NATIONAL INSTITUTE OF PUBLIC HEALTH
AND THE ENVIRONMENT, BILTHOVEN, THE NETHERLANDS

Report no. 719102048

Toxic effects of heavy metals in three worm species exposed in artificially contaminated soil substrates and contaminated field soils.

L. Posthuma and J. Notenboom

July, 1996

This study was carried out at the request and for the account of the Directorate-General for Environmental Protection, for the Direction of Soil and the Direction of Substances, Safety and Radiation of the Ministry of Housing, Spatial Planning and the Environment, within the framework of projectno. 719102, 'Environmental Effect Assessment'.

National Institute for Public Health and the Environment, P.O. Box 1, 3720 BA Bilthoven, The Netherlands
tel. +31-030-2749111, fax +31-30-2742971

MAILING LIST

1 t/m 3. Directoraat-Generaal voor Milieubeheer, Directie Stoffen, Veiligheid en Straling
4 t/m 5. Directoraat-Generaal voor Milieubeheer, Directie Bodem
6. Plv. Directeur-Generaal Milieubeheer, Dr. Ir. B.C.J. Zoeteman
7. Dr. J.M. Roels (DGM/Bo)
8. Drs. C.A.J. Denneman (DGM/Bo)
9. Ir. J.G. Robberse (DGM/Bo)
10. Prof. Dr. C.J. van Leeuwen (DGM/SVS)
11. Dr. J.H.M. de Bruijn (DGM/SVS)
12. Dr. G.P. Hekstra (DGM/SVS)
13. Dr. M.H. Donker (VU, Amsterdam)
14. Dr. J. Faber (IBN-DLO, Wageningen)
15. Dr. C.A.M. van Gestel (VU, Amsterdam)
16. Henzen (TNO-MW, Delft)
17. Dr. N. van den Hoeven (TNO-MW, Delft)
18. Dr. S.P. Hopkin (Univ. Reading, U.K.)
19. Dr. W.Ma (IBN-DLO, Wageningen)
20. Dr. J. Römbke (ECT, Germany)
21. Drs. C.E. Smit (VU, Amsterdam)
22. Dr. J. Vegter (TCB, Den Haag)
23. Dr. H. Vonk (TNO-MW, Delft)
24. Dr. J. van Wensem (TCB, Den Haag)
25. Depot Nederlandse Publikaties en Nederlandse Bibliografie
26. Directie Rijksinstituut voor Volksgezondheid en Milieu
27 t/m 37. Onderzoekers Project Validatie Toxiciteitsgegevens en Risicogrenzen Bodem
38. R. Baerselman (RIVM-ECO/EEB)
39. Drs. H. Boonman (RIVM-ECO/EEB)
40. Dr. G.H. Crommentuijn (RIVM-ACT)
41. Ing. E.M. Dirven-van Breemen (RIVM-ECO/EEB)
42. A.J. Folkerts (RIVM-ECO/EEB)
43. Ir. T. Hamers (RIVM-ECO/BMI)
44. Dr. ir. R.P.J. Janssen (RIVM-ECO/EBB)
45. Dr. W.P.J.G. Peijnenburg (RIVM-ECO/EBB)
46. Ir. C. Roghair (RIVM-ECO/BMI)
47. Dr. J. Struijs (RIVM-ECO/EBB)
48. Dr. ir. F. A. Swartjes (RIVM-LBG)
49 t/m 54. Auteurs
55. SBD/Voorlichting & Public Relations
56. Bureau Rapportenregistratie
57 t/m 58. Bibliotheek RIVM
59. Depot ECO/LWD
60 t/m 80. Bureau Rapportenbeheer

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

This work is a summary report on the activities of many people, of whom we mention:

- Rob Baerselman for performing the experiments with *E. andrei*, and guidance of the practical work of others;
- Hans Boonman for collecting soils and pore waters;
- Liesbet Dirven-van Breemen for performing part of the experiments with the enchytraeids;
- Arie-Jan Folkerts for performing part of the experiments with the enchytraeids and 'archiving' the soil batches;
- Els Smit for exchange of data;
- Rens van Veen for part of the metal analyses, and for development of methods for the micro-analyses of metals in enchytraeids;
- Jeanine van der Velden for performing the accumulation studies with *Enchytraeus crypticus*;
- various laborants of RIVM-LBG and RIVM-LAC for performing soil analyses and part of the metal analyses;
- the participants of the project 'Validatie Toxiciteitsgegevens en Risicogrenzen Bodem' for stimulatory discussions and co-operation.

SUMMARY

The toxicity of chemicals is often determined in standardised laboratory experiments. OECD artificial soil (artisoil) is often used to determine chemical toxicity for soil organisms. This report presents exposure and effect assessments of metals for three worm species (*Eisenia andrei*, *Enchytraeus crypticus* and *Enchytraeus albidus*) in metal contaminated field soils. The species differ with respect to metal sensitivity and ecological niche. Field soils were sampled at geometrically increasing distances from a former zinc smelter, to mimic a concentration-effect curve. The soils were characterised for various physico-chemical parameters. The concentrations of zinc, copper, lead and cadmium were measured, and metal partitioning over solid and liquid phase was determined using total-, 0.01M-$CaCl_2$-extractable- and pore water concentrations.

