test.

DISCUSSION

I N OUR STUDY, mortality in the field experiment was in agreement with the observations in the laboratory experiment. Spurgeon *et al.* (1994), however, found discrepancies in toxicity between laboratory and field conditions. This may be caused by their use of an imitation soil, which was very different from the soil from the polluted area they investigated. Our data indicate that soil pH considerably affects the toxicity of Cu, which is in agreement with Ma (1984, 1988). The low pH itself was not responsible for the observed mortality in plots 4 and 12, because *L. rubellus* is a ubiquitous species with regard to soil pH in the range from 3.8 to 7.0 (Satchell 1955). Mortality was observed in soils that were high in Cu_S. The relationship between Cu_T, soil pH and Cu_S can be described by a two species Freundlich-isotherm (Temminghoff *et al.* 1994)

$$q = K(H^+)^a Cu_S^b \tag{3.2}$$

where q is the absorbed amount [mg kg-1], approximately equal to Cu_T, K is a coefficient, (H^+) is the proton activity in soil solution [mol L^{-1}], and Cu_S is the Cu concentration in a 0.01 M $CaCl_2$ suspension [mg L^{-1}]. According to Eq.(3.2), Cu_S is affected by soil pH and this explains the effect of soil pH on earthworm toxicity.

It is surprising that an increase in Cu_S did not result in an increase in tissue Cu accumulation. However, it is possible that large tissue Cu concentrations could not be observed, because only surviving earthworms were analysed for Cu. Possibly, the high exposure level to dissolved Cu resulted in inactivity of earthworms which caused mortality.

The experiments under laboratory conditions showed that soil moisture significantly affects Cu accumulation only if the soil moisture content is 45% of the dry weight. Generally, earthworms will avoid areas with such high moisture contents (Edwards and Lofty 1977). Soil pH did not affect Cu accumulation significantly.

When levels dissolved Cu are sublethal, Cu accumulation in *L. rubellus* appears to be related with total extractable soil Cu content under both laboratory and field conditions. Under laboratory conditions the Cu accumulation can be described by the one-compartment model (Eq.(3.1)). The accumulation rate varied between soils, because the excretion rates varied considerably. The steady state tissue Cu concentration, however, can be estimated with one equation:

$$Cu_w = 15.41 + 0.25Cu_T \tag{3.3}$$

Comparison of Eq.(3.3) with field data from literature is hampered for the reason of soil spatial variability. Since earthworms avoid exposure to Cu contamination (Streit 1984, Eijsackers 1987), the exposure level to Cu contamination in field situations is uncertain because of soil spatial variability and the horizontal and vertical migration of earthworms. Generally, in literature review, soil spatial variability and the avoidance behaviour cannot be taken into account because the spatially distributed soil samples are mixed before chemical analyses (Ireland 1979, Corp and Morgan 1991, Morgan and Morgan 1991). Large soil Cu concentrations, that probably are avoided by earthworms, will result in the Cu content in the mixed sample being greater than that experienced in the field and result in an underestimation of the concentration factor. Ma *et al.* (1983) sampled earthworms near a zinc-smelting complex and determined a relationship between Cu_w and Cu soil content, taking the decreasing soil Cu content with increasing

distance to the complex into account. They found an equation which is quite similar to Eq.(3.3):

$$Cu_w = 14.88 + 0.344 Cu_{soil} \tag{3.4}$$

where Cu_{soil} is the real total Cu content [mg kg^{-1}]. In our experiments, Cu_w^F was correlated with Cu_T at the locations where the earthworms were sampled. Due to the small horizontal migration of earthworms, the exposure levels could be estimated accurately. The mean Cu_w^F-value in each plot, however, fluctuated considerably during the experiment. According to the one-compartment model (Eq.(3.1)) the tissue Cu concentration changes rapidly when the exposure level changes, hence the decrease in Cu_w^F may be an indication of a decreased exposure. Due to the low soil temperatures, the earthworms may have migrated downwards into a layer that is less contaminated than the plough layer. This is in agreement with Morgan and Morgan (1993) who explained significant seasonal changes in metal concentrations in *L. rubellus* by the downward migration of the worms in unfavourable conditions.

The three main conclusions of this paper are: first, laboratory experiments can be in good agreement with mortality of earthworms at contaminated sites if the experiments are performed in representative soil samples from the site. Toxicity may be related with dissolved Cu.

Second, the accumulation of Cu in *L.rubellus* at contaminated sites may differ from the accumulation under laboratory conditions, because soil temperature in the field affects the exposure to Cu also. This may be attributed to the influence of soil temperature on the depth at which the earthworms are living. At greater depth, soil might be less contaminated compared to the upper layer where epigeic earthworms like *L. rubellus* live normally. The laboratory experiments showed that tissue concentrations will change rapidly when the exposure level has changed.

Third, the spatial variability of Cu_T around the introduction points, in combination with the small horizontal migration activity of earthworms over the duration of the field experiment, has been too small to cause a considerable increase in variance of Cu_w^F. Moreover, s^2_{LAB} was large, although the earthworms were

exposed to a homogeneously contaminated soil. To show effects of spatial variability by comparing the variances of Cu_w needs a large soil spatial variability. Earthworms need much time to sample a relevant part of the contaminated site, whereas our field experiment took only 10 weeks which might have been too short.

ACKNOWLEDGEMENTS

Chapter 4

Heavy metal (Cu, Pb, Zn) accumulation and excretion by the earthworm *Dendrobaena veneta*

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ABSTRACT

To obtain knowledge about heavy metal kinetics in earthworms, we performed accumulation and excretion experiments under laboratory conditions using soils from a heavy metal (Cu, Pb, Zn) contaminated site. To determine heavy metal accumulation rate, earthworms (*Dendrobaena veneta*) were exposed for 1, 2, 3, 7, 14, 28, 56, or 112 days to soil MH (Cu: 815 mg kg^{-1}, Pb: 340 mg kg^{-1}, Zn 225 mg kg^{-1}). After exposure for 28 days to soil LB (Cu 242 mg kg^{-1}, Pb: 109 mg kg^{-1}, Zn 72 mg kg^{-1}) or for 112 days to soil MH, *D. veneta* were transferred to uncontaminated soil and sampled after 1, 2, 3, 7, 14, 28, or 56 days to determine heavy metal excretion rates. Fourteen days after the transfer to the uncontaminated soil, some earthworms were transferred back to soil LB to determine the accumulation after a short recovery period. The Cu accumulation until day 56 could be described by a one-compartment model. At day 112, however, we observed an unexpected further increase in tissue Cu concentration (C_{Cu}). We observed a significant increase in C_{Zn} only at day 112. Excretion of Cu could be described excellently by a two compartment model, the half-life times being $t_{\frac{1}{2};1} \approx 0.36$ days, and $t_{\frac{1}{2};2} \approx 37$ days. Excretion of Pb could be described with a one-compartment model. After a fast initial excretion ($t_{\frac{1}{2}} \approx 0.70$ days), Pb excretion stagnated. The Cu accumulation after a 14-day recovery period appeared to be significantly less than the initial accumulation. The Pb accumulation appeared to be slower after recovery than before.

INTRODUCTION

T HE EXPOSURE OF earthworms to pollutants in contaminated sites is variable, because of soil spatial variability. Despite their small habitats, earthworms in contaminated sites are exposed to various contamination levels (Morgan and Morgan 1991, Edwards 1992, Tomlin 1992). Marinussen and Van der Zee (1996) have shown that soil spatial variability and mobility of organisms lead to considerable variation in exposure to soil contamination. Earthworms in heavy metal contaminated sites are able to avoid contamination (Streit 1984, Eijsackers 1987, Ma 1988). Avoidance behaviour and soil spatial variability can explain the discrepancies between toxicity observed under laboratory conditions and toxicity observed at contaminated sites (Spurgeon *et al.* 1994). Additionally, the relatively large bio-availability of heavy metals in artificially contaminated soil compared to the bio-availability at contaminated sites contributes to these discrepancies.

Tissue heavy metal concentrations may give information about the exposure of earthworms to heavy metals, because earthworms accumulate heavy metals when exposed to heavy metal contaminated soil (Ireland 1979, Ireland and Wooton 1976, Morgan and Morgan 1988, 1993). The amount accumulated depends on soil properties such as the degree of contamination, soil-pH, cation exchange capacity (*CEC*), and organic matter content (*OM*) (Beyer *et al.* 1987, Ma 1982, Ma *et al.* 1983, Morgan and Morgan 1988). Adaptation of earthworms may also occur (Corp and Morgan 1991, Morgan and Morgan 1991). Much research has been done on heavy metal concentration factors (CF: the ratio of tissue heavy metal concentration to soil heavy metal concentration). These CF-values are heavy metal specific and species dependent. Even when these differences are accounted for, the CF-values still vary over a wide range, depending on the contaminated site where earthworms were collected (Ireland 1979, Ash and Lee 1980, Ma *et al.* 1989, Beyer and Cromartie 1985, Beyer *et al.* 1987, Morgan and Morgan 1988). Morgan and Morgan (1991) suggested that food choice plays a role in metal accumulation. Ma (1982) and Terhivuo *et al.* (1994) found differences in accumulation between

earthworms from laboratory experiments and earthworms from contaminated sites. In laboratory experiments, the heavy metal exposure level is well known. In contaminated sites, however, it can only be approximated by analysis of several spatially distributed soil properties. If both earthworm and soil samples are taken at the same point, only the recent exposure and accumulation are known, which may differ from previous levels. The extent to which variation in exposure influences the tissue heavy metal concentration depends on the heavy metal kinetics in earthworms. Whereas knowledge about the heavy metal kinetics in earthworms is needed for the assessment of the impact of soil spatial variability on the exposure to heavy metals in contaminated sites, little is known about these kinetics. Recently, Neuhauser _et al._ (1995) reported on heavy metal (Cd, Cu, Ni, Pb, Zn) uptake- and elimination experiments with _Allolobophora tuberculata_ Eisen in soil from sludge disposal sites and Honeycutt _et al._ (1995) studied Cd uptake and elimination in _Eisenia fetida_. Crossley _et al._ (1995) and Brown and Bell (1995) report on uptake and elimination studies monitoring concentrations in live individuals using radiotracers.

The aim of the present study is a) to determine the accumulation- and excretion kinetics of heavy metals (Cu, Pb, and Zn) for the earthworm _Dendrobaena veneta_ and b) to determine the effect of a short recovery period on the heavy metal accumulation in this earthworm. The latter is a simple simulation of a possible event in contaminated sites. This type of research is quite common for organisms like springtails and isopods (e.g. Hopkin 1989, Janssen _et al._ 1991), but unusual for

TABLE 4.1 _Copper, lead and zinc in soil and porewater, soil pH and porewater pH_

soil	Cu-HNO$_3$ [mg kg^{-1}]	Pb-HNO$_3$ [mg kg^{-1}]	Zn-HNO$_3$ [mg kg^{-1}]	Cu-pore [mg L^{-1}]	Pb-pore [mg L^{-1}]	Zn-pore [mg L^{-1}]	pH-CaCl$_2$ [-]	pH-pore [-]
MH	815±117	340±57	225±51	0.37±0.06	0.06±0.01	0.14±0.06	7.0±0.06	7.0±0.25
LB	242±29	109±23	72±9	0.19±0.01	0.05±0.01	0.05±0.02	7.0±0.00	7.1±0.31
WK[A]	5.7±0.2	15±0.9	9.1±0.2	n.a.	n.a.	n.a.	5.4±0.14	n.a.

[A] pore water of WK soil was not analyzed

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earthworms. The experiments were performed in soil from a contaminated site. Hence, the heavy metal bio-availability in the experiments does not differ from the availability under field conditions. For this reason, the results are more suitable for extrapolation to field situations.

MATERIALS AND METHODS

Design of the experiment

SANDY LOAM SOIL (clay 7%, organic matter (OM; loss on ignition) 3%) was collected at a former wrecking yard in Doetinchem, The Netherlands. Major activities were obtaining Cu by burning cables and breaking up cars and electric motors. At the most contaminated part of the site, the soil was visibly contaminated with ash, metals (scrap-iron, wires, screws, bolts and nuts), plastics and rubber. Contaminated soil was collected at two locations (LB and MH) that differed in heavy metal (Cu, Pb and Zn; Table 4.1) content and where no other contamination was apparent. The two locations were selected after chemical analyses of 20 soil samples. The air-dried soils were sieved separately over a 1-cm sieve to remove large particles and mixed thoroughly to achieve homogeneity. From each soil, four replicates of 15 kg of air-dried soil were mixed thoroughly with distilled water, yielding a moisture content of 25%. The wet soil was put into plastic containers (0.4m×0.3m×0.2m) and 60 earthworms (*Dendrobaena veneta*) obtained from a vermiculturist were added to the soils. The containers were put into a phytotron, adjusted to nine hours daylight and three hours darkness, room temperature at 15°C, and relative air humidity of 85%. The containers were weighed every two days and distilled water was added to compensate for evaporation (approximately 0.1 L day^{-1}). Every 14 days, 0.1 kg of rotten apples and vegetables were supplied on the soil surface as food for the earthworms. The food was not chemically analysed. The initial tissue heavy metal concentrations (C_M) were determined by chemical analysis of 10 earthworms. Exposure to soil MH started later than exposure to soil LB, hence initial concentrations were

determined twice.