Soil characteristics differed between sampling sites. Metal extractability tended to be lower in the field soils in comparison with artisoil with metal salts. Exposure assessment showed that body concentrations of various metals increased less in the smelter soils than in artisoil with similar metal concentrations.

Effect assessments were made by determining concentration-response curves for the dominant metal zinc alone, and for the mixtures of metals present in the soils, at similar soil pH. When expressed by total soil concentrations, toxic effects in field soils were less than in artisoil for *E. andrei*. For *E. crypticus* the opposite was found. *E. albidus* did not perform well in the soils, and was not used further. The predictability of effects in field soil from laboratory toxicity data improved when differences in metal extractability and joint effects of metals were taken into account. Joint effects were judged by application of the concept of relative concentration addition.

It is recommended to take these factors into account when laboratory toxicity data are used to predict effects at contaminated sites or to derive soil quality criteria.

SAMENVATTING

De toxiciteit van chemische stoffen wordt vaak bepaald via gestandaardiseerde laboratorium experimenten. OECD-kunstgrond wordt vaak gebruikt om de toxiciteit van stoffen voor bodemorganismen te bepalen. Dit rapport beschrijft een blootstellings- en effectbeoordeling van metalen bij drie wormensoorten (*Eisenia andrei, Enchytraeus crypticus* en *Enchytraeus albidus*) bij blootstelling aan metaalverontreinigde veldgrond. De onderzochte wormsoorten verschillen wat betreft metaalgevoeligheid en ecologische niche. Veldgronden werden verzameld in een geometrische reeks van toenemende afstand rond een voormalige zinksmelter, om zodoende een concentratie-respons curve voor de veldgronden te kunnen vaststellen. De gronden werden fysisch-chemisch gekarakteriseerd. De concentraties van zink, koper, lood en cadmium werden gemeten, en de partitie over de vaste en vloeibare fase van de grond werd bepaald met behulp van totaal-, en $CaCl_2$-extraheerbare- en poriewater concentraties.

Bodemkarakteristieken verschilden tussen de verschillende monsterlokaties. De beschikbaarheid van metalen in de veldgronden was lager dan in kunstgrond met toegevoegde metaalzouten. De blootstellingsbeoordeling toonde aan dat de lichaamsconcentratie van verschillende metalen minder toenam in de veldgronden dan in kunstgrond bij vergelijkbare metaalconcentraties.

Effectbeoordelingen werden uitgevoerd door het vaststellen van concentratie-effect curves voor het dominante metaal zink apart, en voor het metaalmengsel in de veldgronden, bij gelijke pH. Bij beoordeling op basis van totaalconcentraties bleken de toxische effecten in de veldgrond lager dan in kunstgrond voor *E. andrei*. Voor *E. crypticus* werd het omgekeerde gevonden. *E. albidus* reproduceerde onvoldoende om vergelijkingen te kunnen maken. De voorspelbaarheid van effecten in veldgrond op basis van laboratoriumgegevens nam toe indien rekening gehouden werd met de verschillen in fysisch-chemische metaalbeschikbaarheid en gecombineerde inwerking van de in de veldgond aanwezige metalen. Bij de beoordeling van gecombineerde inwerking is gebruik gemaakt van het concept van relatieve concentratie additie.

Aanbevolen wordt om deze factoren te betrekken bij de beoordeling van toxische effecten op verontreinigde veldlocaties en bij het afleiden van ecotoxicologische risicogrenzen op basis van resultaten uit laboratorium-toxiciteitsstudies.

1. INTRODUCTION

1.1 Scope of the work

Environmental quality criteria, or decisions to take remedial action at contaminated sites, are often based on ecotoxicological risk assessment systems in which standardised toxicity data play a central role. Using standardised laboratory toxicity data for this purpose carries the risk of neglecting important factors that may moderate toxicity in field conditions. Several authors (Van Straalen & Denneman, 1989, Van Gestel, 1992, Van Straalen et al., 1994, Chapman, 1995a, Chapman, 1995b) have pointed out a series of factors that should be taken into account when predicting toxicity in field conditions on the basis of laboratory-collected toxicity data. Major factors are:

- toxicant availability as a function of physico-chemical characteristics of the environment (e.g., Van Gestel, 1992);
- effects of toxicant mixtures (e.g., Weltje et al., 1995)
- heterogeneity in toxicant and species distribution (e.g., Marinussen & Van Der Zee, 1994);
- the relationships between the studied fitness parameter and population growth rate (e.g., Kammenga, 1995);
- the influence of other species (e.g., Posthuma, (in press))
- the duration of exposure (e.g., Posthuma, 1992)
- interspecies differences (e.g., Notenboom et al., 1995; Vaal et al., in prep.).

This paper is part of a larger study project that addresses two questions regarding the Dutch soil ecotoxicological risk assessment methodology, namely (Notenboom & Van Beelen, 1992):
(1) the validity of laboratory single-species toxicity data for situations in the field where exposure differs from the standardised protocol;
(2) the validity of the risk levels, calculated from standardised single-species tests, for field communities.
In the present report, attention focuses on the first question, with special emphasis on toxic effects of metals to earthworms.

1.2 Approach of the present study

In the experiments described in this report worms were exposed under standardised temperature-, pH- and humidity conditions to metal mixtures in soils collected at a contaminated field site. The soil was collected at near a former zinc smelter at various distances from the emission source. Soils were collected in a geometric series, to mimic the classical laboratory concentration-effect approach with field soils.

Due to the standardisation of various abiotic conditions applied in the present work, some potentially moderating factors were ruled out in the present work, so that emphasis was put on

(1) differences in availability of metals between soils, and (2) on the potential contribution of mixture toxicity.

- Differences in metal availability between soils are worked out following current models on metal partitioning over the solid and liquid phase of the soil. Moreover, one should account for biological factors that influence exposure route and bioavailability. Partition coefficients and Freundlich isotherms were used for the comparison of metal partitioning between soils. From these data, the *bio*availability of metals is addressed, in a species-specific and soil-specific way by linking data on metal partitioning with data on body concentrations after exposure and with data on toxic effects. No attempts were made to define the exact speciation of the metals in the soils, since this requires an analytical methodology of great sensitivity and selectivity, which is not (yet) available for soil systems (Ure & Davidson, 1995).
- The contribution of mixture effects was judged in view of recently published data on the effects of metal combinations on soil organisms (Weltje et al., 1995). The findings of the bioavailability analyses were incorporated in the analysis on mixture effects, since the mixture studies were performed in artisoil, viz., with the same species, but under different availability conditions than in Budel soils. It was reasoned that application of the same extraction techniques in the mixture studies would at least partly correct for the differences in metal availability between soils.

Other participants in the project address other species groups, such as micro-organisms, plants and springtails. The preliminary findings for these species have been summarised in progress reports (Notenboom & Posthuma, 1994, Notenboom & Posthuma, 1995), while the publication of detailed reports is in progress. Specifically, the smelter soils used here have also been used for some of the experiments performed by the other project participants. The results have already partly been published elsewhere (see Table 1.1). Comparisons with these data will be made in the discussion section of this report.

Authors	Species	Type of experiment	Use of Budel soils
Van Der Hoeven & Henzen, 1994a	*Lolium perenne* *Vicia sativa*	- (1) comparison of effects in 2 Budel soils	-(1) reference and very contaminated soil was used
	Trifolium pratense	- (2) study of effects with artificially added Cd or Zn	-(2) reference soil as basis
Van Der Hoeven & Henzen, 1994b	*Trifolium pratense*	- soil #1 - #11 *	Approach as here, pH adjusted to ca. 5.5
Van Der Hoeven & Henzen, 1994c	*Sinapis alba*	- soil #1 - #11	Approach as here, pH adjusted to ca. 5.5
Van Der Hoeven & Henzen, 1994d	*Trifolium pratense*	- soil #11	Cd, Zn and Cd/Zn mixtures in soil #11
Posthuma et al., 1994	*Eisenia andrei*	- soil #1 - #11	-(1) at 'natural pH' -(2) at pH adjusted to ca. 5.5
Smit & Van Gestel, in prep.	*Folsomia candida*	- soil #1 - #11	pH adjusted to ca. 6.0

Table 1.1. Published detailed reports on experiments in which toxic effects of metals in standardised toxicity experiments are compared to the series of field soils collected in the vicinity of the former zinc smelter in Budel-Dorplein, for which soil data are summarised in this report. * Soil numbers (#) are explained in chapter 2.1.

1.3 Outline of the paper

Two subjects are distinguished in this report, namely:

(1) a soil-oriented part

(2) a toxic-response oriented part

The first subject concerns all general features with regard to the collection, treatment, storage, and (abiotic) qualifications of the smelter soils. This subject is treated in chapter 2 on the materials and methods, and chapter 3 on the observations. The second subject concerns data collected during experiments with *Eisenia andrei* and *Enchytraeus* species. This subject is also treated in two chapters (4 and 5). In the latter chapters, assessments of exposure and of effects are made. The data collected for the Budel soils are compared to similar data for OECD artificial soil (in this paper further referred to as "artisoil") to which metals are added as metal-salt solution during preparation. This is done to identify (dis)similarities of soil characteristics and of toxic effects between the soil types. Incidentally, comparisons are made with the characteristics of other Dutch soils to illustrate the (dis)similarity of artisoil, Budel soils and other Dutch soils. The latter comparisons are made in more detail in others papers (Janssen et al., 1996, Janssen and Posthuma, in prep.). Eventually (in chapter 6) all data on soil characteristics and toxic effects are integrated, and final conclusions are drawn.