ACCUMULATION RATE   ACCUMULATION AFTER RECOVERY

```
┌─────────────────────┐                    ┌──────────┬──────────┐
│      SOIL MH        │                    │ SOIL LB  │ SOIL LB  │
└─────────────────────┘                    └──────────┴──────────┘

                              15% transferred back
                              after 14 days in soil WK


                         ┌──────────┬──────────┐
           ────────────► │ SOIL WK  │ SOIL WK  │ ◄────────────
  25% transferred to soil WK        └──────────┴──────────┘   100% transferred to soil WK
  after 112 days in soil MH                                   after 28 days in soil LB
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EXCRETION RATE
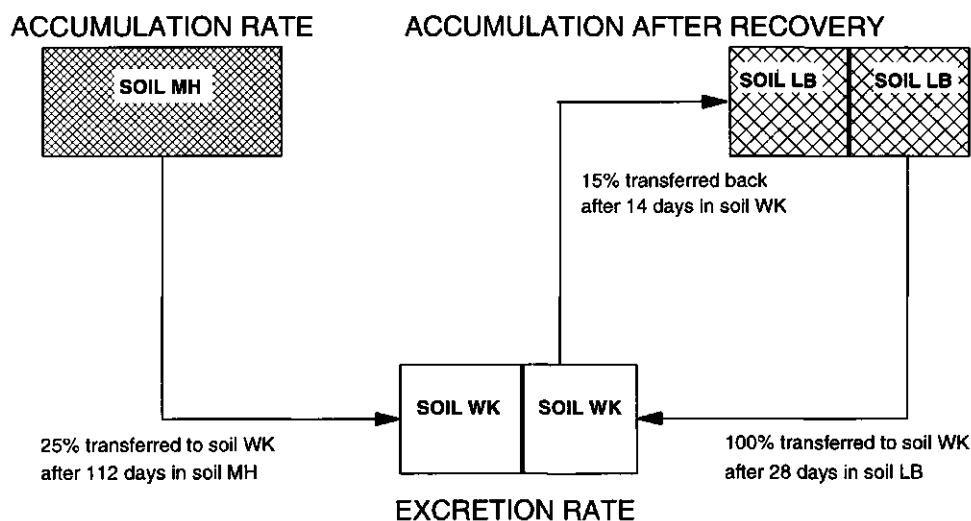
Figure 4.1 _Design of the experiment. At day 0, 60 earthworms were introduced in contaminated soils MH and LB (four replicates). See text for further information._

The heavy metal (Cu, Pb and Zn) accumulation rates were determined in the group of earthworms that were exposed to soil MH (Figure 4.1). Five earthworms from the MH-soils were collected at each of eight sample times (days 1, 2, 3, 7, 14, 28, 56, and 112) for chemical analysis. Data from the first 56 days exposure indicated that tissue heavy metal concentrations had achieved a plateau at day 14. This made us decide to transfer earthworms that remained after sampling at day 112 to uncontaminated soil (WK) to determine the heavy metal excretion rates of this group of earthworms. Soil WK was sampled from an agricultural field near Wageningen, The Netherlands. The soil is a slightly loamy fine sand and low in OM (loss on ignition 3.5%). Three earthworms were collected from the WK soils at each of five sample times (days 1, 2, 3, 7, 14).

After 28 days, all earthworms in LB soils were transferred to soil WK to determine the heavy metal excretion rates in this group of earthworms (Figure 4.1). Five

earthworms were collected from the WK soils at each of eight sample times (days 0, 1, 2, 3, 7, 14, 28, and 56) for chemical analysis. After 14 days recovery, 10 earthworms were transferred back into soil LB (Figure 4.1). Heavy metal accumulation in this group of earthworms was determined after 28 and 56 exposure. The purpose of this part of the experiment was to determine if there was an effect of a recovery period on heavy metal accumulation.

Sampling preparation and chemical analysis
At the end of the experiments, the soil from each container was sampled for chemical analysis. The soil samples were dried in an oven at 40°C for 24 h and sieved to remove aggregates larger than 2 mm. To determine the total extractable soil heavy metal contents, 3 grams soil were equilibrated with 30 ml 0.43 M HNO_3 solution for 20 h in an end-over-end shaker (Houba *et al.* 1989). After centrifugation at 10,000 g for 15 min., Cu, Pb and Zn concentrations in the supernatant were measured on a flame atomic absorption spectrometer (Instrumental Laboratory AA/AE spectrophotometer S11 with Smith-Hieftje background correction only in case of Pb). Soil pH was determined with a glass-calomel electrode in a suspension of 3 grams soil with 30 ml 0.01 M $CaCl_2$ (Houba *et al.* 1989). The 0.01 M $CaCl_2$ extractable heavy metal contents were close to the determination limit (Zn: 0.3 mg kg^{-1}) or below the determination limit (Cu: 0.42 mg kg^{-1}; Pb 0.084 mg kg^{-1} (furnace AAS)). To check the accuracy of the chemical analyses, also a reference soil sample was analysed.

After an experiment had finished, pore-water from the contaminated soils was sampled by centrifugation of soil in Teflon beakers at 8,000 g for 30 min. (Davies and Davies 1963). The pore-water was collected in a bottle through a perforated bottom that was covered by a filter paper. The pore-water was analyzed for Cu and Zn concentrations on the flame AAS, and for Pb concentration on a furnace AAS (Varian SpectrAA 300/400 with Zeeman background correction). The pore-water pH was measured with a glass-calomel electrode.

Earthworms were rinsed with distilled water and put into petri dishes on moist filter papers to empty their gut. The filter papers were changed every day to

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reduce coprophagy. After three days, the petri dishes were put into a freezer (-30°C) to kill the earthworms. Next, the earthworms were individually put in Teflon beakers, lyophilised, and weighed. It is noteworthy that the dry weight did not change significantly for the time of the experiments. After weighing, the earthworms were individually put in digestion tubes, dissolved in 5 ml concentrated HNO_3 and digested at 130°C for two hours. After adding another 2 ml concentrated HNO_3 the solutions were digested at 150°C for one hour. After addition of 2 ml H_2O_2, the solutions were evaporated at 190°C. After cooling down to 90°C, 5 ml 0.2 M HNO_3 was added and heated up to 125°C for a few minutes. After cooling, the solutions were put in test-tubes and analyzed for Cu, Pb and Zn. Cu and Zn were measured on the flame AAS, Pb was measured on the furnace AAS. The determination limits for tissue concentrations were 1.4 mg Cu kg^{-1}, 1.0 mg Zn kg^{-1}, and 0.3 mg Pb kg^{-1}. To check the accuracy of the procedure, BCR certified reference material (CRM 186) was included in the analyses (European Commission 1994). Heavy metal concentrations were within the 95% confidence interval of the certified values.

The significance of differences in means of tissue heavy metal concentrations was tested by ANOVA (Tukey-HSD test) at a significance level $P=0.05$.

RESULTS

Soil analysis

THE RESULTS OF the soil analyses are presented in Table 4.1. The standard deviations of Cu, Pb, and Zn content in the soils MH and LB are large (Coefficient of Variation 12%-23%), whereas the standard deviation of pH is small (less than 1%). Comparison of the CVs for these soil properties in 81 spatially distributed soil samples at the contaminated site and the CVs in the homogenized soils show that the homogenisation procedure was successful (CVs in the field varied between 10% (pH) and 200% (Pb)). The relatively large CVs for heavy metals in soils MH and LB may be an indication that the soils contained

small heavy metal particles. The heavy metal concentrations in the pore-water are very small compared to the total extractable contents. The pH in the 0.01 M $CaCl_2$ suspensions are virtually equal to the pH of the pore-water.

Accumulation

The initial tissue heavy metal concentrations in the group of earthworms that was exposed to soil MH were C_{Cu}: 7.5±2.8 mg kg^{-1} and C_{Zn}: 71±14 mg kg^{-1}; Pb measurements for this experiment failed. After 14 days, C_{Cu} had increased to 66 mg kg^{-1}. For longer times C_{Cu} fluctuated around 60 mg kg^{-1} for 42 days (Figure 4.2*A*). This suggests equilibrium between Cu in soil and Cu in the earthworms over this exposure period. C_{Cu} at day 112, however, was significantly higher than C_{Cu} at day 56. Until day 56, Cu accumulation can be described by a one-compartment model (Atkins 1969):

$$C_{Cu}(t) = C_{Cu}(0) + \frac{\alpha_{Cu}}{k_{Cu}}(1 - \exp(-k_{Cu}t)) \tag{4.1}$$

where t is the exposure time [days], $C_{Cu}(0)$ the initial tissue Cu concentration (7.5 mg kg^{-1}), α_{Cu} the Cu uptake rate parameter [mg kg^{-1} day^{-1}], and k_{Cu} the Cu

TABLE 4.2 *Estimated two compartment model parameters (Atkins 1969) describing the accumulation and excretion of Cu and Pb by D. veneta*

		Cu accumulation soil MH	Cu excretion soils MH and LB	Pb excretion soil LB
A_1			0.68±0.03	0.60±0.06
k_1	[day^{-1}]	0.33±0.05	2.13±0.36	1.71±0.52
A_2			0.32±0.02	n.s.[A]
k_2	[day^{-1}]		0.025±0.005	n.s.[A]
r^2		0.782	0.949	0.794
C_{eq}[B]	[mg kg^{-1}]	61.5±15.5	$C_{Cu}(0)$	3.04±0.11

[A] not significantly different from zero

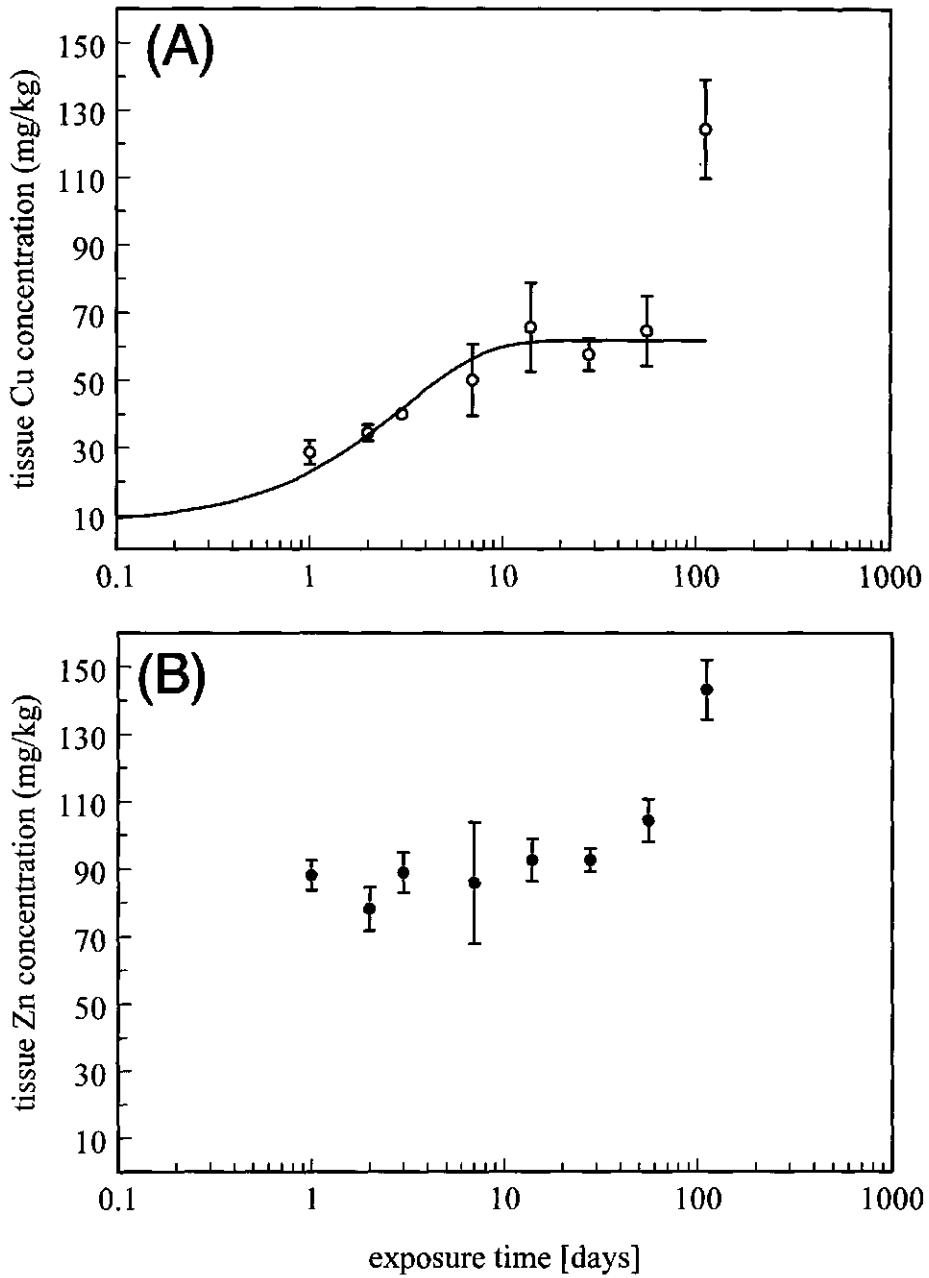[B] estimated heavy metal tissue concentration at equilibrium (t is large)

Figure 4.2 *Cu (A) or Zn (B) accumulation in Dendrobaena veneta exposed to soil MH*

excretion rate parameter [day^{-1}]. We estimated α_{Cu}=17.9±2.4 mg kg^{-1} day^{-1}, and k_{Cu}=0.33±0.05 day^{-1} (R^2=0.782; Table 4.2). This model is in excellent agreement with the data with exception of C_{Cu} at day 112 (Figure 4.2A). Unfortunately, it is unknown what would have been happened to C_{Cu} after day 112 if earthworms would have been exposed for a longer period. Moreover, no intermediate data between day 56 and 112 are available, hence we are not able to fit any (complex) accumulation model to all data.

Until day 56, C_{Zn} did not differ significantly from the initial concentration. At day 56, C_{Zn} was significantly higher than initially, but did not differ from C_{Zn} at other days. At day 112, C_{Zn} had increased to a large extent (Figure 4.2B). Due to lack of sufficient data after day 56 we were unable to fit any accumulation model to these data.

Excretion

The initial tissue heavy metal concentrations in the group of earthworms that was exposed to soil LB were: C_{Cu}: 14.7±2.6 mg kg^{-1}, C_{Zn}: 107±13 mg kg^{-1}, and C_{Pb}: 1.2±0.6 mg kg^{-1}.

The tissue Cu concentration after 28 days exposure to soil LB was 58.9±6.3 mg kg^{-1} (Table 4.3). Seven days after being transferred to soil WK, C_{Cu} had decreased considerably. The tissue Cu concentration after 112 days exposure to soil MH was significantly higher than in earthworms from soil LB (Table 4.3). After 1 day in soil WK, C_{Cu} in the group of earthworms that came from soil MH had decreased considerably. In Figure 4.3, the tissue Cu concentrations are presented after normalisation according to:

$$f_M^i(t_r) = \frac{C_M^i(t_r) - C_{M,\,t=0}}{C_M^i - C_{M,\,t=0}}$$ (4.2)

where $f_M^i(t_r)$ is the fraction remaining heavy metal M in the earthworms from replicate i after t_r days in soil WK, $C_M^i(t_r)$ the tissue heavy metal concentration after t_r days in soil WK, $C_{M,\,t=0}$ is the initial heavy metal concentration, and C_M^i is the

TABLE 4.3 _Cu, Pb and Zn tissue concentrations in D. veneta. Concentrations in the same column are significantly different (P<0.05) if the letters between the parentheses are different (a<b<c<d<e<f). Before transfer to soil WK, earthworms were exposed to soil LB for 28 days or to soil WK for 112 days._

time [days]	$C_{Cu}\pm$s.d [mg kg^{-1}]		$C_{Zn}\pm$s.d [mg kg^{-1}]		$C_{Pb}\pm$s.d. [mg kg^{-1}]		$C_{Cu}\pm$s.d [mg kg^{-1}]		$C_{Zn}\pm$s.d [mg kg^{-1}]	
	in soil LB						**in soil MH**			
-112							7.5±2.8	(a)	71±14	(a)
-28	14.7±2.6	(a)	107±13	(a)	1.2±0.6	(a)				
	transfer to soil WK									
0	58.9±6.3	(f)	106±2	(a)	5.8±0.5	(c)	124±15	(c)	143±9	(b)
1	32.2±2.2	(d)	104±4	(a)	3.7±0.2	(b)	49.5±4.7	(b)	153±5	(b)
2	27.4±2.2	(c,d)	104±8	(a)	2.7±0.3	(b)	49.2±5.2	(b)	160±18	(b)
3	27.5±0.8	(c,d)	106±7	(a)	2.7±0.1	(b)	44.5±5.1	(b)	150±9	(b)
7	23.2±1.9	(b,c)	105±11	(a)	3.3±0.3	(b)	38.4±3.1	(b)	157±8	(b)
14	22.0±1.9	(b,c)	98±6	(a)	3.1±0.4	(b)	35.8±4.1	(b)		
28	22.4±1.9	(b,c)	109±3	(a)	3.1±0.7	(b)				
56	19.1±2.3	(a,b)	140±5	(b)	3.2±0.7	(b)				
	transfer back to soil LB (at t=14)									
42	46.6±2.8	(e)	97±7	(a)	3.6±0.5	(b)				
70	46.5±4.1	(e)	131±14	(b)	6.0±0.8	(c)				

tissue heavy metal concentration at day 0 in soil WK. After 1 day in soil WK, the fraction of Cu remaining had decreased to about 40% in worms from soil LB and to 35 % in worms from soil MH. After 7 days, the fractions were respectively 20 and 25 % (Figure 4.3). The initially fast decrease was followed by a slow decrease in tissue Cu concentration. Fifty six days after the earthworms were transferred from soil LB to soil WK, the tissue Cu concentration was still elevated compared to the initial concentration, but was significantly lower than after 3 days recovery (Table 4.3). The initially rapid excretion rate, followed by a continued but slower rate is a clear indication for a biphase excretion process and could be described

excellently by a two compartment model (Atkins 1969):

$$f_M(t_r) = \sum_{i=1}^{2} A_i \exp(-k_{Cu}^i t_r)$$ (4.3)

where A_i is the coefficient of i^{th} exponential term, k_{Cu}^i is the Cu excretion rate parameter of the i^{th} exponential term [day^{-1}]. The model parameters were estimated with non-linear regression and appeared significant (Table 4.2). The first phase half-life time is in the range from 0.24 to 0.49 days, the second phase half-life time is in the range from 27 to 48 days.

After 28 days exposure to soil LB, C_{Zn} did not differ significantly from the initial concentration, and there was also no decrease in C_{Zn} after the earthworms were
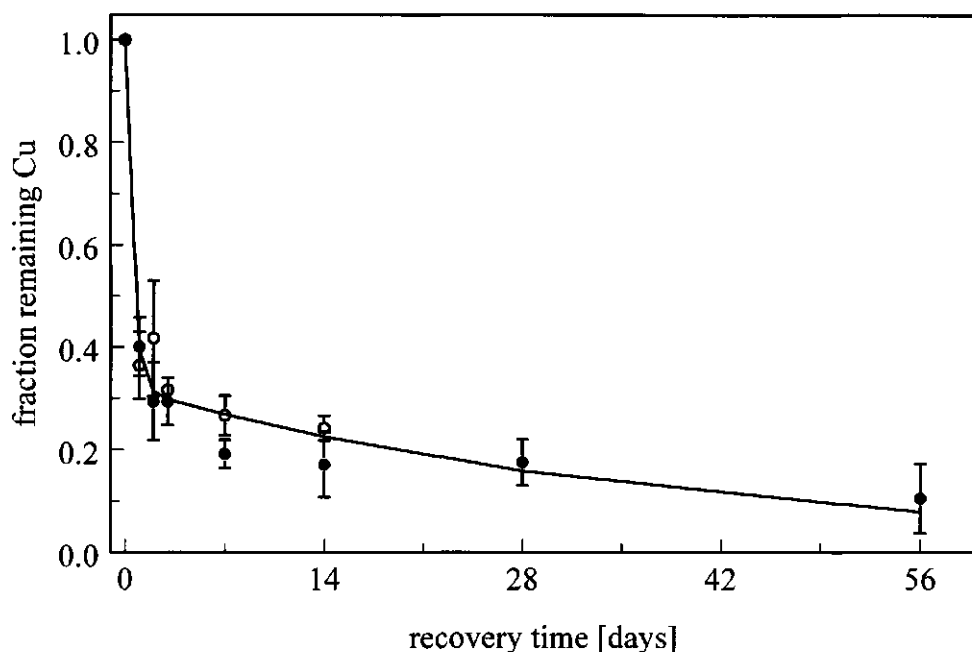

Figure 4.3 *Cu excretion by Dendrobaena veneta after exposure to soil MH (○) or to soil LB (●). Note: earthworms from soil MH were exposed for 112 days before transfer to uncontaminated soil, whereas earthworms from soil LB were exposed for 28 days.*

transferred to soil WK (Table 4.3). After 56 days in soil WK, C_{Zn} was significantly larger than at any other day. Since the mean dry weight at day 56 was about 110% of the initial mean dry weight, the unexpected increase in C_{Zn} could not be explained by loss of weight. C_{Zn} in the earthworms from soil MH did not decrease significantly during the 7 days in soil WK and was not determined at day 14 (Table 4.3).
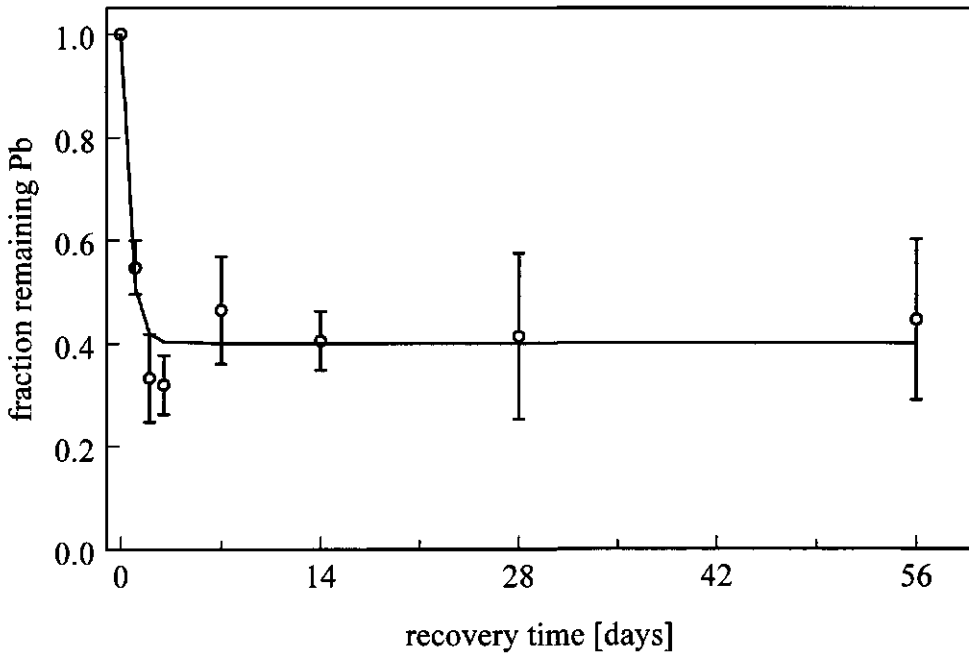
Figure 4.4 *Pb excretion by Dendrobaena veneta after exposure to soil LB*

After 28 days exposure to soil LB, C_{Pb} in the earthworms had increased to 5.8±0.5 mg kg^{-1} (Table 4.3). After 1 day in soil WK, the remaining fraction Pb had decreased to 55% and stagnated at this level. Fifty six after the transfer to soil WK, C_{Pb} was significantly larger than the initial tissue Pb concentration, but did not differ significantly from C_{Pb} after 1 day in soil WK. The excretion can be described by a one-compartment excretion model (Atkins 1969):

$$f_M(t_r) = A_1 \exp(-k_{Pb} t_r) \qquad\qquad\qquad (4.4)$$

The model parameters were estimated by non-linear regression (Table 4.2 and Figure 4.4). The half-life time of Pb is in the range from 0.25 to 1.0 days. Since C_{Pb} stagnated at the level of day 1, the model is of limited value.

Exposure after recovery-period

Tissue heavy metal concentrations were determined after 28 and 56 days in soil LB (Table 4.3). After 28 days in soil LB, C_{Cu} had increased from 22.0 ± 1.9 mg kg^{-1} to 46.6 ± 2.8 mg kg^{-1} which is significantly less than C_{Cu} exposure before recovery. C_{Zn} at day 28 did not differ significantly neither from C_{Zn} before recovery, nor from C_{Zn} after the 14 days recovery period. C_{Pb} at day 28 was significantly less than C_{Pb} before recovery. After 56 days in soil LB, C_{Cu} did not differ significantly from C_{Cu} at day 42 (Table 4.3). Both C_{Zn} and C_{Pb} had increased significantly. At day 70, C_{Zn} was significantly higher than C_{Zn} before recovery, whereas C_{Pb} was very similar to C_{Pb} before recovery (Table 4.3).