1.4 Aims and approach

In this research we aimed to:

- determine the performance of three worm species in laboratory conditions and in metal-contaminated field soils;
- compare the toxic effect levels of various metals found in the field soil with toxic effect levels found in other soils (in particular OECD-artificial soil), viz. comparison of concentration-response curves in various soils, and focusing on the *magnitude* of differences;
- unravel the possible causes of the reproduction effects in the contaminated field soil, viz. focus on *explanation* of the relative role of metal toxicity and other soil factors.

We aimed at both looking at differences *per se*, and at their causes, to be able to extend the conclusions to other soils and species. In this research, straightforward interpretation of performance data is hampered by the action of various factors simultaneously (e.g., concentration- and availability differences for various metals, mixture effects, and effects of other soil factors), of which the relative importance is unknown. Therefore, various lines of reasoning are explored in the section where soil- and toxicity data are integrated.

2. SOIL CHARACTERISTICS: SAMPLING AND METHODS

2.1 Soil sampling

Metal-contaminated soils were sampled on 11 sites on March 11, 1993, in a metal-pollution gradient around the zinc factory of Budelco (the former Kempensche Zinkmaatschappij) near Budel-Dorplein (NL). The soils are further abbreviated as #1 to #11. Site pollution has been caused by ca. 90 years smelting of zinc ores. An electrolytic process has been in operation since 1973. For details on the history of site pollution, see Posthuma, 1992). The sampled soils are used by research groups at TNO (Delft), Vrije Universiteit (Amsterdam) and RIVM, for research with plants and micro-organisms, collembolans, and micro-organisms and earthworms, respectively. In this report, soil characteristics are summarized, and the results of experiments with earthworms in these soils are given.

Soil sample sites were located at different distances Northeast of the smelter stack. Metal concentrations in the soil were expected to show an exponential decrease with distance. Distances between sampling sites were chosen on a linear gradient in a series of approximately exponentially increasing distances, so as to obtain soils with a geometrically decreasing sequence of metal concentrations. At some of the locations chosen on the basis of an area map, however, sampling was impossible due to fences. In such cases, sampling sites were chosen near selected locations.

At each distance, 5-7 sub-sites were randomly selected to collect soil. This was done to reduce variation in soil conditions other than metal pollution. The litter layer at the sites was removed, and the top 10 cm of the soil was collected until ca. 40 - 60 kg of field-wet soil was collected. The most distant location was used as reference site. The sub-samples collected at each distance were transported to the laboratory. In addition to the 11 sampling locations, the sampling location 'pomp' (further mentioned: #P), mentioned in Posthuma et al. (1994), was also used in some experiments. This site is located slightly eastward of the linear gradient #1 - #11, in an isolated pine stand near the factory. Since this soil was sampled earlier (December, 1992) for pilot observations, the pre-treatment of soil #P may be different in details from the treatment of the other soil batches.

Local site characteristics differ between sites (Table 2.1). The soil from site #1 differed from all other soils. The soil at that location consists of a thick debris-soil mixture. Specimens of *Alnus* spec. stood up till the stem in the soil, and the soil contained trash (glass, empty paint containers, etc.).[1] Information on local soil characteristics was obtained from the appropriate sections of the 'Bodemkaart van Nederland', and is summarized in Table 2.2.

---

[1] The site characteristics of site #1 are given in this report, to enable comparisons with data from other participants in the project who used this soil in their experiments; references to these works are given elsewhere. This soil has not been used for experiments with worms. This implies that the data of soil #1 have also not been used for the derivation of, for example, Freundlich isotherms.

#	*km**	*n***	*Dominant genera in vegetation*	*Remarks*
1	0.4	5	*Alnus, Festuca, Poa*	Debris-soil cover
2	1.1	7	*Betula, Quercus, Pinus, Poa, Festuca, Erica*	4 from *Pinus*-sites, 3 from *Betula*-sites
P	1.2	4	*Pinus*	not in linear gradient
3	1.6	6	*Betula, Pinus, Alnus, Festuca, Poa, Deschampsia*	3 from *Pinus*-sites, 3 from *Betula*-sites
4	2.6	7	*Pinus*	open area with *Pinus*- stands
5	5.0	6	*Pinus*	open area with *Pinus*- stands
6	6.6	4	*Pinus, Calluna*	near motorway
7	7.9	5	*Pinus, Quercus*	2 from *Pinus*-stands, 2 from *Quercus*-stands
8	9.6	4	*Pinus, Quercus, Deschampsia*	2 from *Pinus*-stands, 2 from *Quercus*-stands
9	12.0	4	*Pinus, Betula, Quercus, Erica*	3 from *Pinus*-stands, 1 from *Betula*-stand
10	15.0	5	*Pinus, Betula, Quercus, Deschampsia*	3 from *Quercus*-stands, 2 from *Pinus*-stands
11	20.7	6	*Pinus, Erica*	

Table 2.1. Summary of local site characteristics in the sampled gradient around the Budel zinc smelter.
* 'km' indicates the distance from the smelter, ** 'n' indicates the number of sub-sampling sites

The soil typology data show that the soils from the Budel gradient are not exactly similar, but still largely homogeneous (except for soil #1). The presence or absence of a podzol profile is the largest difference of soil typology between sampling sites. A layer rich in organic matter, with or without iron and aluminium bound to it, is present in the soils with a podzol-profile (#1, P, 5, 7, 9, 10, 11). In some soils the sand and the humus fractions are coated with a layer of iron, as a result of soil formation in humid, reducing conditions.

The water content of the soil batches directly after sampling ranged from ca. 6 to 17 percent (w/w). To prepare the soils for storage, soil batches were air dried in a thin layer at room temperature for ca. 3 weeks, to a water content between 1 and 1.8% (w/w). Thereafter, the soil batches were homogenised in a concrete mill and sieved (4 mm mesh). During homogenisation, the water content was adjusted to 2% by adding demineralised water. The soils were stored until use at 5 ^{o}C, in loosely closed containers in the dark[2]. The Budel soils treated in this way are further referred to as 'batches'.

2.2 Characteristics of the soil batches

Soil characteristics (except for the metals) were determined following standard protocols, derived from Steur & Heijink (1991).

2.2.1 pH

The pH(KCl) of the Budel soils was measured after the 3-weeks drying period. The pH(KCl) and pH(H_2O) were measured again in the soil batches rewetted to 2% humidity, ca. 2 months after sampling. To measure pH, 20 g of each batch was weighed into PVC bottles, and 50 ml

---

[2] Since early 1995, after the experiments, the soil batches are stored at room temperature.

deionised water or 1M KCl was added. The suspensions were shaken for 2 hrs. The next day pH values were measured in clear supernatant.

#	Soil code	Soil type	Texture name (Dutch)
1	Hn23	veldpodzolgrond	lemig fijn zand
P	Hn21	veldpodzolgrond	leemarm en zwak lemig fijn zand
2	Zn21	vlakvaaggrond	leemarm en zwak lemig fijn zand
3	Zd21	duinvaaggrond	leemarm en zwak lemig fijn zand
4	Zd21	duinvaaggrond	leemarm en zwak lemig fijn zand
5	Hd21	haarpodzolgrond	leemarm en zwak lemig fijn zand
6	Zd21	duinvaaggrond	leemarm en zwak lemig fijn zand
7	Hd21	haarpodzolgrond	leemarm en zwak lemig fijn zand
8	Zn21	vlakvaaggrond	leemarm en zwak lemig fijn zand
9	Hn21/Hd21	veldpodzolgrond/haarpodzolgrond	leemarm en zwak lemig fijn zand
10	Hn21	veldpodzolgrond	leemarm en zwak lemig fijn zand
11	Hd21/Zd21	haarpodzolgrond/duinvaaggrond	leemarm en zwak lemig fijn zand

Table 2.2. Summary of local soil characteristics in a gradient around the Budel zinc smelter, with a podzolic or sandy soil type, generally with a texture of fine-sand poor in loam (further characterisations according to the Dutch nomenclature, following Steur & Heijink, 1991).

Soil type descriptions are (in Dutch):

- veldpodzolgrond: podzolgrond (inspoelingshorizont: B-horizont) met amorf humus in de duidelijke podzol-B, hydromorfkenmerk: zonder ijzerhuidjes, de humushoudende bovengrond is 0-30 cm.
- vlakvaaggrond: zandgrond zonder minerale eerdlaag (vaaggrond), hydromorfkenmerk: zonder ijzerhuidjes.
- duinvaaggrond: zandgrond zonder minerale eerdlaag (vaaggrond), hydromorfkenmerk: met ijzerhuidjes, zonder bruine laag in de positie van een B-horizont.
- haarpodzolgrond: podzolgrond (inspoelingshorizont: B-horizont) met amorf humus in de duidelijke podzol-B, hydromorfkenmerk: met ijzerhuidjes, de humushoudende bovengrond is 0-30 cm.

Texture characteristics of the Dutch nomenclature are:

- 'leemarm zand': 0-10 % clay
- 'zwak lemig zand': 10-17.5 % clay
- 'ijn zand': M50 between 50-210 μM

2.2.2 Water retention characteristics

Water contents of Budel soil batches were measured at pF=0 and pF=2, resp. the maximum water holding capacity and the field capacity. The obtained values were used to judge the physiological availability of water in Budel soil batches in comparison with artisoil. The 'physiological availability' of water in soils not only depends on the total water content of the soil, but also on the water retention characteristics of soils. Water retention characteristics may differ both among the 11 Budel soils, and between the Budel soils and artisoil.