DISCUSSION

THESE EXPERIMENTS WERE performed to gain insight into the accumulation- and excretion rates of Cu, Pb and Zn for earthworms. The purpose was neither to investigate effects of soil properties on heavy metal accumulation, nor to determine the toxicity of the heavy metals for *D.veneta*. Both soil MH and soil LB were collected from a site that was heavily contaminated with Cu and Pb. Nevertheless, we did not observe any earthworm mortality during the experiments. This was not unexpected since the soil pH is high and it is known that the heavy metal toxicity increases when soil pH decreases (Ma 1984, Lee 1985, Ma 1988). The experiments were unusual, because they were performed with homogeneously mixed soils from a contaminated site. Generally, sewage sludge is added to soil (Andersen 1979, Ireland 1979,

Hartenstein _et al._ 1980, Ma 1988) or soil is artificially contaminated (Ma 1984, Streit 1984, Spurgeon _et al._ 1994).

D.veneta accumulated Cu during exposure to soils MH and LB. Ireland (1979) found no accumulation in _D. veneta_ that were collected in the winter months from a site that consisted of sewage sludge deposited on bare soil, although the soil copper content was 252 ± 5 mg kg^{-1}. In soil MH, a transient plateau was achieved within 14 days. This is in agreement with Neuhauser _et al._ (1995) who found a transient plateau for both Cu and Zn in _Allolobophora tuberculata_. In the literature no more data are available which enable a comparison of the Cu accumulation rate in _D. veneta_ with Cu accumulation rate in other earthworm species. Belfroid _et al._ (1994) observed a steady state condition for chlorobenzene accumulation in earthworms (_Eisenia andrei_) within 4 days. In their experiments, the earthworms were exposed to chlorobenzenes via food. It is plausible that an inevitable exposure via food leads to a steady state faster than an exposure to contaminated soil. Janssen _et al._ (1991) found a steady state of Cd accumulation in the carabid _Notiophilus bigattatus_ within 7 days after exposure to Cd contaminated food. In other soil arthropods (pseudoscorpion _Neobisium muscorum_, oribatid mite _Platynothrus peltifer_, and collembolan _Orchesella cincta_), they did not observe a steady state in accumulation after exposure for 30 days.

Although soil MH has a larger Cu content than soil LB, the earthworm tissue Cu concentration after 28 days exposure did not differ significantly in one soil from the other. This is not in agreement with the general idea that tissue Cu concentration is positively correlated with the total soil Cu content (Ma 1982, Morgan and Morgan 1988, Neuhauser _et al._ 1995). The pore water Cu concentration in soil MH and soil LB differed also considerably, hence the equal values of C_{Cu} can not be related to the pore water Cu concentrations. The Cu concentration in 0.01 M CaCl$_2$ solutions were below the detection limit. Although extraction with 0.01 M CaCl$_2$ is useful for the estimation of availability of heavy metals for plant uptake (Novozamsky _et al._ 1993), this procedure does not seems to be useful for the estimation of Cu availability for earthworms.

Both C_{Cu} and C_{Zn} at day 112 differed significantly from the steady state that was

apparently achieved after 14 days. This can not be attributed to a change in (dry) weight, because the dry weight of our earthworms did not change significantly for the duration of the experiment. There is also no doubt about the reliability of the chemical analysis, because both Cu and Zn concentrations in the CRM reference samples were within the 95%-confidence intervals. Hence, there must be a worm related factor that explains these unexpected changes. Neuhauser *et al.* (1995) found a similar unexpected second shift in C_{Zn} in the earthworm *A. tuberculata* between day 56 and day 112 whereas C_{Cu} had decreased considerably. Belfroid *et al.* (1994) found a comparable second shift when they exposed earthworms (*E. andrei*) to multiple doses of chlorobenzenes via contaminated food. Neither study, however, explained this unexpected increase. Generally, earthworms are able to regulate Cu and Zn (Ireland 1979, Lee 1985, Morgan and Morgan 1988). Possibly, this regulation mechanism is damaged due to the prolonged chronic exposure to heavy metal contaminated soil, which causes a significant increase in both C_{Cu} and C_{Zn} at day 112 compared with the tissue metal concentrations at day 56. When transferred to uncontaminated soil, however, the earthworms appeared to be able to eliminate the Cu rather quickly, which does not support this suggestion. The kinetic process over 112 days is complex, and it seems to us that it changed between days 56 and 112. Until day 56, Cu accumulation can be described very well by a one-compartement model. Since we do not have intermediate data between days 56 and 112, we are not able to model the mechanism after day 56.

The Cu excretion is a biphasic process (two-compartment model) as was also found by Neuhauser *et al.* (1995) for *A. tuberculata*. Only 14 days were required until about 80% of the accumulated Cu was eliminated by *D. veneta*. Using the model parameters in Eq. 4.3, we estimated that the earthworms need a recovery period of about half a year before their tissue Cu concentration would have reached the initial level.

Unlike Cu excretion, Pb excretion is initially a fast process that involves approximately 60% of accumulated lead and that is followed by a steady state for the remaining time of the experiment (until 56 days). This is in agreement with

Neuhauser _et al._ (1995) who observed a plateau after a rapid decrease in C_{Pb} in _A. tuberculata_ between days 0 and 7. Both Pb and Cu are bound to chloragosomes (Ireland 1978) which may explain the slow loss of Cu in the second phase. According to Hopkin (1989), Pb can be stored permanently in waste nodules which can be too large to be excreted by earthworms (Lee 1985) and which may explain the steady state in C_{Pb} after 7 days in soil WK.

After a 14 days recovery period, earthworms that were exposed again to soil LB did not accumulate Cu and Pb to the same extent as during the first exposure. This could be an indication that _D. veneta_ physiologically adapted within a relatively short period (28 days). We do not know, however, if individual earthworms are able to adapt to heavy metal contamination so fast. Corp and Morgan (1991) showed that larger amounts of heavy metals had accumulated in native earthworms _Lumbricus rubellus_ than in earthworms that were introduced in the contaminated soil and exposed for 31 days, which could be an indication for physiological adaptation. Our accumulation experiments, however, show that exposure for 31 days may be too short to allow a comparison of the Cu accumulation in earthworms in contaminated sites with the Cu accumulation in introduced earthworms, as was also suggested by Corp and Morgan (1991).

The experiments with _D. veneta_ were performed to estimate the impact of soil spatial variability on heavy metal accumulation in earthworms living in contaminated sites. The results show that tissue heavy metal concentrations change rapidly when the exposure level changes, since both accumulation- and excretion rates are large. Moreover, the equilibrium heavy metal tissue concentration after a recovery period differs from the equilibrium after direct exposure. On the other hand, earthworms that are exposed chronically to contaminated soil for a long period show a decreased ability of Cu and Zn regulation. Hence, we conclude that soil spatial variability has to be taken into account when comparing heavy metal accumulation under field conditions with heavy metal accumulation under laboratory conditions.

ACKNOWLEDGEMENTS

Chapter 5

Effect of Cd or Pb addition to Cu contaminated soil on tissue Cu accumulation in the earthworm *Dendrobaena veneta*

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Submitted to: *Ecotoxicology and Environmental Safety*

ABSTRACT

Generally, soil heavy metal contamination consists of a mixture of heavy metals. Soil chemical properties and interaction with other pollutants in soil affect the external heavy metal bio-availability. Moreover interaction with other pollutants accumulated in organisms may change the toxicity of each pollutant. Therefore, we tested the hypotheses that addition of Cd or Pb to Cu contaminated soil would lead to an increase in tissue Cu accumulation in the earthworm *Dendrobaena veneta*, caused by (i) induction of metallothionein by Cd, or (ii) an increase in Cu concentration in soil solution due to the exchange of adsorbed Cu for Pb. Tissue heavy metal concentrations were determined after exposure in contaminated soils for 3 or 21 days. Considerable amounts of Cu, Cd and Pb were accumulated, indicating that these heavy metals were available for uptake by *D. veneta*. Both Cd and Pb, however, did not significantly affect tissue Cu accumulation.

INTRODUCTION

EARTHWORMS ARE KNOWN to accumulate heavy metals when exposed to heavy metal contaminated soil. Soil properties affect the heavy metal accumulation, where pH, CEC (Cation Exchange Capacity) and OM (Soil Organic Matter content) are the most relevant ones (Ma 1982, Ma *et al.* 1983, Beyer *et al.* 1987). Earthworms from contaminated sites show a large variation of the values of concentration factors (CF, ratio tissue heavy metal concentration to soil heavy metal concentration) (*e.g.* Ireland 1983, Terhivuo *et al.* 1994). Contamination at field sites is spatially variable and therefore exposure of an individual earthworm to contaminants varies with time and space. Within a group of earthworms inhabiting a contaminated site the exposure differs, depending on both the degree of spatial variability of contamination and the earthworm migration activity as has been suggested by Marinussen and Van der Zee (1996). Spatial variability together with specific food choices and earthworm ecology (Morgan and Morgan 1991, Edwards 1992, Tomlin 1992, Morgan and Morgan 1993), seasonal variability in earthworm activity (Lee 1985) and earthworm avoidance behaviour (Eijsackers 1987) may explain the large variation in CF values for different species in different contaminated sites. Marinussen *et al.* (1996c) found that the CFs in earthworms (*Dendrobaena veneta*) introduced in a heavy metal contaminated field site were in excellent agreement with CFs in *D. veneta* exposed to soil from this site under laboratory conditions. They found a decrease in CF with increasing soil Cu concentration under both conditions, and concluded that the earthworms exposure in a contaminated field can be predicted provided that soil sampling density is sufficiently high. From an ecotoxicological point of view it is important that field observations are in agreement with data obtained by laboratory experiments, which is generally not the case (Lofs-Holmin 1980, Terhivuo *et al.* 1994, Spurgeon and Hopkin 1995). According to the OECD guidelines (OECD 1984) to test the toxicity of soil contamination under laboratory conditions, earthworms (*Eisenia fetida*) are introduced to freshly contaminated artificial soil. Although there are good reasons for standardization, the OECD

mixture (10% peat, 20% kaolin clay and 70% sand) can not be considered to be a soil and there are at least three soil chemical aspects that hamper translation of OECD toxicity test results to a toxicity of heavy metal contaminated soils. First, heavy metals in soils are adsorbed to the solid phase it takes time before sorption equilibrium is achieved, which will at least to some extent affect the bioavailability of heavy metals in soil. Second, humic substances in soil ecosystems are heterogeneous in metal ion adsorption properties (*e.g.* Wit 1992) and peat may be a poor model for the large variety of different organic substances that is present in soil ecosystems. Third, there is a large variety of clay minerals that differ considerably in their structure and therefore in their chemical and physical properties. For example, kaolinite is a clay that has a specific surface area of about 1-40 m² g^{-1}, whereas for example montmorillonite may have a specific surface area of 6-800 m² g^{-1} (Bolt *et al.* 1978). The CEC of clay is related to the specific surface area, hence the CEC of montmorillonite is much larger than the CEC of kaolinite.

Generally, contamination in heavy metal contaminated sites consists of several heavy metals, whereas in many toxicological laboratory experiments organisms are exposed to only one heavy metal (Streit 1984, Ma 1988, Gestel *et al.* 1993, Spurgeon *et al.* 1994), sometimes at varied pH-levels (Ma 1984). Since the sorption capacity of soil is limited to the CEC, heavy metal cations compete for sorption sites. The affinity to soil organic matter, which is an important adsorbent in sandy soils, is different for different heavy metals. For example, whereas Cu and Pb show strong affinity to soil organic matter, Cd and Zn show less affinity (Stevenson 1982). Hence, in soils contaminated with a mixture of heavy metals, adsorption-interactions may change the bio-availability of each heavy metal compared to soils contaminated with only one heavy metal. The toxicity of heavy metal polluted soil for earthworms increases with decreasing pH (Ma 1984, Bengtsson *et al.* 1986, Marinussen *et al.* 1996a) which suggests that the heavy metal availability is determined by heavy metal concentrations in soil solution. Tissue Cu accumulation data, however, do not support this suggestion as observed in a laboratory study by Marinussen *et al.* (1996a). They found that tissue accumulation

increased with increasing total extractable soil Cu concentration and did not observe a significant influence of pH on tissue Cu accumulation. This suggests that Cu availability for uptake is not determined by the Cu present in soil solution, and thus the availability for uptake should be different from the availability for toxic effects. Possibly, pH does not only influence Cu sorption, but might also affect the earthworm heavy metal uptake mechanism, so that a decrease in pH results in a decreased ability to accumulate Cu or any other heavy metal. In the present study, our intent is to affect the Cu concentration in soil solution by adding a varied amount of Pb to soils contaminated by Cu, to test the hypothesis that Cu availability for uptake by the earthworm *Dendrobaena veneta* is independent of the Cu concentration in soil solution. We expected that addition of Pb would change the Cu speciation in soil.

Interaction with other pollutants accumulated in organisms may change the toxicity of each pollutant (Hansen and Lambert 1987). Exposure to Cd may induce the production of metallothionein (MT) (Berger and Dallinger 1993, Dallinger 1994, Berger *et al.* 1995). These metal-binding proteins play a role in regulating the intracellular availability of essential metals (Cu and Zn) and nonessential metals (Cd) (Roesijadi 1994). Accumulation of Cd may lead to an increase in Cu binding capacity of earthworms as was hypothesized by Morgan *et al.* (1993). In the present study, this hypothesis was tested by adding different amounts of Cd to Cu contaminated soils before earthworms *D. veneta* were exposed to the soils and tissue Cu concentration was determined. Addition of Cd to Cu contaminated soil will not lead to a significant Cu desorption, since the affinity of Cd to soil organic matter is small compared to the affinity of Cu.

MATERIALS AND METHODS

U NCONTAMINATED SANDY LOAM soil (7% clay, 3% organic matter (OM, loss on ignition) was sampled at a field site in Doetinchem, The Netherlands. The air-dried soil was sieved over a 1 cm sieve to remove

large particles and mixed thoroughly to achieve homogeneity. Subsamples were sieved to remove aggregates larger than 2 mm before chemical analyses to determine heavy metal (Cu, Cd and Pb) concentrations and soil pH. Next, heavy metal nitric salts were added to eight portions of 5 kg each to establish one Cu level (addition of 250 mg kg^{-1}) and four Cd levels (addition of 0, 4, 8, and 16 mg Cd kg^{-1}) or five Pb levels (addition of 0, 200, 400, 600, and 800 mg Pb kg^{-1}). These Cd and Pb levels include the intervention values according to the Dutch soil protection legislation. According to the Dutch legislation, the functional properties of soil are in serious danger if any pollutant concentration exceeds its intervention value (Ministerie van VROM 1994). The Cd intervention value for the soil of our experiments is 8 mg kg^{-1}, and the Pb intervention value is 375 mg kg^{-1}.

Nine months after the addition of the metal salts, four replicates of 1 kg air-dried soil of each treatment were mixed thoroughly with distilled water, yielding a moisture content of 20% (kg kg^{-1}). The wetted soils were put into plastic 6 L jars and stored in a phytotron at 15°C for 10 days to get a desorption equilibrium. Next, in each jar 0.1 kg apple sauce was mixed thoroughly with the soil as a food source for earthworms (*D. veneta*). After incubation in uncontaminated soil for one week, 15 earthworms obtained from a vermiculturist were put into each jar. To hamper escaping, adhesive tape (0.05 m) was attached to the rim of each jar (Fig. 5.1). The phytotron was adjusted to sixteen hours daylight and eight hours darkness, room temperature at 15°C, and relative air humidity of 85%. The jars were weighed every two days and distilled water was added to compensate for evaporation. After 3 and 21 days, four earthworms were sampled from each jar to determine tissue heavy metal (Cu, Cd and Pb) concentrations. Earthworms were rinsed with distilled water and kept for 3 days in petri dishes on moist filter papers to empty their gut. The filter papers were changed every day to reduce coprophagy. Earthworms were lyophilised and analysed for Cu, Cd and Pb following the procedure as was described by Marinussen *et al.* (1996b). Initial tissue heavy metal concentrations were determined in 4 earthworms after the incubation in uncontaminated soil. We did not observe any change in the earthworms dry weight between days 3 and 21.
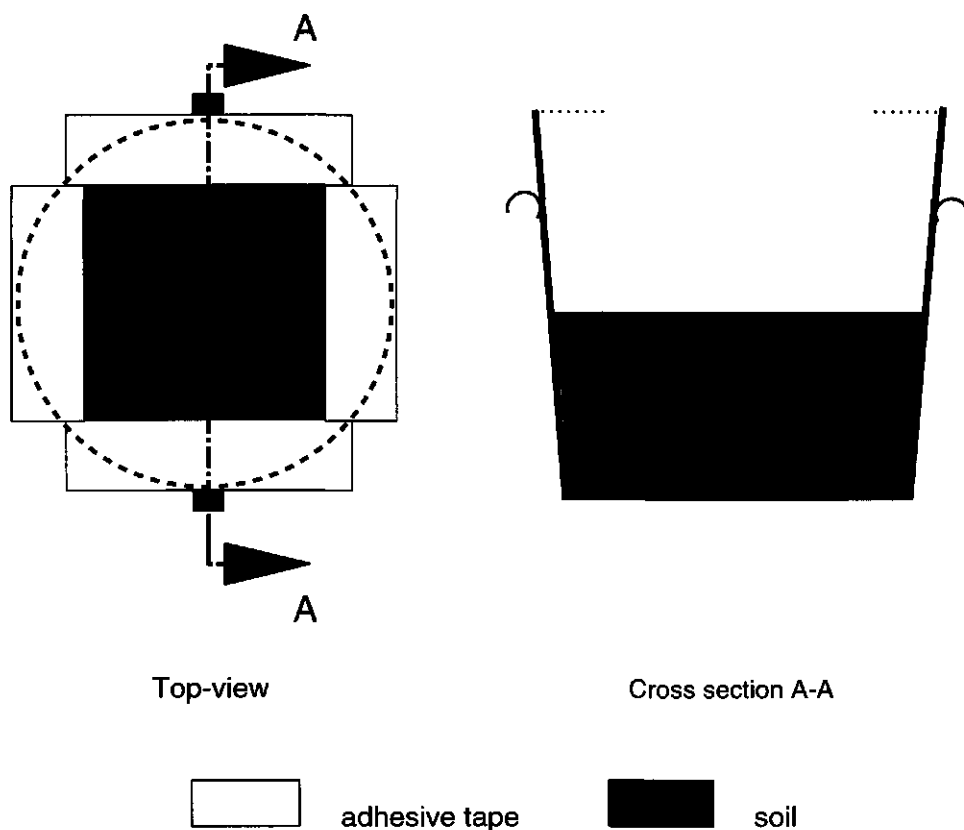
A

A

Top-view   Cross section A-A

☐ adhesive tape   ■ soil

Figure 5.1 *Schematic drawing of the jars in which earthworms were exposed to soil.*

At the end of the experiment, after all earthworms were removed, subsamples were taken from each soil determine total extractable soil heavy metal (Cu_T, Cd_T and Pb_T) concentrations and soil pH. The soil samples were dried in an oven at 40°C for 24 h and sieved to remove aggregates larger than 2 mm. Three grams soil were equilibrated with 30 ml 0.43 M HNO_3 solution for 20 h in an end-over-end shaker to determine the total extractable Cu, Cd and Pb content (Houba *et al.* 1989). After centrifugation at 2,000 *g* for 15 min., Cu, Cd and Pb concentrations in the supernatant were measured on a flame atomic absorption spectrometer

(Instrumental Laboratory AA/AE spectrophotometer S11 with Smith-Hieftje background correction only in case of Pb). Soil pH was determined with a glass-calomel electrode in a suspension of three grams soil with 30 ml 0.01 M $CaCl_2$ equilibrated for 20 h in an end-over-end shaker (Houba _et al._ 1989). After centrifugation at 2,000 _g_ for 15 min., Cu, Cd and Pb concentrations in the supernatant (Cu_S, Cd_S and Pb_S) were measured on the flame atomic absorption spectrometer. These heavy metal concentrations are an indication of the amount which is available for plant uptake (Novozamsky _et al._ 1993) and may be useful as indication for earthworm availability too.

The effect of Cd or Pb addition on tissue heavy metal accumulation was tested by ANOVA (analyses of variance, Tukey-HSD test) at the significance level _P_=0.05 (Kleinbaum _et al._ 1988).

RESULTS AND DISCUSSION

TOTAL EXTRACTABLE Cu, Cd and Pb concentrations in soil are presented in Table 5.1, where code Pb-0 refers to the same treatment as code Cd-0. The total extractable Cu concentrations in the different treatments were in the same order of magnitude (267.2 ± 9.42 mg kg^{-1}), five different soil Pb concentrations (Pb-0, Pb-1, Pb-2, Pb-3, and Pb-4) and four different soil Cd concentrations could be distinguished (Cd-0, Cd-1, Cd-2, and Cd-3). The pH in the soils was 5.4 ± 0.1 and Cu_S varied hardly (1.1 ± 0.2 mg kg^{-1}) which implies that neither the addition of Cd nor the addition of Pb affected the Cu sorption. In the Cd-soils, Cd_S increased slightly from 0.6 ± 0.3 mg kg^{-1} (Cd-1) to 2.3 ± 0.5 mg kg^{-1} (Cd-3). In all samples, Pb_S could not be detected and seemed totally adsorbed.

Three days after introduction in the group of Cd-soils, only tissue Cd concentrations (Cd_w) in soil Cd-3 and Cd-0 differed significantly. After 21 days exposure in the Cd-soils, however, four significantly different Cd_w levels could be distinguished with Cd_w linearly proportional to Cd_T (Fig. 5.2_A_). Despite these

TABLE 5.1 *Total extractable Cu, Cd and Pb concentrations in soil.*

soil	$Cu_T \pm sem$ [mg kg^{-1}]	$Cd_T \pm sem$ [mg kg^{-1}]	$Pb_T \pm sem$ [mg kg^{-1}]
Cd-0	270±4	0.4±0.3	57±2
Cd-1	255±5	3.3±0.2	56±1
Cd-2	278±6	6.8±0.2	61±2
Cd-3	260±6	12.0±0.5	70±6
Pb-0	see Cd-0	see Cd-0	see Cd-0
Pb-1	264±6	0.6±0.05	239±6
Pb-2	268±3	0.4±0.09	426±5
Pb-3	284±3	0.3±0.12	655±8
Pb-4	258±8	0.5±0.13	798±15

significant differences in tissue Cd accumulation, we did not observe differences in tissue Cu accumulation. After 3 days exposure in this group of soils, tissue Cu concentrations (Cu_w) in the different treatments were similar. After 21 days, Cu_w in soil Cd-3 was significantly smaller than in soil Cd-0, which disagrees with our hypothesis (Fig. 5.2*B*). Suzuki *et al.* (1980) showed the induction of Cd-binding proteins in earthworms *Eisenia foetida* that were exposed for 30 days to five different Cd concentrations in composted sewage sludge containing about 100 mg Cu kg^{-1} dry compost and Cd concentrations ranged from 1.3 to 511 mg kg^{-1}. They found that the Cu in the earthworm remained at a constant level for the five different treatments, however. We suggest that the Cu availability for accumulation is low in the compost due to the high organic matter content of compost and the strong affinity of Cu to organic matter. Unfortunately, Suzuki *et al.* (1980) did not give data on Cd_w, hence we do not know at which internal Cd level the metal-binding protein was induced. In literature, no data are available about threshold levels of Cd_w above which metal-binding proteins are induced. Hence, we do not know whether the internal Cd concentrations in our earthworms were too low to induce MTs, or whether the induced MTs were not
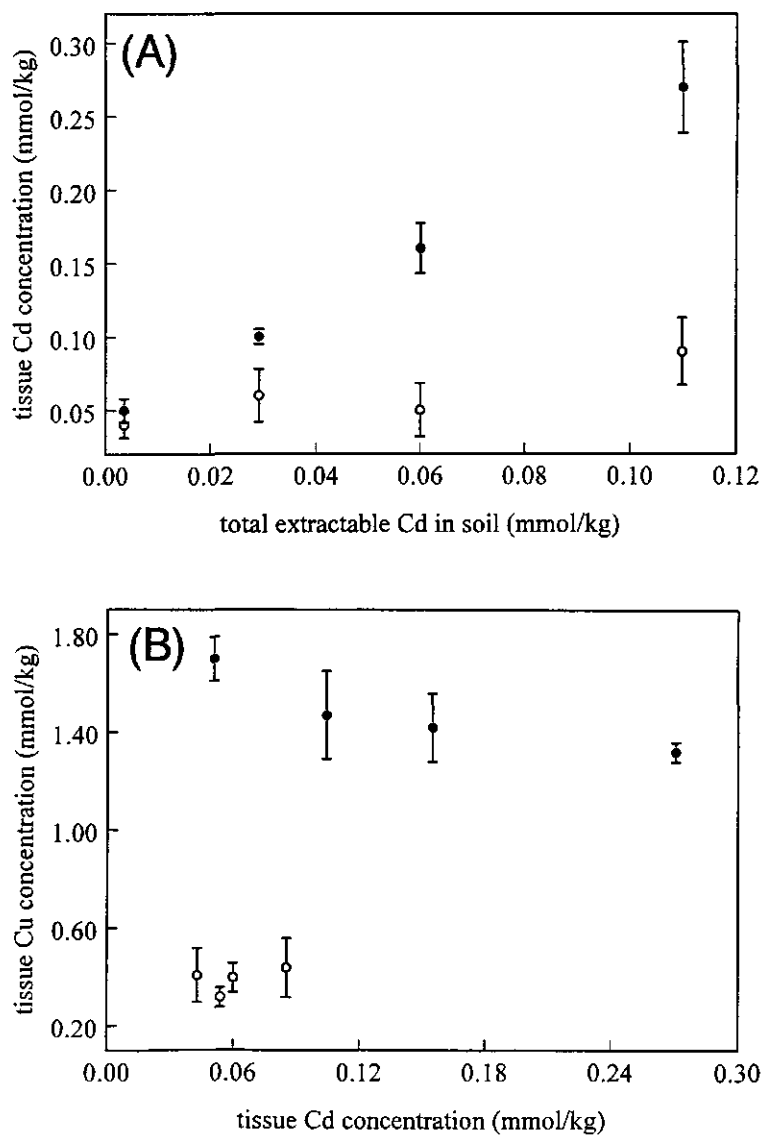
Figure 5.2 *Tissue Cd (A) or Cu (B) concentrations in earthworms Dendrobaena veneta after exposure to soil containing a mixture of Cu and Cd contamination; open circles=tissue heavy metal concentration 3 days after introduction, solid circles=tissue heavy metal concentration 21 days after introduction.*

capable to bind Cu, which would contradict the general idea that MTs are able to bind Cd, Zn and Cu (e.g. Kägi and Schäffer 1988), or whether the Cu availability in our soils was too low.

After 21 days in soil Cd-3, Cd_W was 30 mg kg^{-1}, which is considerably less than 115 mg kg^{-1} as was found by Gestel *et al.* (1993) in earthworms *E. foetida* exposed for 21 days to artificial soil containing 10 mg Cd kg^{-1}. This may suggest that Cd accumulates faster in *E. foetida* than in *D. veneta*. However, Gestel *et al.* used artificial OECD soil, in which the heavy metal bioavailability may be larger than in natural soils as used in our study.

In the group of Pb-soils, tissue Pb accumulation increased proportionally with increasing total extractable soil Pb concentration. The considerable Pb accumulation in earthworms indicate that Pb is available for uptake by earthworms. Five significantly different tissue Pb concentrations (Pb_W) could be distinguished after 21 days (Fig. 5.3*A*), whereas after 3 days only tissue Pb accumulation in soil Pb-0 was significantly less than tissue Pb accumulation in the other Pb-soils and other differences in Pb_W were not statistically significant. Addition of Pb did not significantly affect tissue Cu accumulation, except in soil Pb-4, where, after 21 days, earthworms contained significantly less Cu than earthworms in soil Pb-0 (other differences were not statistically significant, Fig. 5.3*B*). After the exposure experiments had been finished, we measured adsorption isotherms in a 0.01 M $CaCl_2$ suspension for a concentration range of 0-1000 mg Cu or Pb kg^{-1} at a solid:solution ratio of 1:10. Both Cu and Pb appeared to be adsorbed to a large extent, more than 95% of the added amount of these heavy metals was adsorbed. A Pb addition of 1000 mg kg^{-1} to soil containing 270 mg Cu kg^{-1} resulted in an increase in Cu desorption from 13.2 to 14.3 mg kg^{-1}. This difference seems too small to result in an increase in tissue Cu accumulation.

Whereas the Cd accumulation was obviously too small to affect the total accumulation of the three heavy metals, the contribution of Pb to the heavy metal accumulation in the Pb-soils was considerable. This is not that surprising, since in the Cd-soils the total extractable Cu concentration was excessive compared to the total extractable Cd concentration, whereas in the Pb-soils Cu_T and Pb_T were
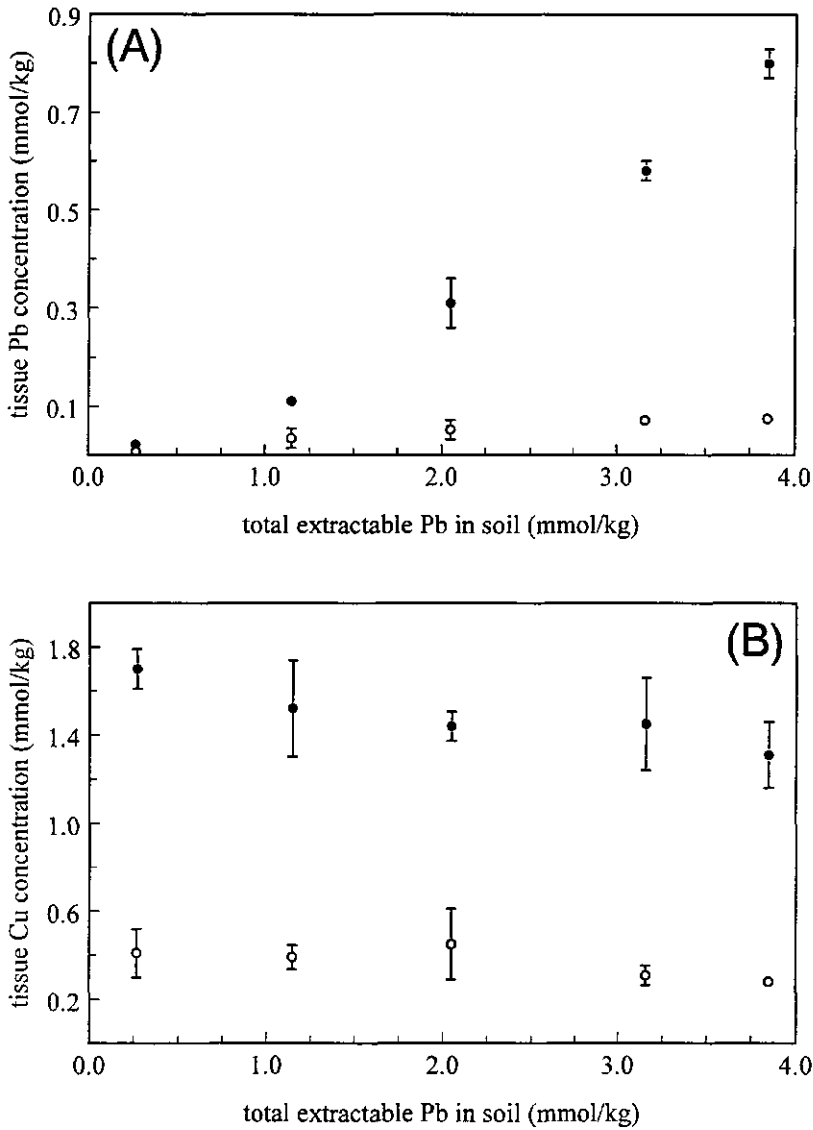
Figure 5.3 _Tissue Pb (A) or Cu (B) concentrations in earthworms Dendrobaena veneta after exposure to soil containing a mixture of Cu and Pb contamination; open circles=tissue heavy metal concentration 3 days after introduction, solid circles=tissue heavy metal concentration 21 days after introduction._

TABLE 5.2 *Ratio Cu [mmol kg^{-1}] to Cd [mmol kg^{-1}] or Cu to Pb [mmol kg^{-1}] in soil and earthworms Dendrobaena veneta. When marked with an asterisk, the ratio in earthworms at day 21 was significantly different from the ratio at day 3 (Student's t-test, P<0.05). tr.code=treatment code, see also Table 5.1.*

		$Cu_W:Cd_W$ *after exposure for*		
	$Cu_T:Cd_T$	**3 days**	*or*	**21 days**
Cd-0	683±375	10.1±3.6		34.0±4.7*
Cd-1	139±8	7.4±3.2		14.2±1.1*
Cd-2	73.5±2.4	6.8±3.1		9.2±0.5
Cd-3	37.3±1.2	5.2±1.0		4.9±0.6

		$Cu_W:Pb_W$ *after exposure for*		
	$Cu_T:Pb_T$	**3 days**	*or*	**21 days**
Pb-0	15.6±0.6	84.2±70.3		81.4±24.4
Pb-1	3.6±0.07	14.2±6.3		13.7±1.8
Pb-2	2.0±0.03	9.0±1.4		4.8±1.0*
Pb-3	1.4±0.03	4.5±1.1		2.5±0.5*
Pb-4	1.1±0.02	3.8±0.5		1.6±0.2*

in the same order of magnitude (Table 5.2). The considerable tissue Pb accumulation, however, did not lead to a proportional decrease in Cu accumulation, which suggests that the storage mechanisms of these metals do not compete with each other.

Neither the ratio $Cu_W:Cd_W$ nor the ratio $Cu_W:Pb_W$ reflect the ratio Cu_T to Cd_T or Pb_T respectively (Table 5.2), although for Pb the differences were smaller than for Cd. In the Cd-soils, the ratios $Cu_W:Cd_W$ were very much smaller than the ratios $Cu_T:Cd_T$, indicating that Cd was accumulated to a relatively higher extent than Cu. Despite a significant tissue Cu and Cd accumulation between days 3 and 21 in the Cd-soils, the ratio $Cu_W:Cd_W$ at days 3 and 21 only increased significantly in soils Cd-0 and Cd-1, suggesting that in these soils Cu was accumulated in a higher rate than Cd, whereas in the two other Cd-soils, Cu and Cd were

accumulated in about the same rates. The initial ratio $Cu_w:Cd_w$ was 8.0 ± 4.1. In the Pb-soils, the ratios $Cu_w:Pb_w$ decreased significantly in soils Pb-2, Pb-3 and Pb-4 between days 3 and 21 (Table 5.2), indicating that in this period in these soils, Pb was accumulated at a higher rate than Cu. The initial ratio $Cu_w:Pb_w$ was 55.5 ± 28.4, and it is obvious that the ratio tended to decrease rapidly after the earthworms were exposed to the Pb-soils.

Some earthworms were found dead at the soil surface of all treatments after 1, 2 or 3 days exposure (Fig. 5.4). While sampling the earthworms at the third day, we found some dead earthworms _in_ the soils too. Mortality was largest in soils Pb-3 and Pb-4. Despite a significant Cd, Cu and/or Pb accumulation between days 3
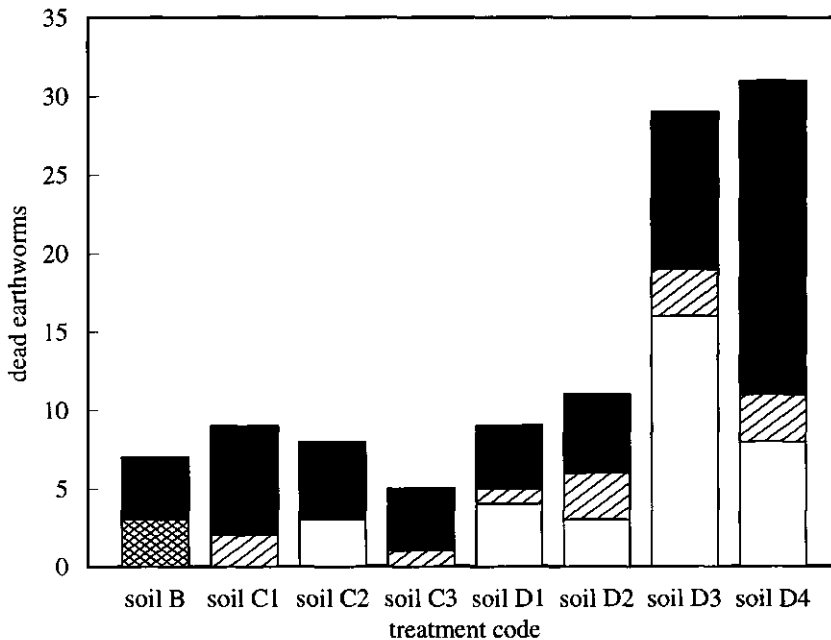

Figure 5.4 _Mortality of earthworms during the first 3 days of the experiments. Earthworms were found at the soil surface at day 1 (OPEN), day 2 (HATCHED), day 3 (CROSS-HATCHED) or in the soil at day 3 (SOLID)._

and 21, we found dead earthworms neither at the soil surface in this period, nor *in* the soils while sampling at day 21. Twelve earthworms (5%) escaped and were found dead on the floor of the phytotron between days 3 and 21. At day 21, it appeared that thirty earthworms (12.5%) were missing. It is unclear whether they died or escaped. More than 95% of the earthworms that survived 3 days exposure in soils Pb-3 and Pb-4 were still alive at day 21. This surprising observation was also done by Spurgeon *et al.* (1994), who observed a large decrease in earthworm mortality (*Eisenia foetida*) after one week in soils containing 1000 mg kg^{-1} Cu or 2000 mg kg^{-1} Zn, whereas large but not complete mortality occurred in these soils in the first week. They suggest that "the main toxic effect of metals was exerted via uptake across the body wall, rather than trough dietary metal assimilation". Data on (sub)lethal effects of Pb to earthworms is limited. Lee (1985) mentioned that very high concentrations of Pb are necessary to affect earthworms growth and reproduction. Bengtsson *et al.* (1986) observed high earthworm mortality (*Dendrobaena rubida*) in soils where Pb concentrations in the cerebral ganglion of surviving earthworms were relatively high (>200 mg kg^{-1}), while Pb concentrations in muscles were low (<50 mg kg^{-1}). Their study indicates that heavy metal tissue concentrations may not correlate to observed adverse effects. Detoxification mechanisms protect earthworms against heavy metal toxicity by reducing the internal bioavailability of heavy metals.