2.2.3 Weight loss on ignition, proportion of clay, carbon content

Weight loss on ignition (LOI%) was measured to estimate the organic matter (OM%) content. To measure LOI%, samples of each soil batch were dried overnight at 105 °C. Weight loss was measured after heating the oven-dried soil for 4 hrs. at 500 °C. Weight loss due to loss of particle-bound water was considered negligible. LOI% may, however, slightly over-estimate the true organic matter content. The total carbon content (C%) was determined with a Carlo Erba elemental analyser. The proportion of clay was determined by a soil sieving method.

2.2.4 *Metal analyses*

Various metal fractions were determined for each Budel soil. This was done, since the total metal concentration is usually not correlated to bioavailability, e.g. for plants (Gupta & Aten, 1993) or worms (Gonzalez et al., 1994). In addition to the total concentrations, various extraction techniques have been proposed to characterise (potentially) bioavailable fractions. We chose two methods out of the series of possible extraction methods that have been proposed (e.g., Van Loon & Barefoot, 1995). It is *a priori* obvious that the methods chosen do not describe the bioavailability of the metals exactly. They have been chosen, since they may be a better approximation of the available fraction for various organisms than the total concentration, and since they operate on the basis of relatively easy techniques that can routinely be applied for risk characterisation of contaminated soils. For these reasons they have been frequently applied as the so-called functionally- or operationally defined techniques for bioavailability approximation, in contrast to exact measurements of speciation (Ure & Davidson, 1995). All metal concentrations were determined with AAS-techniques (either flame or graphite furnace, depending on the metal concentrations present).

- Total metal concentrations. Samples of the soil batches were dried at 80 °C, and homogenised by milling before analyses. Total metal concentrations were determined after oxidation of the homogenised samples in heated *Aqua regia* (a mixture of HNO_3 and HCl, 3:1) at high pressure (150 psi). Total metal concentrations were expressed in $mg.kg^{-1}$ dry wt.

- Pore water concentrations. Pore water concentrations were measured in pore water collected in three differently treated samples of Budel soils. Firstly, pore water (PW1) was collected from Budel soils with storage as the sole treatment. To this end, ca. 3 kg of field-wet soil was packed directly after sampling in closed plastic vials, which were stored at 5 °C. After 3 months, on June 8th, 1993, the vials were acclimated for two days at room temperature. Pore water was collected by centrifugation over a paper filter at ca. 7000 rpm for 20 min., and acidified to a pH-value below 2. Secondly, pore water (PW2) was collected from the stored soil batches that were only rewetted prior to the reproduction studies with worms (see e.g. par 4.3.1). Pore water was collected 1.5 week after rewetting soils to ca. 20 percent water (w/w). Thirdly, pore water (PW3) was collected from soil batches that were not only rewetted for reproduction studies, but for which the pH was adjusted to ca. 5.5 with $CaCO_3$. Of the three types, metal concentrations in PW3 are the most relevant for the reproduction studies with *Eisenia andrei* and *Enchytraeus crypticus* reported here, since these studies were performed in Budel soils treated in this way. Metal concentrations of zinc, copper, lead and cadmium in the pore water were measured by AAS, and were expressed in $mg.l^{-1}$.

- Extractable concentrations. The extractable concentrations of metals were determined in neutral-salt extracts collected in two differently treated samples of Budel soils. Extracts were made by shaking 10 g of the soil batches in 100 ml of 0.01*M* $CaCl_2$ for 17 hrs at 20 ^{0}C. After shaking, the extracts were filtered over a 0.45 µm filter, and concentrated HNO_3 was added to obtain a $pH \leq 2$. Replicate extracts were made from each soil batch, and the obtained metal concentrations were averaged before further use.

Firstly, neutral-salt extracts (type $CaCl_2$-1) were made from the stored soil batches without pH adjustment. Secondly, neutral-salts extracts (type $CaCl_2$-2) were made from the stored soil batches in which the pH was adjusted to ca. 6 by addition of $CaCO_3$, the optimum pH for experiments with the springtail *Folsomia candida*. The latter data, collected for zinc only, were kindly provided by C. E. Smit, Vrije Universiteit Amsterdam. Of the two types, the metal (i.e., zinc) concentrations measured in type $CaCl_2$-2 are most relevant for the reproduction experiments reported here, since these studies were performed in Budel soils treated in this way.

The neutral-salt extractable concentration is considered to be a measure of the sorption strength of the solid phase (e.g., Gupta & Aten, 1993) and it is considered to express the lower limit of the amount of labile sorbed metal (Gooddy et al., 1995). Different authors have used various neutral salts for the extraction of metals from soils (e.g., Boekhold, 1992). The obtained values for the sorption strength depends on the extraction conditions (temperature, shaking time, soil to liquid ratio) and the concentration and type of the neutral salt solution (e.g., Chardon, 1984, Boekhold, 1992). Comparison of soils can, thus, best be made on the basis of a single, operationally clearly defined extraction technique. We chose to use 0.01*M* $CaCl_2$, since this extractant is often used, recommended (Pepels & Lagas, 1993), and since it has also been applied to assess metal extractability in artisoil (Weltje et al., 1995).