CONCLUSIONS

THIS HAS BEEN the first study in which the effect of Cd or Pb on tissue Cu accumulation in earthworms was investigated. It was hypothesized that exposure to Cd would increase the earthworms ability to bind Cu, which would lead to an increase in tissue Cu accumulation. Lead would affect the Cu availability for uptake by soil chemical interactions. Considerable tissue accumulation of Cd or Pb indicated that these metals were available for uptake by earthworms in the soils. Nevertheless, the earthworm tissue Cu concentration

remained at approximately a constant level in all treatments, which implies that there was not an obvious effect of Cd or Pb addition to Cu contaminated soil on tissue Cu accumulation. Dutch intervention values were our starting-points to establish the Cd or Pb-levels. We recommend further research at higher Cd levels to test the hypothesis that addition of Cd to Cu contaminated soils stimulates tissue Cu accumulation. Since we were confronted with a considerable mortality in soils containing more than 600 mg Pb kg^{-1}, we think their is no point to perform these experiments at higher Pb contamination levels.

ACKNOWLEDGEMENTS

Chapter **6**

Cu accumulation in the earthworm *Dendrobaena veneta* in a heavy metal Cu, Pb, Zn) contaminated site compared to Cu accumulation in laboratory experiments

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Submitted to: *Environmental Pollution*

ABSTRACT

Earthworms (*Dendrobaena veneta*) were exposed to heavy metal contaminated soil from a field site under both laboratory and field conditions. In the laboratory study, earthworms were analysed for Cu after 2 weeks exposure. The tissue Cu concentration (Cu_w) increased proportionally with the total extractable soil Cu concentration (Cu_T) in soils that contained less than 150 mg Cu kg^{-1}. In earthworms exposed to soils containing more than Cu_T=150 mg kg^{-1}, however, we observed no further increase in Cu_w. In a 64 m² field plot at a contaminated site, 81 soil samples were taken and analysed for Cu, Pb, Zn and pH. In June 1994, a thousand earthworms homogeneously distributed in the plot and sampled after 1, 2 and 5 weeks to analyse for Cu. Additionally, two thousand earthworms were introduced in September 1994 and sampled after 1, 2 and 4 weeks. At locations where earthworms were found, the soil Cu concentration was estimated by disjunctive kriging. Cu concentration factors (ratio Cu_w to Cu_T) in the field experiment were in excellent agreement with those of the laboratory experiment. This indicates that with a sufficiently high sampling density, it may be feasible to predict earthworm Cu accumulation in the field using a concentration factor determined in the laboratory.

INTRODUCTION

EARTHWORMS ARE KNOWN to accumulate heavy metals when exposed to heavy metal contaminated soil (e.g. Ash and Lee 1980, Lee 1985). Soil properties affect the heavy metal accumulation where pH, CEC (Cation Exchange Capacity) and OM (Soil Organic Matter content) are the most relevant ones (Ma 1982, Ma *et al.* 1983, Beyer *et al.* 1987). Like other soil invertebrates, earthworms are able to regulate Cu and Zn to a certain extent (Ireland 1979). Earthworms from contaminated sites show a large variation of the values of the concentration factor (CF, ratio tissue heavy metal concentration to soil heavy metal concentration) (Lee 1985, Terhivuo *et al.* 1994). Several suggestions have been forwarded to explain the variation. Eijsackers (1987) mentioned avoidance-behaviour and Marinussen *et al.* (1996a) suggested an effect of soil spatial variability. Specific food choices and earthworm ecology also play a role in the exposure to contaminated soil (Morgan and Morgan 1991, Morgan and Morgan 1993). Marinussen *et al.* (1996a) showed that tissue Cu accumulation in earthworms (*Lumbricus rubellus*) introduced to a Cu contaminated arable field was in good agreement with tissue Cu accumulation in earthworms (*L. rubellus*) exposed to soil from the arable field under laboratory conditions. For the time of their experiments, however, migration of earthworms was small as was also the spatial variability of the contamination. After their study, it remains questionable if an extrapolation from laboratory observations to field situations can be made when exposure levels in the field vary with time and space as is the case in contaminated field sites. Marinussen *et al.* (1996b) showed that tissue Cu accumulation in earthworms (*Dendrobaena veneta*) exposed to Cu contaminated soil under laboratory conditions can be described by a one-compartment model. In their study, a plateau was achieved within 14 days at tissue Cu concentration 60 mg kg^{-1} in earthworms that were exposed in soil containing 815 mg Cu kg^{-1}. They also showed that under laboratory conditions earthworms that were transferred to uncontaminated soil lost approximately 60% of the accumulated Cu within 1 day and 75% of the accumulated Cu within 7 days. With this knowledge on Cu

kinetics, we would suggest that tissue Cu concentrations in earthworms inhabiting a Cu contaminated field site may considerably vary in time and space. The degree of variation will depend on both the degree of spatial variability of soil Cu contamination and the migration pattern of earthworms.

The aim of the present study is (i) to determine Cu concentration factors (CF, ratio tissue Cu concentration to soil Cu concentration) for earthworms *Dendrobaena veneta* exposed to soil sampled from a contaminated site, and (ii) to determine Cu concentration factors for earthworms that were introduced into a heavy metal contaminated field site. We wish to assess whether tissue Cu accumulation in earthworms inhabiting heterogeneously contaminated field sites can be predicted using results from laboratory studies. Since this kind of research has never been reported about before, we wish to decide whether such a prediction is impossible, or whether it is possible providing that the soil sampling density is sufficiently high.

MATERIALS AND METHODS

Laboratory experiment

SANDY LOAM SOIL (clay content is 7%, soil organic matter content (OM) is 3% (loss on ignition)) was collected at a former breaking-up yard in Doetinchem, The Netherlands. At three locations differing with regard to the heavy metal content (Cu, Pb and Zn), amounts of approximately 20 kg soil were sampled. The air-dried soils were sieved separately over a 1 cm sieve to remove large soil particles and mixed thoroughly to achieve homogeneity. Subsamples from the soils were sieved to remove aggregates larger than 2 mm before chemical analyses. To obtain ten different Cu contamination levels, subsamples of the soils were mixed in different ratios. From each mixture, three replicates of 2 kg of air-dried soil were mixed thoroughly with distilled water, yielding a moisture content of 25% by mass. The wetted soil was put into jars and 10 earthworms (*Dendrobaena veneta*) obtained from a local vermiculturist were
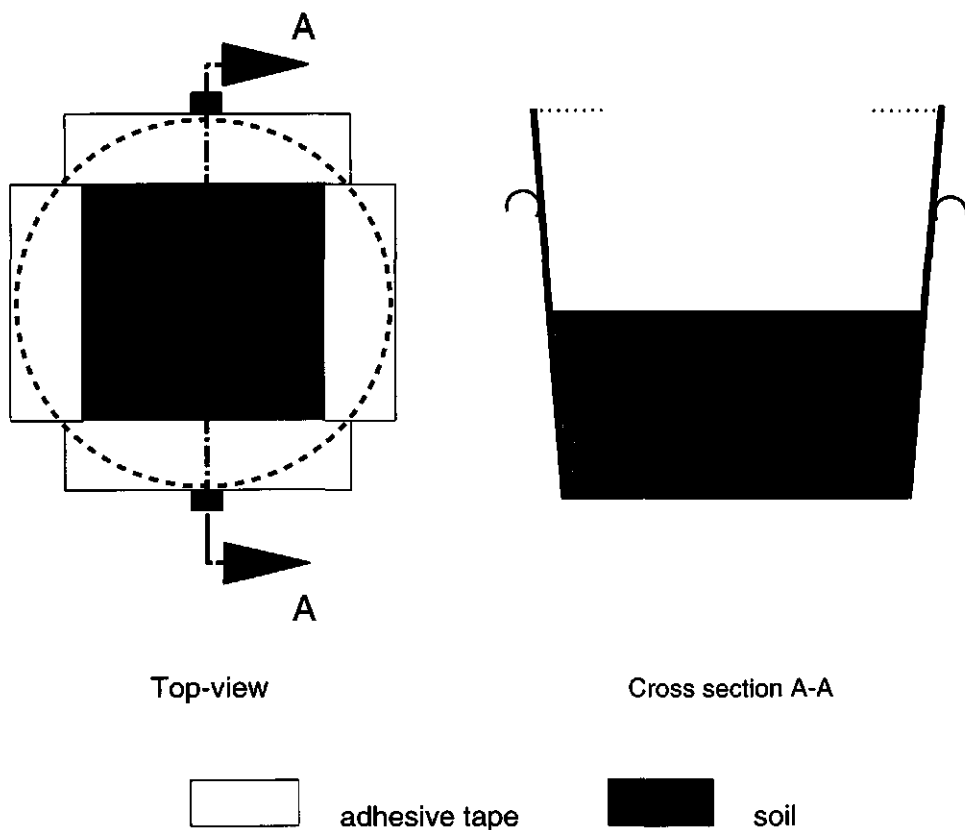
	A		

Top-view   Cross section A-A

☐ adhesive tape   ■ soil

Figure 6.1 *Jars that were used in the laboratory experiment.*

added to the soils. Adhesive tape of 5 cm width was attached to the rim of each jar to prevent earthworms from escaping (Fig. 6.1). The thirty jars were put into a phytotron, adjusted to eighteen hours daylight and six hours darkness, room temperature at 15°C, and relative air humidity of 85%. The jars were weighed every two days and distilled water was added to compensate the evaporation (approximately 0.1 L day^{-1}). The initial tissue Cu concentration (Cu_w) was determined by chemical analysis of 10 earthworms. After 14 days, five earthworms were sampled, rinsed with distilled water and kept for 3 days in petri dishes on moist filter papers to empty their gut. The filter papers were changed every day to reduce coprophagy. Earthworms were lyophilised and individually

analysed for Cu by flame AAS (Instrumental Laboratory AA/AE spectrophotometer S11). The determination limit of tissue Cu concentration was 1.4 mg Cu kg^{-1}. To check the accuracy of the chemical analyses a reference earthworm sample was included in the analyses. The reference sample was obtained by grinding 1 kg of oven-dried earthworms obtained from a vermiculturist. The reference sample was calibrated by analysing for Cu both this reference sample and BCR certified reference material (CRM 186, European Commission 1994). After earthworms were sampled, soils were sampled to determine the total extractable Cu (Cu_T), Pb (Pb_T) and Zn (Zn_T) contents by equilibration of three grams of air dried soil with 30 ml 0.43 M HNO_3 solution for 20 h in an end-over-end shaker (Houba *et al.* 1989). Soil pH was determined with a glass-calomel electrode in a suspension consisting of three grams soil with 30 ml 0.01 M $CaCl_2$ solution equilibrated for 20 h in an end-over-end shaker (Houba *et al.* 1989). To check the accuracy of the chemical analyses, also a reference soil sample was analysed.

Field experiment

Soil samples were taken from the top layer (0-20 cm) at a contaminated plot in the site that was mentioned before. The soil sampling scheme was a squared 8x8 grid with 1 m node distance, resulting in 81 soil samples at 64 m^2. It appeared afterwards that some earthworms that were introduced in the plot had accumulated more Cu than we expected from the soil sample analyses. Therefore, we additionally sampled 4 consecutive layers of 5 cm thickness (0-5 cm, 5-10 cm, 10-15 cm, 15-20 cm) at 9 locations (Fig. 6.3*A*) hypothesizing that the degree of contamination was decreasing by depth. Soil samples were analysed for total extractable heavy metal contents (Cu_T, Pb_T, and Zn_T) by equilibration of three grams of air dried soil with 30 ml 0.43 M HNO_3 solution for 20 h in an end-over-end shaker. Copper extraction with 0.01 M $CaCl_2$ is useful for the estimation of Cu toxicity for earthworms (*Lumbricus rubellus*; Marinussen *et al.* 1996a). Therefore, three grams air dried soil were equilibrated with 30 ml 0.01 M $CaCl_2$ solution for 20 h in and end-over-end shaker (Houba *et al.* 1989) and the Cu concentration

(Cu_S) in the supernatant was measured after centrifugation of the suspension at 2,000 g for 15 minutes. The fraction of Cu_T that is measured as Cu_S is pH dependent (Temminghoff *et al.* 1994). Before centrifugation, soil pH was determined in the suspension. The presence of earthworms is related to soil pH (Satchell 1955) as well as the earthworm Cu toxicity in sandy soils is affected by soil pH (Ma 1984, Marinussen *et al.* 1996a) even though soil pH does not seem to affect tissue Cu accumulation directly (Ma *et al.* 1983, Marinussen *et al.* 1996a).

In June 1994, a thousand earthworms obtained from a local vermiculturist were introduced to the field plot. We choose the species *D. veneta*, after convincing ourselves that this species was absent at the field-site. At each of four homogeneously distributed locations, 300 (at the contaminated part of the plot: Figs 6.3A-6.3C, bottom-right and top-left) or 200 earthworms were introduced. Earthworms were sampled after 1, 2 and 5 weeks by trembling the soil using a garden fork. This sampling method was preferred to adding toxic chemicals like formalin (Raw 1959, Ash and Lee 1980) which may cause adverse effects like avoidance of sampled locations, decreased earthworm activity or earthworm mortality. Each time, we aimed to sample introduced earthworms at about 30 different positions. However, we were not able to find earthworms at so many locations. This may be due to the warm weather (mean soil temperature 21°C), and the little amount of rain fall (33 mm) in this period, which resulted in low soil moisture in the upper layer. It is very likely that this caused earthworms to migrate downwards which made them inaccessible for our sampling method. Because we were not able to find earthworms at many different locations, we performed the experiment once more in September 1994. We changed the spatial distribution of the earthworms: about 100 earthworms were introduced at each of 20 homogeneously distributed locations. We aimed to sample earthworms after 1, 2, or 4 weeks. We expected that earthworms would migrate in the field-site, which would enable us to collect earthworms at several differently contaminated locations. Sampled earthworms were rinsed with distilled water, kept for 3 days in petri-dishes on moist filter papers and individually analysed for Cu by flame AAS. At each location where earthworms were sampled, the soil Cu concentration

was estimated by disjunctive kriging (Yates _et al._ 1986, Webster and Oliver 1989) using the software GEOPACK (Yates and Yates 1990). Disjunctive kriging is an interpolation procedure which takes spatial variability into account when estimating spatially correlated soil properties at unsampled locations.

RESULTS AND DISCUSSION

SOIL COPPER CONCENTRATIONS in soils used in the laboratory experiment varied between 12 and 350 mg kg^{-1} (Table 6.1) and soil pH was 7.0 in all treatments. The initial tissue Cu concentration in the group of earthworms from the laboratory study was 12.4±1.1 mg kg^{-1}. Under laboratory conditions, the tissue Cu concentration after 2 weeks exposure was linearly related with Cu_T until Cu_T exceeded 150 mg kg^{-1}. A further increase in Cu_T did not lead to a significant increase in Cu_W (Fig. 6.2). The data of Marinussen _et al._ (1996b) appear to be in good agreement with the data of this study, as is shown in Fig. 6.2. In their accumulation experiments under laboratory conditions, a plateau at 55 mg Cu kg^{-1} was achieved 2 weeks after introduction of the earthworms.

Figures 6.3A and 6.4 show a considerable spatial variability of the Cu contamination in the field plot, and only part of the soil samples was considerably contaminated with Cu (mean: 0.19 g kg^{-1}, range: 0.008-2.2 g kg^{-1}), Pb (mean: 0.16 g kg^{-1}, range: 0.09-1.8 g kg^{-1}), and Zn (mean: 0.074 g kg^{-1}, range: 0.008-0.43 g kg^{-1}) (Figs 6.3A-6.3C). Soil in the upmost 5 cm layer of plot A (0-5 cm) contained significantly more Cu than soil in the other layers at all except three (II, III and VII, Fig. 6.4) locations. Soil Cu concentrations generally decreased with increasing depth. Soil samples containing a large amount total extractable Cu were also high in Cu_S as can be seen by comparison of Figures 6.3A and 6.3D. Soil samples outside the heavily contaminated area of the plot were low in Cu_S. Soil pH varied between 5.1 and 7.7 (mean: 6.6), which is generally not too low for earthworms (Satchell 1955). The earthworm _D. veneta_ is a poorly known species that may be acid tolerant or not. But even acid intolerant species have been found in soils with

TABLE 6.1 *Soil heavy metal contents and tissue Cu concentrations in earthworms Dendrobaena veneta 14 days exposed to contaminated soils under laboratory conditions; s.d.=standard deviation.*

TREATMENT	soil Cu-HNO$_3$±s.d. [mg/kg]	soil Pb-HNO$_3$±s.d. [mg/kg]	soil Zn-HNO$_3$±s.d. [mg/kg]	earthworm CuW±s.d. [mg/kg]
WK[A]	13	17	10	12±1
A	9±1	19±1	9±1	14±2
B	25±4	27±2	13±1	19±4
C	68±9	49±5	20±2	25±4
D	84±11	57±6	28±6	28±7
E	94±10	62±5	31±2	39±13
F	73±1	51±1	40±4	44±6
G	102±19	66±9	46±1	47±13
H	227±36	128±18	86±11	54±14
I	223±16	126±8	69±4	58±12
J	310±39	170±19	114±33	52±17

[A] Before exposure to contaminated soil, earthworms were kept for 1 week in soil WK.

pH 4.7 (Satchell 1955), so that the soil pH as such will not be a reason to earthworms to abandon the plot.

In the heavily contaminated part the plot (bottom right, Figs. 6.3A-6.3C), we only found a few earthworms, whereas outside this area many earthworms emerged when we trembled the soil which suggests that the earthworms preferred to inhabit the less polluted area and avoided to inhabit significantly heavy metal contaminated soil. Avoidance behaviour was already observed in laboratory experiments (Eijsackers 1987), but has never been shown in field experiments. From our experiments, it can not be concluded whether Cu_T or Cu_S motivated earthworms to leave the contaminated area, since both Cu_T and Cu_S are high in the specific area. We did not found dead earthworms, which suggests that the earthworms were able to leave the area before accumulating a lethal amount of Cu.
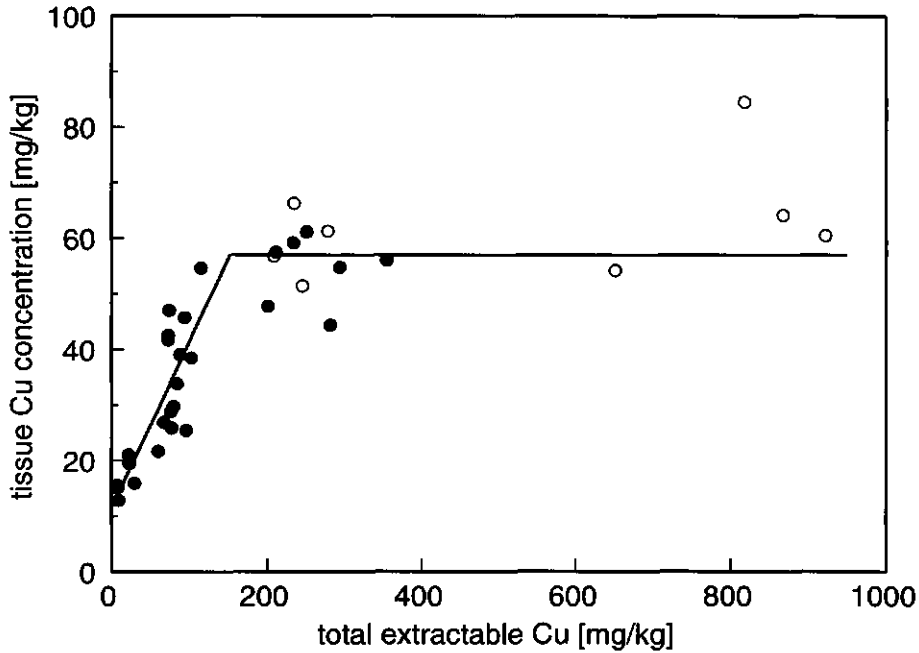
Figure 6.2 _Tissue Cu concentration after 14 days exposure under laboratory conditions to Cu contaminated soil; solid circles=data obtained by our study, open circles=data obtained by laboratory study by Marinussen et al. (1996b)._

In the experiment that was started in June, we also sampled some native earthworms (not identified), that were treated as the sampled _D. veneta_ and consequently analysed for Cu. Also in September and October, a few native earthworms emerged, but these were neither sampled nor analysed. Tissue Cu concentrations in the earthworms sampled from the plot are summarized in Table 6.2. Among the earthworms that were sampled after introduction in June, there were only 3 (8%) _D. veneta_ and 13 (35%) native earthworms in which Cu_w considerably exceeded 60 mg kg^{-1} (Fig. 6.5, A and B). Among the _D. veneta_ that were sampled after introduction in September, there were only 13 (16%) _D. veneta_ in which Cu_w exceeded 60 mg kg^{-1} (Fig. 6.5C). The large Cu_w-values of the few worms are obviously in contrast with the results of the laboratory experiment
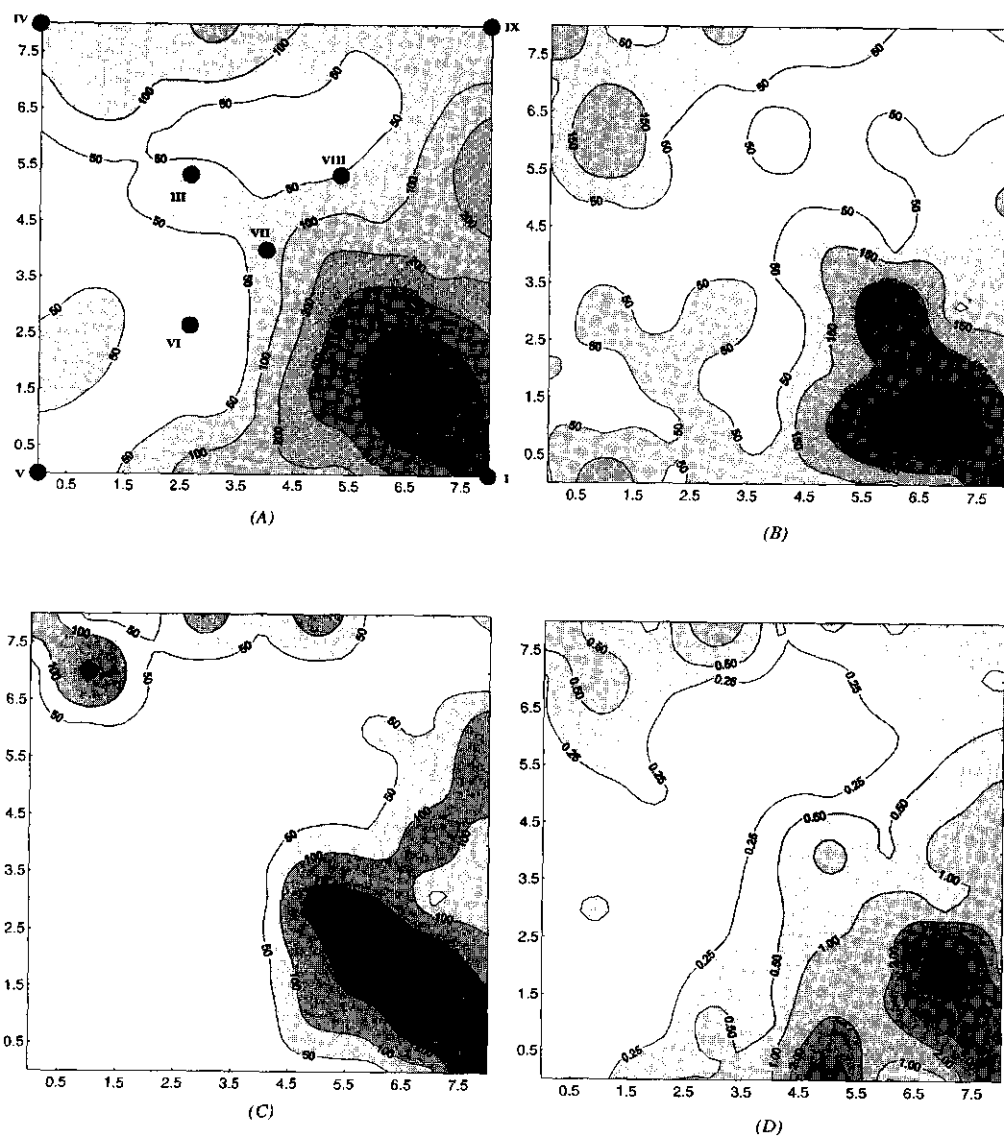
83

Figure 6.3 *Heavy metal contamination of the top-layer (0-20 cm) in the field plot; (A) contourgraph total extractable Cu in soil [mg kg^{-1}], (B) contourgraph total extractable Pb in soil [mg kg^{-1}], (C) contourgraph total extractable Zn in soil [mg kg^{-1}], (D) 0.01 M CaCl$_2$ extractable Cu in soil [mg kg^{-1}]. The number at the bottom and left axis are distances [m]. In (A), the solid circles indicate the locations at which 4 consecutive layers were sampled; see text for details.*
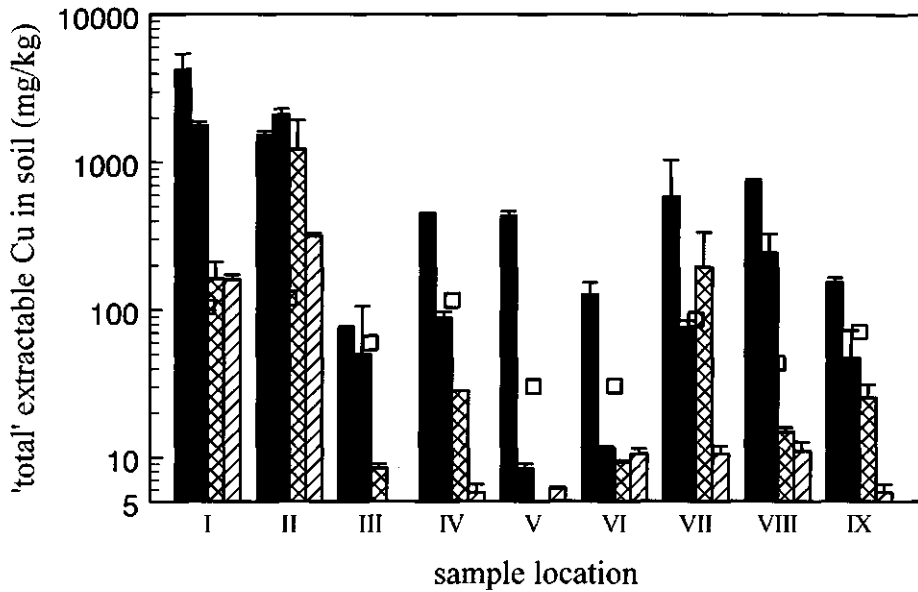
Figure 6.4 _Total extractable Cu gradient at 9 locations in the field plot; solid=0-5 cm, grey=5-10 cm, cross-hatched=10-15 cm, hatched=15-20 cm, open square=estimated Cu_T._

where earthworms seemed to achieve a maximum level at 55 mg kg^{-1}. It may be worthwhile to mention large spatial variability of accumulated Cu. For example, two earthworms found at almost the same position contained 57 and 142 mg Cu kg^{-1} respectively. Such a big difference in tissue Cu concentration in earthworms that were found in the close environment of each other suggests that the heterogeneity of the soil contamination was very large.

Temperature and rain fall in June/July (mean 21°C, total rain fall 33 mm) differed considerably from September/October (mean 13°C, total rain fall 142 mm). These factors, however, do not seem to influence tissue Cu accumulation, since histograms of Cu_W in earthworms introduced in June and histograms of Cu_W in

TABLE 6.2 *Tissue Cu concentrations in earthworms sampled from a field plot in a heavy metal (Cu, Pb, Zn) contaminated site. In June 1994, a thousand earthworms Dendrobaena veneta were introduced and in September 1994, another two thousand earthworms Dendrobaena veneta were introduced.*

date	exp. time [weeks]	species	number	Cu_w min [mg kg^{-1}]	Cu_w max [mg kg^{-1}]	Cu_w mean [mg kg^{-1}]	Cu_w stds[B] [mg kg^{-1}]
20 June	1	*D. veneta*	11	18	291	69	75
27 June	2	*D. veneta*	12	23	73	36	14
19 July	5	*D. veneta*	14	21	45	33	6
20 June	?	native[A]	15	33	114	69	27
27 June	?	native	15	34	163	65	30
19 July	?	native	7	21	42	29	7
19 Sept.	1	*D. veneta*	29	17	108	41	16
26 Sept.	2	*D. veneta*	28	29	93	48	16
10 Oct.	4	*D. veneta*	24	27	142	56	23

[A] not identified native species

[B] sample standard deviation

earthworms introduced in September are quite similar (Fig. 6.5, *A* and *C*). Also the histogram of Cu_w in the 37 native earthworms is quite similar to both former histograms (Fig. 6.5*B*), which suggests that Cu availability was the same for the different species. Generally, tissue Cu accumulation in the earthworm population exposed in the contaminated was in agreement with laboratory observations, with the exception of some discrepancies with the apparent maximum of 55 mg kg^{-1}. In the field experiments, earthworms (*D. veneta* or unidentified native earthworms) were found at 119 different locations. At each location, at most two earthworms were sampled and analysed for Cu. Total extractable soil Cu concentrations at these locations were estimated by disjunctive kriging and were in the range from 25 to 1450 mg Cu kg^{-1}, but most earthworms were found at locations where soil contained less than 60 mg Cu kg^{-1} (Fig. 6.6). Cu concentration factors (CF, ratio Cu_w to Cu_T) tended to decrease when Cu_T increased, which was also mentioned by Hopkin (1989). Concentration factors from the field study are generally in excellent agreement with CFs from our laboratory study and also
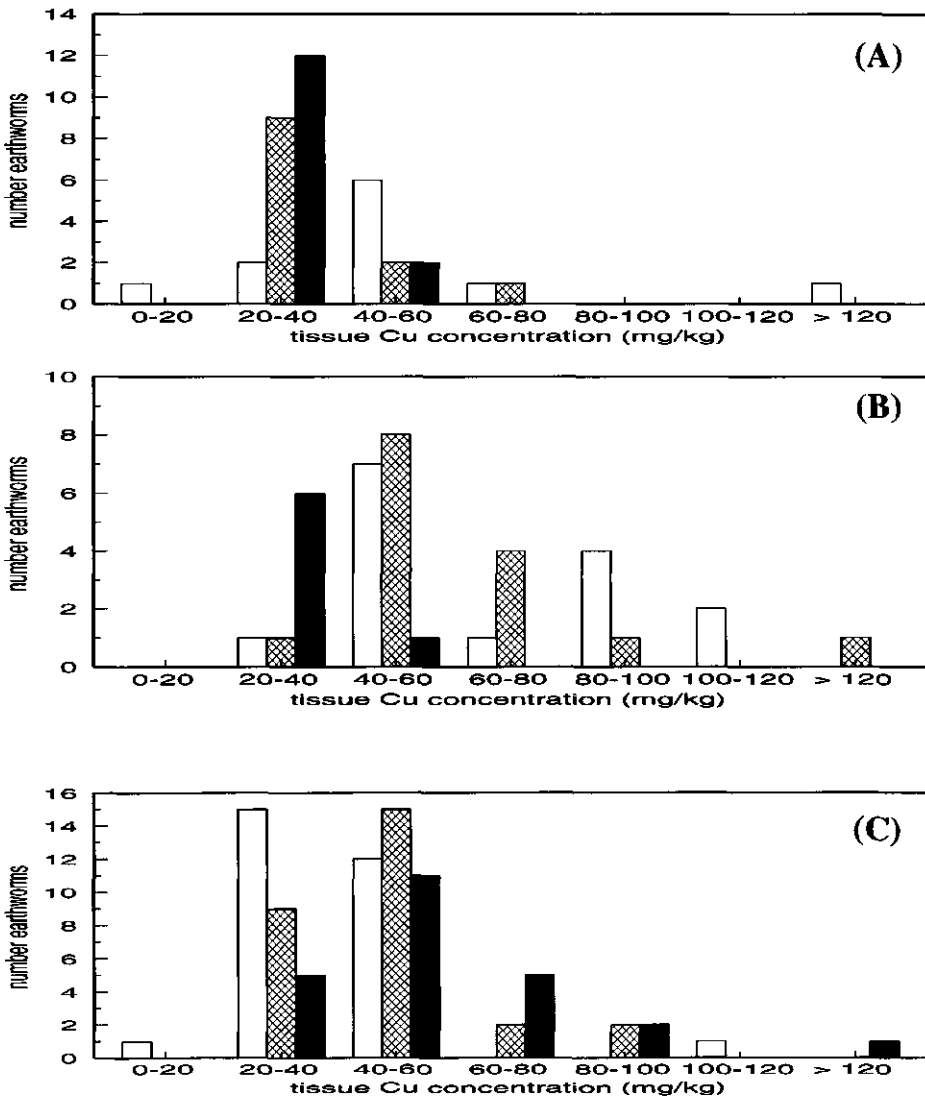
Figure 6.5 *Tissue Cu concentrations in earthworms collected from the field plot; (A) Dendrobaena veneta introduced in June 1994, (B) native earthworms sampled in June or July 1994, (C) Dendrobaena veneta introduced in September 1994; empty=after 1 week; narrow cross-hatched=after 2 weeks; solid (in (A) and (B))=after 5 weeks; solid (in (C))=after 4 weeks.*
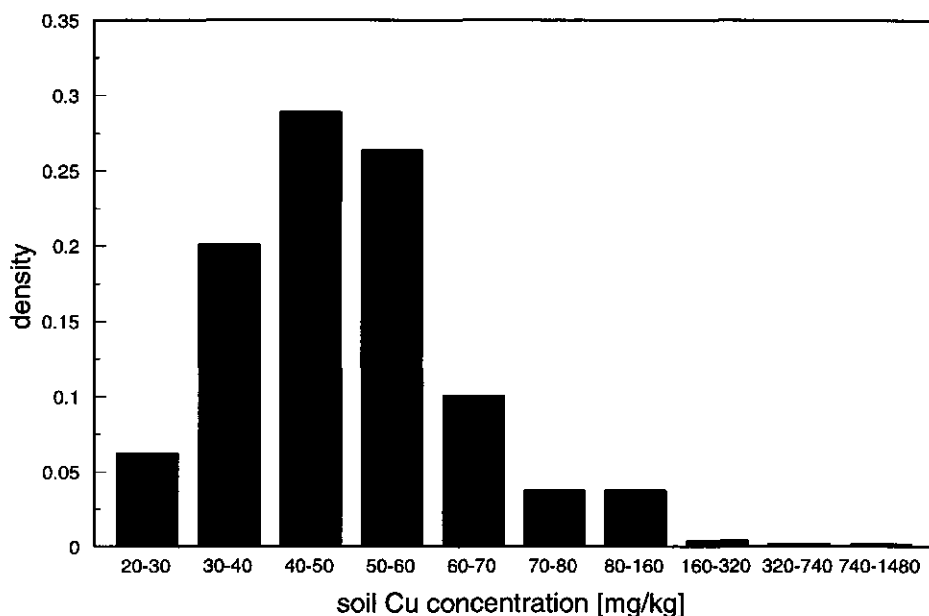
Figure 6.6 *Total extractable soil Cu concentrations in the top-layer (0-20 cm) at positions where earthworms were found in the contaminated site.*

with CFs from laboratory studies by Marinussen *et al.* 1996b (Fig. 6.7). At low Cu_T (10 or 30 mg kg^{-1}), however, earthworms seemed to accumulate less Cu under laboratory conditions than under field conditions. At any Cu_T level, we found CF values in a wide range, the highest one sometimes being 2 or 3 times larger than the smallest one. This may be due to an overestimation of Cu_T at some locations, indicating that the spatial variability of the contamination is large. Soil from the upper layer (0-5 cm) contained significantly more Cu at 6 out of 9 sampled locations (Fig. 6.4). Hence, soil Cu concentrations in the samples that were taken over the entire top layer may give a misleading impression of exposure of *D. veneta* which feeds on raw humus (Piearce 1972) and therefore is likely inhabiting the upper 10 cm layer of soil. This suggestion is supported in Figure 6.4, where we show that in general, the estimated Cu_T is smaller than Cu_T in the upmost 5 cm layer.
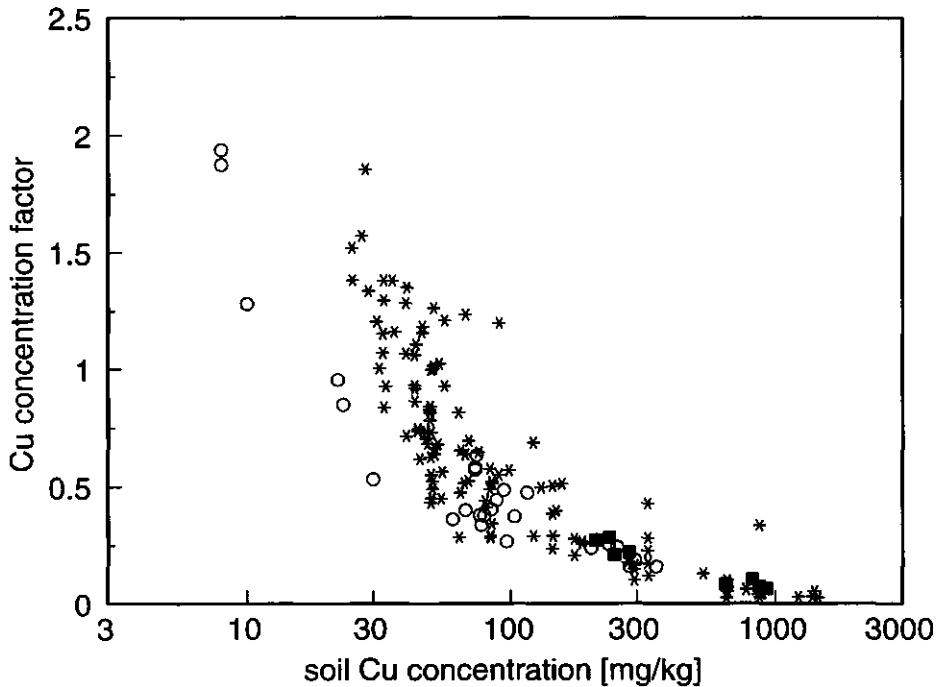
Figure 6.7 _Concentration factors (CF: ratio tissue Cu concentration to total extractable soil Cu concentration) in earthworms exposed under field conditions and in earthworms exposed under laboratory conditions; open circles=present laboratory study, solid circles=laboratory study by Marinussen et al. 1996b, asterisks=present field study._

CONCLUSIONS

THE STUDY CONFIRMED earlier works by Marinussen _et al._ (1996 b,c) that Cu accumulates in _D. veneta_. Under laboratory conditions Cu accumulation appears to stagnate at Cu_w 55 mg kg^{-1}. However, for a few of the sampled earthworms in the field study, this apparent maximum was exceeded. The soil sampling density in the field study was extremely high (1.2 sample m^{-1}), which enabled us to estimate accurately the value of Cu_T at positions

where earthworms were sampled. Cu concentration factors (ratio Cu_W to Cu_T) in the field experiment were in excellent agreement with those of the laboratory experiment indicating that the sampling density was sufficiently high to estimate exposure of individual earthworms despite the large spatial variability of soil Cu concentration. This result suggests that it is in principle feasible to predict the heavy metal exposure of earthworms in a field site using heavy metal contents in soil and relationships between tissue heavy metal concentration and soil heavy metal concentration that have been determined in a laboratory study.

ACKNOWLEDGEMENTS

Chapter 7

Earthworm tissue Cu accumulation and mortality in contaminated field sites: computer simulations

I N THE RESEARCH described in the earlier chapters, earthworms were exposed in soil contaminated with heavy metals under both laboratory and field conditions. Experiments under various laboratory conditions showed that earthworm tissue Cu accumulation is associated to the total extractable soil Cu concentration, whereas neither soil pH nor soil moisture, and neither soil Cd nor soil Pb concentrations affected tissue Cu accumulation. Mortality of earthworms in Cu contaminated soil was related to the 0.01 M $CaCl_2$ extractable soil Cu concentration.

This Chapter intends to illustrate effects of soil spatial variability on tissue Cu accumulation and mortality of earthworms by computer simulations. Values of model parameters were derived from observations in the experiments described in this thesis. Since observations in field experiments were in excellent agreement with observations in laboratory studies (Chapters 3 and 6), it is legitimate to use model parameters that were determined under laboratory conditions in the computer simulations.

SIMULATION MODEL

T HE COMPUTER MODEL described in Chapter 2 was adapted to the objectives of this study.

- In Chapter 2, living areas of different size were moved through a Cd contaminated field. In this Chapter, each computation concerned only one

earthworm living area of 10 m by 10 m (this was 3 m by 3 m), because I intend to illustrate the effects of spatial variability at the habitat scale of earthworms.

- To simulate migration, the area was divided into cells of 0.33 m by 0.33 m resulting in 961 cells. Earthworms moved every day at random from one cell to an adjacent cell, where two constraints were taken into account: (i) earthworms were not allowed to leave the area; and (ii) earthworms were enabled to try to avoid locations where the Cu contamination exceeds a specific threshold level (see below). If an individual had moved into a cell where soil Cu contamination exceeds the threshold level, it tried to find an adjacent cell where the Cu contamination is smaller than the threshold level. If this avoidance behaviour failed, however, the individual died.
- In the computer simulations of this Chapter, 20 individuals were introduced at each of 36 homogeneously distributed locations instead of 250 individuals in the middle of a living area.

MODEL PARAMETERS

migration-rate of earthworms

THE MIGRATION-RATE of earthworms was set equal to 0.3 m day^{-1}. To illustrate the migration pattern in the computer-model, 5000 earthworms were introduced in the middle of an unpolluted 33 m by 33 m area. After 365 days (one year!) the relative population density in each cell was calculated according to:

$$pd(x,y) = \frac{nw(x,y)}{5000} 100\% \qquad (7.1)$$

where $pd(x,y)$=population density in the cell with coordinates x and y, $nw(x,y)$=number of earthworms in this cell. It is known that the average dispersion-rate of an earthworm population is about 10-15 m year^{-1} (Ma, personal
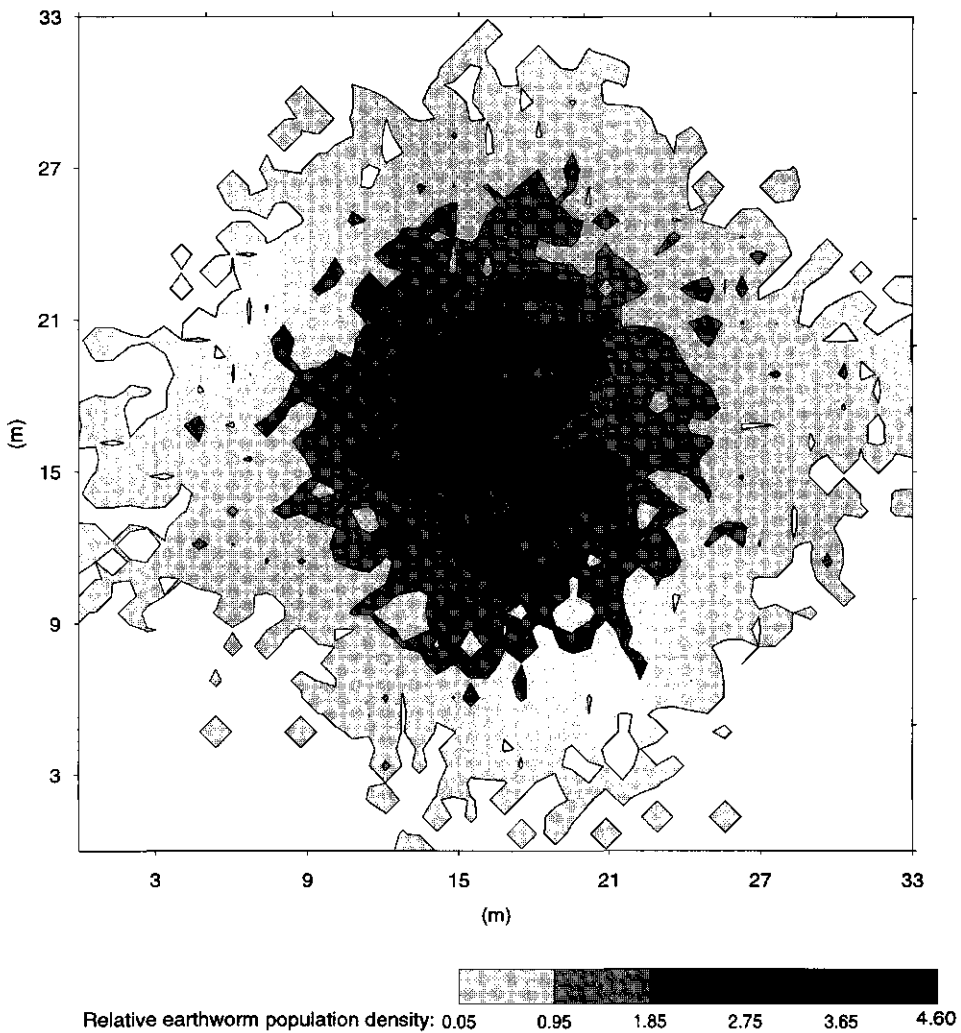
Figure 7.1 *Computer simulation of earthworm dispersion in uncontaminated soil: contourgraphs of the relative earthworm population density 365 days after introduction of five thousand earthworms in the middle of the 33 m by 33 m area.*

communication). As is illustrated in Figure 7.1, the earthworm migration in the computer-model results in a population dispersion rate which is in agreement with this common knowledge.

avoidance and mortality

Soil pH does not affect tissue Cu accumulation, but considerably affects mortality of earthworms in Cu contaminated soils (Chapter 3). The increased mortality with decreasing soil pH suggests that mortality is associated with Cu concentrations in the soil solution. In laboratory studies that have not been described in this thesis, earthworms (*Dendrobaena veneta*) were exposed in various soil samples from a heavy metal contaminated site. In these soils, the 0.01 M $CaCl_2$ extractable Cu (Cu_s) was determined. Cu_s is well correlated with the Cu availability for plant uptake (Novozamsky *et al.* 1993) and therefore is an indicator for the amount of Cu in soil solutions. Soil samples were put in jars and earthworms were put on the soil surface (method described in Chapter 6). Generally, they moved immediately into the soil to avoid exposure to day-light. However, in soils where Cu_s exceeded 0.3 mg L^{-1}, however, earthworms remained at the soil surface for a long time despite exposure to day-light. Finally, some died at the soil surface of these soils, whereas all the others were found dead in these soils 3 days after introduction.

In the computer simulations, this avoidance behaviour and mortality is simulated by giving earthworms the opportunity to escape from locations where Cu_s exceeds 0.3 mg L^{-1} to any adjacent cell. Earthworms died after being exposed for three consecutive days to Cu contamination that exceeds this threshold level.

tissue Cu accumulation

Tissue Cu accumulation can be described by a one-compartment model (Chapter 4):

$$Cu_w(t) = Cu_w(0) + \frac{\alpha_{Cu}}{k_{e,Cu}}(1 - \exp(-k_{e,Cu}t)) \tag{7.2}$$