There are two ways to express the results of neutral salt extraction. On the one hand, when the extraction is used to characterise metal sorption in soils collected in the field, such as in the Budel soils, the extractable metal concentrations are expressed in $mg.kg^{-1}$ dry wt (see, e.g., Janssen et al., 1996, Gooddy et al., 1995), or as percentage of the total metal concentration that can be extracted. This is the common expression form, considering that the method aims to characterise labile binding to the solid phase. On the other hand, when neutral salt extraction is used to determine the sorption isotherm of a metal in a soil, various sub-samples of that soil are shaken in neutral salt solutions with various concentrations of the metal added in the form of metal salts (see, e.g., Boekhold, 1992, Chardon, 1984). Metal sorption to the soil solid phase is then often expressed as the decrease of the metal concentration in the salt solution. The decrease is recalculated as the metal concentration bound to the solid phase ($mg.kg^{-1}$ dry wt), whereas the metal concentration remaining in the extract after shaking is expressed as $mg.l^{-1}$. The latter values can, however, often not be recalculated towards $mg.kg^{-1}$ or % from the literature data.

To enable comparisons with different types of literature data, thus, 0.01*M* $CaCl_2$ extractable concentrations were expressed both as $mg.kg^{-1}$ dry wt (or %) for comparisons with contaminated field soils, and as $mg.l^{-1}$ extract for comparison with sorption isotherms of some soils..

In the present study, the measurements of the different metal concentrations were made parallel, viz. the initial steps were made with different soil samples from each batch of Budel soil. This implies that pore water concentrations are incorporated in the $CaCl_2$ extract measurements, and that the 0.01*M* $CaCl_2$- extractable- and the pore water concentrations are incorporated in the total concentration measurements. The alternative procedure is subsequent extraction of pore water, neutral salt extraction, and measurement of rest-metals in the solid phase. We chose to use parallel extraction in view of the hypothesised exposure of worms, which is determined by pore water concentrations only, or by a combined exposure route through pore water and the easily extractable metals, rather than by the extractable concentration alone (see Janssen and Posthuma, in prep.). The differences between subsequent and parallel extraction may be negligible for pore water extracted amounts versus neutral salt extraction, but it may be relevant when total- and neutral salt concentrations are determined.

2.2.5 *Distance-concentration relationships*

The metals were expected to show a geometric decrease of soil concentrations with increasing distance. This was tested by fitting linear distance-concentration relationships to *ln*-transformed data with the formula:

Equation 1: $\ln C = A * \ln D + B$

with D (distance) in km, C (concentration in soil, extract or pore water) in $mg.kg^{-1}$ dry wt or $mg.l^{-1}$, and A and B metal specific constants. Next to testing significance of concentration changes, linear regression models were used to interpolate metal concentrations at sites not actually sampled in the field. This is necessary for the interpretation of the reproduction studies with the worms (see Chapter 5). Since the reproduction studies were performed in rewetted (20% w/w) soil batches at pH ca. 5.5, the regression models for metal concentrations measured as 'total', in PW-3 and in $CaCl_2$-2 (rewetted, pH adjusted soils) are the most relevant for the interpretation of toxicity studies performed with the worms (see Chapter 4 and 5).

2.2.6 *Metal partitioning calculations*

The toxic effects of the metals for exposed organisms depend on the (bio)availability of the metals, which in turn depend (at least partly) on the sorption to the soil solid phase. The sorption of metals to the soil solid phase was characterised using total-, extractable-, and pore water concentration data. These data were used to calculate partition coefficients and Freundlich isotherms, since physico-chemical characteristics of metal sorption are often published in these forms.

To compare our data with literature data on metal partitioning in contaminated field soils, we calculated site-specific partition coefficients for soils #2-11 using total- and pore water concentrations, by:

Equation 2: $$K_P = \frac{[Me]_{solidphase}}{[Me]_{porewater}} \qquad (\mathrm{l.kg^{-1}})$$

(square brackets indicate concentrations in mg.kg^{-1} or mg.l^{-1})

The soil/solution partition coefficient reflects the tendency of an element to bind to the surface of soil particles, high values indicate strong sorption. Application of the formula to the collected data resulted in 3 partition coefficients for zinc, copper, lead and cadmium for each Budel soil, based on PW1, PW2 or PW3 resp. in de denominator. In addition, the K_p averaged over the soils #2-11 was calculated for each metal and pore water type.