where $Cu_w(t)$=earthworm tissue Cu concentration after t days exposure [mg kg^{-1}], α_{Cu}=Cu uptake-rate [mg Cu kg^{-1} day^{-1}], and $k_{e,Cu}$=Cu excretion-rate parameter [day^{-1}]. In Chapter 4, the uptake- and excretion-rate were determined: α_{Cu}=17.9±2.4 mg Cu kg^{-1} day^{-1}, and $k_{e,Cu}$=0.33±0.05 day^{-1}. The uptake-rate is associated with the Cu availability for uptake by earthworms. In Chapter 6, it was shown that Cu_w increased proportionally with the total extractable soil Cu concentration. Therefore, the Cu uptake-rate can be written as:

$$\alpha_{Cu} = k_{a,Cu}Cu_T \tag{7.3}$$

where $k_{a,Cu}$=uptake-rate parameter [day^{-1}], and Cu_T=total extractable soil Cu concentration [mg kg^{-1}]. Assuming that the excretion-rate parameter is a constant, the uptake-rate parameter can be calculated from tissue Cu concentrations that are at equilibrium with soil Cu concentration (t is relatively large) according to:

$$\frac{k_{a,Cu}}{k_{e,Cu}} = \frac{Cu_w(t) - Cu_w(0)}{Cu_T} \tag{7.4}$$

Earthworm tissue Cu concentration at equilibrium with soil Cu concentration is:

$$Cu_w = Cu_w(0) + \frac{k_{a,Cu}}{k_{e,Cu}}Cu_T \tag{7.5}$$

In soil MH (Chapter 4), $k_{a,Cu}$ was approximately 0.02 day^{-1}, whereas in soil LB $k_{a,Cu}$ was approximately 0.07 day^{-1}. In both artificially contaminated soils (Chapter 5) and in soils of the laboratory experiments described in Chapter 6, $k_{a,Cu}$ was approximately 0.1 day^{-1}. Therefore, $k_{a,Cu}$ was set equal to 0.1 day^{-1}. The Cu excretion-rate parameter in earthworms exposed in contaminated soils was set equal to 0.33 day^{-1}, which is equal to the excretion-rate parameter in soil MH (Chapter 4).

SPATIALLY VARIABLE SOIL CONTAMINATION

THREE DIFFERENTLY COPPER contaminated random-fields were generated using the computer program GSTAT, developed by Pebesma (1995). The fields differed among others in spatial variability of total Cu concentration (Figure 7.2). In *NEUS* the range of correlation was 15 m, in *PUKKELS* 8 m, and in *SPROETEN* 5 m. In *NEUS*, total soil Cu concentration is high throughout the field. In *PUKKELS*, there is a significant soil Cu contamination at spatially distributed small areas, whereas in *SPROETEN*, there are only a few hot-spots. The soil Cu concentrations in soil were lognormally distributed. The mean soil Cu concentration was highest in *NEUS*, whereas the

TABLE 7.1 *Total soil Cu concentrations and Cu concentrations in soil solutions in three computer generated random fields.*

	NEUS	*PUKKELS*	*SPROETEN*
Cu_T [mg kg^{-1}]			
mean	363	233	147
median	361	215	115
std (CV)	81.2 (22%)	105 (45%)	94.5 (64%)
range	187-615	75-726	37-761
Cu_S [mg kg^{-1}]			
mean	0.43	0.25	0.16
median	0.40	0.211	0.12
std (CV)	0.19 (44%)	0.15 (59%)	0.14 (85%)
range	0.112-1.26	0.068-1.342	0.02-1.23

coefficient of variation (CV) was largest in *SPROETEN* (Table 7.1). The latter implies that the soil heterogeneity in *SPROETEN* is relatively largest. Median values in both *PUKKELS* and *SPROETEN* was considerably smaller than the mean-values, which is typical for lognormally distributed data.

Copper in soil solution was estimated using a Freundlich adsorption equation:

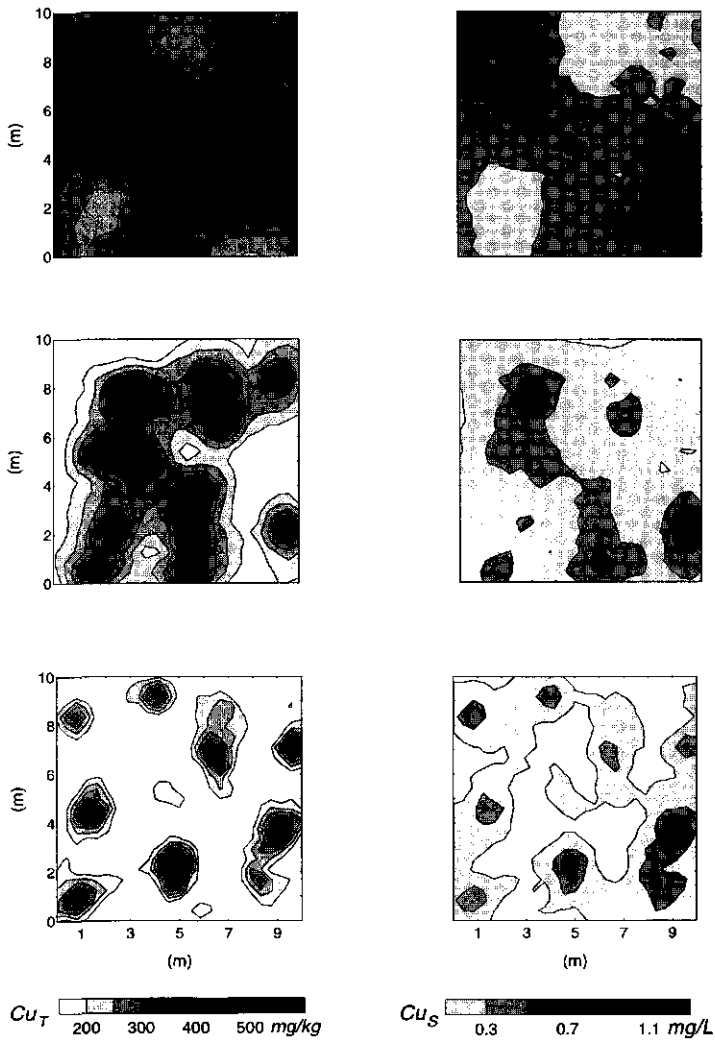**Figure 7.2** *Contourgraphs of total soil Cu concentrations (left hand site) and Cu concentrations in soil solution (right hand site) in three computer generated random fields; from the top to the bottom, field are: NEUS, PUKKELS, and SPROETEN.*

$$q = K_F (H^+)^{-0.5} Cu_S^{\ n} \tag{7.6}$$

where q=adsorbed Cu [mg kg^{-1}], K_F=Freundlich adsorption parameter, Cu_S=Cu concentration in soil solution [mg L^{-1}]. K_F and n were arbitrarily set equal to 2.5 and 0.9 respectively. One random-field of soil pH was used to calculate Cu_S in every field *NEUS*, *PUKKELS* and *SPROETEN* (Figure 7.2). Soil pH varied between 4.3-5.8, and the mean was 5.0. In each field, the coefficient of variation for Cu_S was larger than the coefficient of variation for Cu_T (Table 7.1), which implies that spatial variability of soil pH increased the heterogeneity of soil contamination.

RESULTS OF COMPUTER SIMULATIONS

I N *NEUS*, THE mortality-rate was large from the beginning (76% after 1 week), whereas in both *PUKKELS* and *SPROETEN* mortality started slowly (Table 7.2). As a result of migration, the mortality increased as a function of time. After one year, 100% of the introduced earthworm died for the *NEUS* scenario, whereas in both *PUKKELS* and *SPROETEN* only few earthworms remained alive. As is illustrated in Fig. 7.3, earthworms were mainly found at locations that were low in Cu_S (compare Figs 7.2 and 7.3). The initial differences between mortality in the three fields disappeared in the long run. Even for the *SPROETEN* scenario, where the mean-value of Cu_S was considerably smaller than 0.3 mg L^{-1}, most earthworms died eventually.

Tissue Cu concentrations (Cu_W, Table 7.2) were different in the different fields. The mean tissue Cu concentration in each field and its standard deviation varied only slightly as a function of time. Differences in spatial variability of soil contamination resulted in different variations in tissue Cu accumulation as indicated by the coefficient of variation. In *NEUS*, the coefficient of variation was 12%, in *PUKKELS* 28%, and in *SPROETEN* 30%. These CVs are considerably smaller than the CVs for Cu_T, which implies that earthworms smooth to some extent the spatial variability.

TABLE 7.2 _Tissue Cu accumulation in earthworms and mortality of earthworms introduced at 20 locations in three differently contaminated plots. At each location 36 earthworms were introduced (720 in each plot). Earthworms died after exposure for 3 consecutive days to Cu in soil solution that exceeded 0.3 mg L^{-1}. For more details, see text._

data after _t_ weeks	in _NEUS_	in _PUKKELS_	in _SPROETEN_
Cu_W [mg kg^{-1}] **t=1**			
mean±s.d.	94.8±12.2	72.5±21.6	50.6±15.1
range	76-145	38.5-134	28.4-142
mortality (%)	76	25	13
Cu_W [mg kg^{-1}] **t=2**			
mean±s.d.	103±12.9	77.4±23.0	55.6±17.7
range	82-142	40.7-146	31.5-173
mortality (%)	80	32	19
Cu_W [mg kg^{-1}] **t=6**			
mean±s.d.	105±12.7	77.7±23.3	56.0±16.8
range	85-142	40.8-138	33.2-130.4
mortality (%)	90	53	34
Cu_W [mg kg^{-1}] **t=18**			
mean±s.d.	106±9.7	76.4±20.2	55.8±17.1
range	86-119	40.8-132	32.7-144
mortality (%)	98	82	58
Cu_W [mg kg^{-1}] **t=54**			
mean±s.d.		78.1±19.0	57.7±16.4
range		42.5-116	37.4-119
mortality (%)	100	97	90

Estimations of the mean tissue Cu concentrations can be calculated using Eq. (7.5). The mean exposure was set equal to the median value of Cu_T, because the mean value overestimates the mean exposure, since the Cu_T data were lognormally distributed. The estimated mean tissue concentrations were: 135 mg kg^{-1} (_NEUS_), 87 mg kg^{-1} (_PUKKELS_), and 53 mg kg^{-1} (_SPROETEN_). In both the _NEUS_ scenario and the _PUKKELS_ scenario, the mean earthworm tissue concentrations (Table 7.2)
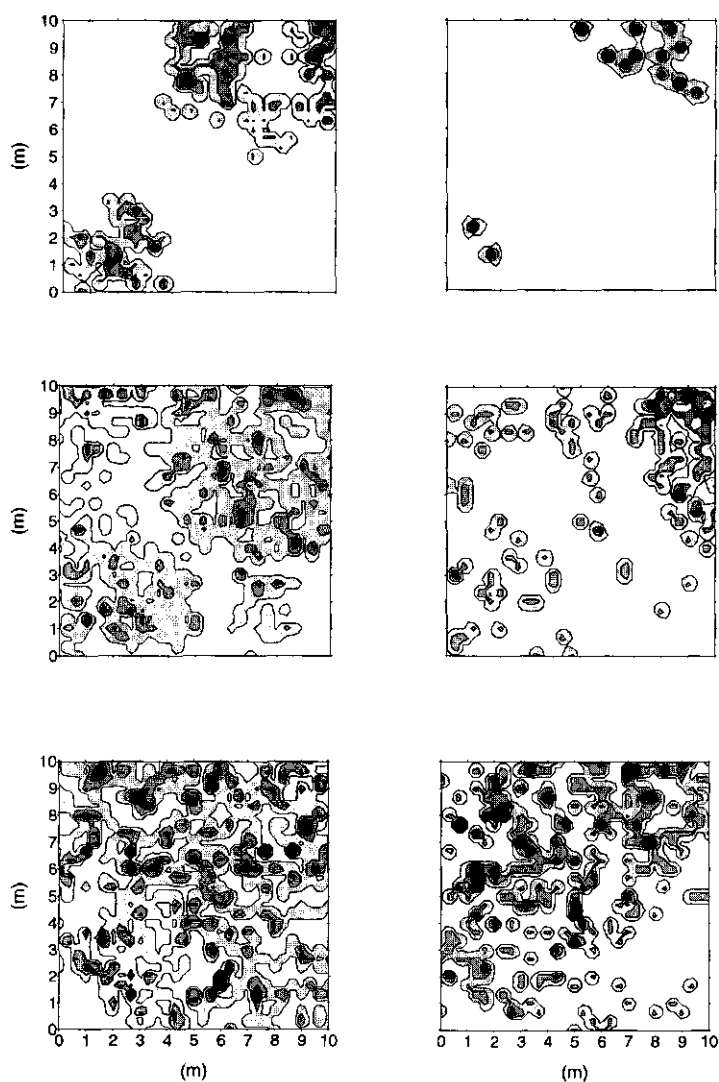
Figure 7.3 *Spatial distribution of earthworms in three soil Cu contaminations that differed in spatial variability; relative earthworm population density 1 week (left hand site) or 18 weeks (right hand site) after introduction; black indicates that there were relatively many earthworms; from the top to the bottom, fields are: NEUS, PUKKELS and SPROETEN.*

were smaller than these estimations, which can be explained by mortality of earthworms that are exposed to high soil Cu concentrations.

From the results of these computer simulations, it can be concluded that due to migration of earthworms even small volumes of contamination in the habitat may lead to adverse effects at the population level. I am aware that the results of the calculations probably change considerably when earthworm reproduction is also accounted for. For example, the earthworm population in the *SPROETEN* scenario might be able to withstand the Cu contamination, and the population density will only decrease a little instead of a full eradication as was suggested by the computer simulations. However, effects of spatial variability of soil contamination on earthworm reproduction are unknown: it is unknown whether or not temporal variation in exposure affects the earthworm reproduction, and it is unknown whether or not earthworms in contaminated fields depose their cocoons in soils that are safe for juveniles to grow up.

CONCLUSIONS and RECOMMENDATIONS for FURTHER RESEARCH

THE COMPUTER SIMULATIONS, that were supported by the successful extrapolation of data obtained by laboratory studies to the field scale, show that even small volumes of hazardous contamination in the habitat of organisms may lead to adverse effects at the population level. The Dutch legislation on soil quality criteria does not account for this effect of spatial variability. The present soil quality criteria may be useful when concerning diffuse soil contamination, but seem to disregard the possible effects of point sources in habitats of soil dwelling organisms. Further research is recommended to integrate the ecology of organisms in the risk assessment of soil contamination for soil-ecosystems. In as far as Dutch Environmental legislation intends to provide a scientific link between 'effect' and proposed or actual standards, it seems that the ecology of organisms should also be taken into account.

Chapter 8

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Samenvatting

Speciaal voor hen die niet "in het vak" zitten, zoals dat zo mooi heet, heb ik achteraan een begrippenlijst toegevoegd waarin ik vaktermen die in deze samenvatting voorkomen uitleg. Deze vaktermen zijn in de samenvatting eenmalig cursief gedrukt. Voor de eenvoud zijn de termen uitgelegd in de geest van dit proefschrift en niet in algemene termen. Ik neem aan dat vakgenoten hiervoor alle begrip zullen hebben.

HET NEDERLANDSE BODEMBESCHERMINGSBELEID is er onder andere op gericht mens en milieu te beschermen tegen negatieve effecten van bodemverontreiniging. Dit beleid heeft reeds geleid tot immense bodemsaneringsoperaties, waarvan "Lekkerkerk" en "de Volgermeerpolder" bij U waarschijnlijk bekend in de oren zullen klinken. Op nog veel meer lokaties in Nederland is de bodem verontreinigd, maar gelukkig niet overal in dezelfde mate van ernst. Van bodemverontreiniging is al sprake indien van één of meer stoffen het *gehalte* in de bodem hoger is dan van nature verwacht mag worden. Bodemverontreiniging leidt niet per definitie tot ongewenste effecten op mensen of milieu. Pas bij overschrijding van de *interventiewaarde* is, volgens de Nederlandse overheid, sprake van een ernstige verontreiniging van bodem of grondwater. Interventiewaarden zijn wettelijk vastgelegde criteria die mede gebaseerd zijn op *ecotoxicologisch* onderzoek in laboratoria. Kleine bodemdieren, zoals regenwormen, springstaarten en pissebedden, worden daarbij aan verontreinigde grond blootgesteld. Binnen een tijdsbestek dat varieert van een paar dagen tot een paar weken, wordt het gehalte verontreiniging in grond vastgesteld waarbij duidelijk negatieve effecten aan het betreffende organisme optreden. Hierbij kijkt men naar groei en ontwikkeling, naar de voortplanting, of naar *mortaliteit*. Deze laboratoriumgegevens worden dus mede gebruikt om interventiewaarden af te leiden.

Laboratoriumproeven echter geven geen reëel beeld van wat er in de praktijk op een verontreinigde locatie zo allemaal kan spelen. De verschillen in omstandigheden, waarvan ik er drie wil noemen, lijken dusdanig cruciaal dat

daardoor het risico van een bodemverontreiniging voor een *bodemecosysteem* niet zonder meer voorspeld kan worden op basis van ecotoxicologische laboratoriumexperimenten. Ten eerste is er in het veld sprake van *ruimtelijke variabiliteit* van bodemverontreiniging. Als gevolg hiervan varieert de blootstelling van een organisme aan verontreiniging als functie van tijd en plaats, terwijl in labororiumexperimenten organismen langdurig aan één gehalte worden blootgesteld. Bovendien wordt slechts een fractie van de populatie blootgesteld aan verontreiniging, waardoor ook slechts een fractie effecten hiervan ondervindt. Ten tweede hebben allerlei chemische eigenschappen van de bodem invloed op de toxiciteit van een verontreiniging. Die chemische eigenschappen verschillen voor iedere grond en veranderen bovendien als functie van tijd en plaats. Eenvoudige correcties zijn vaak onvoldoende om verschillende gronden onderling met elkaar te vergelijken. Ten derde is in het veld meestal sprake van een mengsel van verontreinigende stoffen en niet, zoals in standaard laboratorium testen, van één verontreinigende stof.

IN HET KADER van dit proefschrift heb ik effecten van verschillen tussen laboratorium- en veldomstandigheden onderzocht aan de hand van studies naar *accumulatie* van zware metalen in regenwormen. Resultaten van dit onderzoek staan in de hoofdstukken 3 t/m 6. In hoofdstuk 2 beschrijf ik echter eerst een algemeen onderzoek over effecten van ruimtelijke variabiliteit op blootstelling van organismen. Door middel van computersimulaties laat ik zien, dat als gevolg van ruimtelijke variabiliteit van bodemverontreiniging, binnen een groep individuen die in hetzelfde gebied leven, grote verschillen kunnen optreden in blootstelling. Slechts een bepaald percentage van de individuen bleek zodanig in aanraking met de verontreiniging te komen dat het hiervan nadelige effecten zou kunnen ondervinden. Dit percentage bleek evenredig met de verhouding tussen het oppervlak (volume) van de bodemverontreiniging en het oppervlak (volume) van het gebied waar het (denkbeeldige) organisme leefde.

De proeven met regenwormen (hoofdstukken 3 t/m 6) hadden de volgende resultaten:

(i) onder laboratorium omstandigheden is de accumulatie van koper in regenwormen een snel proces, waarbij binnen drie dagen na de blootstelling een duidelijke verandering in het kopergehalte in wormen meetbaar is (H4). De accumulatie is gerelateerd aan het kopergehalte (H3, H6) in de bodem en wordt niet beïnvloed door de *pH* van de bodem (H3), de bodemvochtigheid (H3), of interacties met cadmium of lood (H5).

(ii) onder laboratorium omstandigheden is de *excretie* van koper en lood in regenwormen een snel proces, waarbij binnen één dag na overplaatsing naar schone grond wormen ca. 60% van de geaccumuleerde hoeveelheid koper of lood uitgescheiden hebben.

(iii) op basis van (i) en (ii) mag verwacht worden dat het kopergehalte in wormen in een verontreinigd gebied zich snel aanpast aan veranderingen in het omgevingsgehalte (door verplaatsing van de wormen). De resultaten van onderzoek naar accumulatie van koper in wormen in een verontreinigd terrein spreken dit zeker niet tegen. De accumulatie bleek over het algemeen in overeenstemming met verwachtingen op basis van de data uit de laboratoriumexperimenten en op basis van gemeten kopergehaltes in grond, ondanks duidelijke ruimtelijke variabiliteit van koperverontreiniging (H3 en H6).

(iv) de pH van de bodem had een duidelijk effect op de mortaliteit van regenwormen; bij gelijkblijvend *totaalgehalte* gingen meer wormen dood bij lage pH dan bij een hoge pH; waarnemingen in het veld zijn volledig in overeenstemming met waarnemingen in de laboratoriumproeven (H3).

(v) accumulatie van cadmium en lood is evenredig met het totaalgehalte in grond (H5).

IN HOOFDSTUK 7 zijn opnieuw een aantal computersimulaties uitgevoerd, gebaseerd op de resultaten van al het voorafgaande experimentele werk. Aan de hand van 3 sterk vereenvoudigde rekenvoorbeelden wordt geïllustreerd dat relatief kleine volumina bodemverontreiniging op de lange termijn kunnen leiden tot het volledig uitsterven van de regenwormenpopulatie. Ik kom tot de conclusie dat waarnemingen in het veld in overeenstemming kunnen zijn met

verwachtingen op basis van laboratoriumexperimenten mits laboratoriumexperimenten model staan voor omstandigheden in veldsituaties. Ik beëindig het proefschrift met een aanbeveling voor verder experimenteel onderzoek. Dit zou gericht moeten zijn op het voorspellen van effecten van kleinschalige verontreiniging in een bodemecosysteem op bodemorganismen, waarbij rekening gehouden wordt met de *ecologie* van deze organismen.

Begrippenlijst

Accumulatie: het metaalgehalte in het weefsel van de regenworm is verhoogd ten gevolge van blootstelling aan verontreinigde grond; accumulatie heeft plaats in regenwormen die meer metalen binnen krijgen dan zij (kunnen) verwijderen; wanneer de hoeveelheid die er per dag in het weefsel bijkomt even groot is als de hoeveelheid die er per dag uit het lichaam verwijderd wordt is het evenwichtsgehalte bereikt.

Bodemecosysteem: de betekenis van ecosysteem in het algemeen is: "het geheel van planten- en dierengemeenschappen in een territorium, beschouwd in hun wisselwerking met de milieufactoren"; met bodemecosysteem wordt dit alles beperkt tot de bodem.

Ecologie van de regenworm: (leer van de) betrekkingen tussen de regenworm en de omgeving waarin hij leeft.

Ecotoxicologie: studie naar de giftigheid van stoffen voor organismen; ecotoxicologie onderscheidt zich van humane toxicologie, waarbij de giftigheid van stoffen voor mensen het onderwerp van studie is.

Excretie: uitscheiding van metalen uit het lichaam van regenwormen; wormen verwijderen het teveel aan metalen dat zij binnen krijgen door excretie; dit is de enige wijze waarop metalen uit het lichaam verwijderd kunnen worden.

Gehalte: hoeveelheid metaal in grond (of in wormen), uitgedrukt in milligram metaal per kilogram grond (of worm).

Interventiewaarde: wettelijk vastgelegd criterium ten aanzien van gehaltes van stoffen in de bodem en het grondwater; wanneer het gehalte van een stof in de bodem of grondwater de interventiewaarde overschrijdt is er, volgens de Nederlandse overheid, sprake van een "ernstige of dreigende vermindering (...) van de functionele eigenschappen die de bodem heeft voor mensen, flora en fauna"; interventiewaarden zijn wetenschappelijk onderbouwd en gebaseerd op humaan- en ecotoxicologisch onderzoek.

Mortaliteit: sterfte; in ecotoxicologisch onderzoek kan de mortaliteit variëren van 0 tot 100%; het percentage geeft aan hoeveel procent van de organismen een bepaalde blootstelling niet heeft overleefd.

pH: zuurgraad; een lage pH komt overeen met 'zuur'; de pH van water is ongeveer 7; de pH van azijn is ongeveer 3,5.

Ruimtelijke variabiliteit: het gehalte van metalen (of de pH, of het kleigehalte, etc.) is op ieder plekje in de bodem binnen het verontreinigd terrein anders;

Totaalgehalte: de totale hoeveelheid metaal in een kilogram grond; metaal in grond wordt ten dele gebonden aan vaste bodembestanddelen en een ander gedeelte is opgelost in het grondwater; het totaalgehalte is de som van het vastgelegde hoeveelheid en de opgeloste hoeveelheid.

Levensloop

Mari (Martinus Petrus Johannes Cornelius) Marinussen werd op 14 april 1963 geboren in het Brabantse Reek, gemeente Schaijk. In 1981 behaalde hij het VWO-diploma aan het Kruisheren Kollege te Uden. In 1982 begon hij de studie Weg- en Waterbouwkunde aan de Hogere Technische School te 's-Hertogenbosch. In 1986 rondde hij deze studie af met een afstudeeropdracht, waarvoor hij in samenwerking met Stephan Gruijters tot in de kleine uurtjes werkte aan computersimulaties van het schoonspoelen van een mestgoot in een kalvermesterij.

Na één jaar bij De Ruiter Milieutechnolgie (Halfweg) als uitvoerder milieukundige projecten gewerkt te hebben, begon hij in 1987 aan de studie Milieuhygiëne aan de Landbouwuniversiteit Wageningen. De Harmonisatiewet dreigde zijn ambities te verstoren, maar dank zij een noodwetje kreeg hij alsnog de gelegenheid om in 4 jaar het volledige programma te doorlopen. Hij deed twee afstudeervakken, beide op de vakgroep Bodemkunde en Plantenvoeding. Tijdens een 5-maands afstudeervak Bodemscheikunde onderzocht hij, wederom samen met Stephan Gruijters, onder begeleiding van Han de Wit en Maarten Nederlof, de affiniteitsverdelingsfuncties van organische stof in de bodem voor zware metalen. Tijdens een 5-maands afstudeervak Bodemhygiëne en Bodemverontreiniging onderzocht hij, onder begeleiding van Sandra Boekhold, de toepassing van geostatistiek bij bodemverontreiniging.

Naast zijn studie was hij actief bij o.a. jongerenvereniging Unitas en theater 't Hemeltje. In 1989 zat hij in de Commissie Algemene Introductiedagen, die een programma samenstelt voor aanstaande eerstejaars ter voorbereiding op hun studententijd in Wageningen.

In 1992 studeerde hij af en werd aansluitend aangesteld als Assistent in Opleiding bij de vakgroep Bodemkunde en Plantenvoeding, de laatste 17 maanden met 0.8 weektaak. Het promotie-onderzoek heeft geleid tot dit proefschrift.

Tijdens zijn aanstelling was hij lid van het bestuur van de vakgroep en gedurende 2 jaar van het bestuur van het Wagenings AiO en OiO Overleg, dat o.a. formeel door het College van Bestuur van de Landbouwuniversiteit gevraagd wordt advies uit te brengen inzake beleidsvoornemens betreffende AiO's en OiO's.

Sinds november 1996 is hij als tijdelijk medewerker bij de vakgroep Bodemkunde en Plantenvoeding belast met een aantal onderwijstaken.

Nawoord

Het was rond de jaarwisseling '91-'92 toen ik van Sandra Boekhold, namens de sollicitatiecommissie, mocht vernemen dat ik haar op mocht volgen als AiO bij de vakgroep Bodemkunde en Plantenvoeding. Direct na mijn afstuderen op 31 januari 1992 begon ik, en dat is inmiddels dus al weer 5 jaar geleden. Heel ambitieus ging ik de problematiek van ruimtelijke variabiliteit bij het inschatten van risico's van bodemverontreiniging te lijf. Het was hard werken, soms. Het zat ook lang niet altijd mee. Het bedenken van een goede hypothese bleek geen garantie voor het welslagen van experimenten met regenwormen. Die beesten zijn eigenwijs en beschikken tot mijn stomme verbazing in beperkte mate over intelligentie. Maar om een lang verhaal kort te maken: het onderzoek heeft geresulteerd in dit proefschrift. En dat geeft aan dat er ook een heleboel proeven in hun opzet geslaagd zijn. In al die tijd heb ik van verschillende mensen ondersteuning gehad, zowel privé als op het werk, en een aantal van hen wil ik hier bij naam noemen.

Op de allereerste plaats (buitencategorie) wil ik Marie-Louise noemen. Zoveel steun, zoveel vertrouwen, zoveel liefde, zoveel waardering, zoveel meedenken, zoveel luisteren en zoveel opbeuren, meelachen en meehuilen. Het is bijzonder, heel bijzonder. Poekie: ik hou van je.

Mijn maatjes-op-het-werk: Frans, Sjoerd en Erwin. Frans, jij bent voor mij een bijzondere promotor. Ik heb grote waardering voor jouw vertrouwen in mij en hecht aan het persoonlijke van onze relatie. Sjoerd, dank zij jou werden de manuscripten langzaam maar zeker rijp voor publicatie. Jij bekeek de getallenbrij vanuit een ander perspectief dan ik en wist mij daarmee te inspireren tot hernieuwde wetenschappelijke creativiteit. Erwin, met praktische problemen kon ik altíjd bij jou terecht. Je tips waren zinvol. In de tijd dat we op de Marijkeweg samen op één kamer zaten bleek al snel dat we op dezelfde golflengte spraken en dachten. Ons project met springstaarten was een succes en een artikel daarover is, na veel "discussie", geaccepteerd. Wanneer gaan we weer naar Loburg, jongens?

Collegae van het ondersteunend en beheerspersoneel: Willem M. en Arie B., helaas is het niks geworden met de maïsplanten die we in het najaar van 1992 van

de Wildekamp gehaald hebben. Wat een enorme klus. Dankjewel voor jullie inzet aldaar. Ook daarna stonden jullie altijd klaar als ik weer eens ter elfder ure iets kwam vragen. "Alles kan", zei jij dan Willem. Tof. Jaap, jongen, jammer dat je geen koffie meer drinkt in de vakgroepsruimte met ons samen. Ik vind je een toffe peer, ook jij had aan een half woord meer dan genoeg. Lekker recht door zee, dat mag ik wel. Willeke, fijn dat ik je auto zo af en toe heb mogen lenen. Gerdine, Egbert, Toos, Arie van den B., Henk, en alle andere analisten: bedankt voor het verzetten van een grote hoeveelheid analysewerk en voor het begeleiden van de studenten die bij mij een afstudeervak deden. Egbert, zullen we samen nog eens een weddenschap afsluiten om een kratje? Dan drinken we die piljes wél samen op, oké? Simon de knutselaar: Ajax wordt wel weer eens de beste en Vitesse wint waarschijnlijk nooit meer van jouw FC Wageningen. Zal het gras straks in het Arnhemse stadion beter groeien dan in het Amsterdamse? Kees, je bent een goeie beheerder en hebt de zaakjes goed voor mekaar. Bedankt voor de vlotte afhandeling van zaken. Meestal valt er wel wat met je te regelen, vooral als tussen jouw regels door geluisterd wordt. Herma, ook jij bedankt voor het vlotte afhandelen van zaken en het regelen van de koffie, de thee en de oudejaarsviering. Jij was voor mij op de vakgroep de rots in de branding. Het vakgroepsuitstapje naar Terra Nova en het nieuwe Ajax stadion was één van de dingen die we samen tot stand gebracht hebben. Ik vind het leuk om met jou dat soort dingen te doen, we begrijpen elkaar. Leden van de activiteitencommissie (Willem M., Willeke, Herma en Sjoerd), ik hoop dat we nog veel leuke dingen samen gaan organiseren. De promovendi van de vakgroep wens ik veel succes met het proefschrift.

Mijn werk is mede ondersteund door enthousiaste studenten, die een afstudeervak "Bodemhygiëne en Bodemverontreiniging" deden. Hen wil ik op deze plaats bedanken voor de vaak plezierige samenwerking: Anne Schoen, Arlienke Ouwehand, Aroena Lakhi, Boris van Seeters, Debby Berends, Gerben Mol, Gijs-Jan van Hoorn, Jan Hilbert, John van Zelst, Lydia Bouwman, Mariet Hefting, Monique Riphagen, Ronald Vledder en Sander van Beusekom, bedankt!

Ik wil ook alle mensen bedanken die kritische vragen stelden of kanttekeningen plaatsten naar aanleiding van één van mijn lezingen op symposia en dergelijke. Daar haalde ik veel inspiratie uit en moed om verder te gaan. Verder bedank ik

de leden van de leescommissie en de personen die zich bereid hebben verklaard te opponeren tijdens de openbare verdediging van mijn proefschrift.

Medebestuursleden van het Wagenings AiO en OiO Overleg, met name Corné, Henrieke, Jolanda, Anne, Erna, Gonneke en Marie-Louise: we hebben samen de vinger aan de pols gehouden en het College van Bestuur van goed onderbouwd advies voorzien inzake haar beleid ten aanzien van AiO's. En we hebben lekker veel frustraties uitgewisseld. Goeie begeleiders zijn zeldzaam en zijn tevens een voorwaarde om binnen 4 jaar te promoveren. Helaas neemt het aantal promovendi met een studentenstatus nog steeds toe. Ik ben blij dat ons College daar voorlopig van heeft afgezien.

Het gezin waar ik uit kom: ons pa en ons ma, ik heb het proefschrift aan jullie opgedragen als dank voor jullie blijk van belangstelling en medeleven en jullie onvoorwaardelijke steun in mijn leven. Henk en Christine, broer en zus, bedankt voor het kritisch lezen van de samenvatting. Jullie commentaar en de brieven naar aanleiding van de samenvatting was hét duwtje in de rug dat ik nodig had opdat ik het proefschrift begin november bij de leescommissie kon inleveren. Henk, samen met jou naar Ajax kijken zal ik niet gauw opgeven, ook al heb ik het nog zo druk. Het is veel te leuk, samen met jou op de tribune.

Vrienden en vriendinnen! Wytske: jij hebt me overtuigd dat dit werk echt iets voor mij was toen ik daar nog aan twijfelde. Het onderzoek zelf interesseerde jou niet zo, wel hoe ik er mee bezig was. Je hebt me regelmatig geholpen door mijn problemen te relativeren en als ik met didactische problemen zat, kon ik bij jou terecht. Ruud, Ellen, Ger, Gerrie, Stephan en Carin: we kaarten weinig de laatste tijd. Dat potje komt zo nooit vol en voor zo weinig geld als er nu in zit kunnen we alleen bij Kees Kroket uit eten gaan. Maar, zonder gekheid, het is fijn om al zo lang zulke lieve vriendjes te hebben. Stoffer, jij weet uit eigen ervaring waar je als AiO zoal tegenaan loopt. De uitgebreide gedachtenwisselingen over ons beider werk weet ik zeer te waarderen. Het was inspirerend en dat moeten we d'r daarom in houden, ook al hebben we straks ander werk. Ik ben blij dat jij paranimf wilt zijn. Joost, druk, druk, druk, druk. Jij kan erover meepraten. Heerlijk om met jou aan de telefoon te hangen, te praten over het werk, over vriendschap en liefde. Het brengt rust en orde in mijn hoofd. Samen schijnen we altijd veel te moeten drinken. Dat was al toen je nog in Wageningen woonde

(even een borreltje in 't Gat en vervolgens tegen sluitingstijd de kroeg uit worden gezet), dat was zo in Gent waar we in no-time een fles Schelvispekel halfleeg hadden in hotel 'Den IJzer' en dat is iedere keer als ik je in Maastricht opzoek of jij bij mij komt. Overigens is Tunesië een land waar ik nog een keer naar toe zou willen gaan, om samen met jou in Hôtel '20 Mars' de koffie comme moi of comme toi te drinken. Ik ben blij dat ik een fles whisky aan jou verloren heb met een weddenschap en ik hoop nog lang vriendjes met jou te zijn. Fijn dat ook jij paranimf wilt zijn. Mieke, een jaar lang iedere week een half uurtje meppen op de squashbaan was heerlijk afreageren. Marianne, in de kroeg was het iedere keer weer anders. Lollig, diepzinnig, zwaarmoedig, of licht. Maar ik kwam er iedere keer weer uitgerust en ontspannen vandaan. Jouw liefde voor Ierland is naar mij overgeslagen. Wim en Corry, bij jullie voel ik me altijd welkom. Dank jullie wel voor jullie gastvrijheid en onvoorwaardelijk medeleven. Olivier, bedankt voor het ontwerpen van de omslag.

En zo kan ik nog een tijdje doorgaan, maar dat doe ik niet. Als je hierboven niet genoemd wordt, wil dat niet zeggen dat je onbelangrijk voor me bent. Mijn vermoeidheid is groot, ik ga straks met Marie-Louise op vakantie: heerlijk. Na vijf jaar is het AF. Allemaal bedankt!

Wageningen, december 1996

Mari Marinussen

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