To compare our data with literature data on sorption isotherms, as applied for e.g. artisoil, we applied the Freundlich isotherm formula:

Equation 3: $$C_S = K_f C_W^{\frac{1}{n}},$$

with C_W = concentration in the aqueous fraction (mg.l^{-1})
C_S = concentration in the soil (mg.kg^{-1})
K_f = sorption constant (l.kg^{-1})
n = shape parameter of the Freundlich curve

Estimates of the sorption constant and the shape parameter were obtained by linear regression in a double logarithmic plot of C_W versus C_S, viz.:

Equation 4: $$\log C_s = \log K_f + \frac{1}{n}\log C_W$$

Sorption isotherms were calculated with the total metal concentration as a measurement of C_S, and with either (1) the pore water concentrations PW1 to PW3, or (2) the 0.01*M* CaCl2-extractable concentrations $CaCl_2$-1 and $CaCl_2$-2 as measurement of C_W.

The Freundlich isotherm has also been calculated for metal sorption in toxicity experiments with artisoil, which is a medium prepared by mixing highly homogeneous constituents. For the derivation of the Budel-soil Freundlich isotherms we implicitly assumed that the soils are homogeneous over the whole sampling range. Inhomogeneities in soil characteristics between the Budel soils, that may affect metal partitioning, are considered to be randomly distributed. This assumption means that local, random sources of variation cause ‘noise’ on the isotherm, without affecting isotherm parameters. This may not be true in practice, in particular for metals for which partitioning is strongly affected by local soil factors. This aspect will be discussed in section 3.

2.2.7 *Similarity of K_p and K_f*

The average K_p calculated for a series of differently contaminated field soils from a gradient (here soils #2-11) equals the K_f of that soil if the parameter *n* of the Freundlich isotherm equals unity, and if variation of soil factors is random or negligible. If *n* not equals unity, then metal partitioning over solid and liquid phase changes with increasing metal concentrations in the soil, due to saturation of sorption sites.

2.3 Correlation analyses

To identify the soil characteristics that may influence the performance of exposed organisms in a systematic way, correlation analyses were applied. Correlations among variables were calculated by Pearson product-moment correlations (Sokal & Rohlf, 1981). Data were logarithmically transformed when necessary, to obtain homogeneity of variances.

3. SOIL CHARACTERISTICS: OBSERVATIONS

3.1 Soil properties

Abiotic characteristics of the field soils are summarized in Table 3.1 (pH, clay%, C%, LOI%, humidity measurements).

#	D km	pH (KCl) (1)	pH (KCl) (2)	pH (H_2O) (3)	pH(KCl) (4)	Clay %	C %	LOI %	W1 %	W2 %	W3 %	pF0 %	pF2 %.
1	*0.4*	*4.68*	*4.9*	*5.6*	*4.7*	*1.99*	*1.11*	*1.88*	*16.7*	*12.3*	*4.46*	*29*	*12*
2	1.1	5.04	5.2	5.7	4.9	1.43	2.39	3.55	11.2	8.0	3.15	34	20
P	1.2	n.d.	3.2	4.7	4.1	1.79	2.83	4.11	n.d.	n.d.	n.d.	n.d.	23
3	1.6	3.84	4.0	4.8	4.0	1.25	1.72	2.83	8.6	5.9	2.70	7	4
4	2.6	5.14	4.5	5.0	4.3	1.24	1.44	2.41	6.3	3.6	2.74	18	5
5	5.0	3.05	3.1	3.8	3.1	1.26	2.33	3.63	10.2	7.0	3.20	34	11
6	6.6	3.02	3.0	3.7	2.9	1.48	3.63	6.38	13.1	9.8	3.35	43	20
7	7.9	3.06	3.2	3.8	3.2	1.27	3.12	4.46	13.8	10.2	3.64	35	12
8	9.6	2.93	3.0	3.7	2.9	1.31	2.92	4.26	13.3	8.8	4.55	36	10
9	12.0	2.88	2.9	3.8	2.9	1.26	4.30	5.50	17.0	13.0	4.01	38	18
10	15.0	3.16	3.3	3.8	3.3	1.55	2.46	4.68	12.3	9.9	2.38	35	13
11	20.7	2.97	3.5	3.0	3.4	1.15	2.41	3.53	10.1	6.9	3.18	32	11

Table 3.1. Abiotic characteristics of Budel soils after sampling, and in storage conditions (after homogenisation, drying and sieving).

= site code; D=Distance from smelter; (1): pH(KCl) of field soils directly before pore water collection; (2) pH(KCl) of field soil batch after drying to ca. 2 % water (w/w); (3): pH(H_2O) of stored soil batch; (4): pH(KCl) of stored soil batch; C: % carbon; LOI = loss on ignition; W1= water content (% w/w) of field soil in vessels used for pore water collection; W2= water content (% w/w) of soils after pore water collection; W3= extractable pore water content (% w/w), (W1-W2=W3); pF0= mass-% water (dry wt basis) of stored soil batches at maximum water holding capacity; pF2= *ibid.* at field capacity.

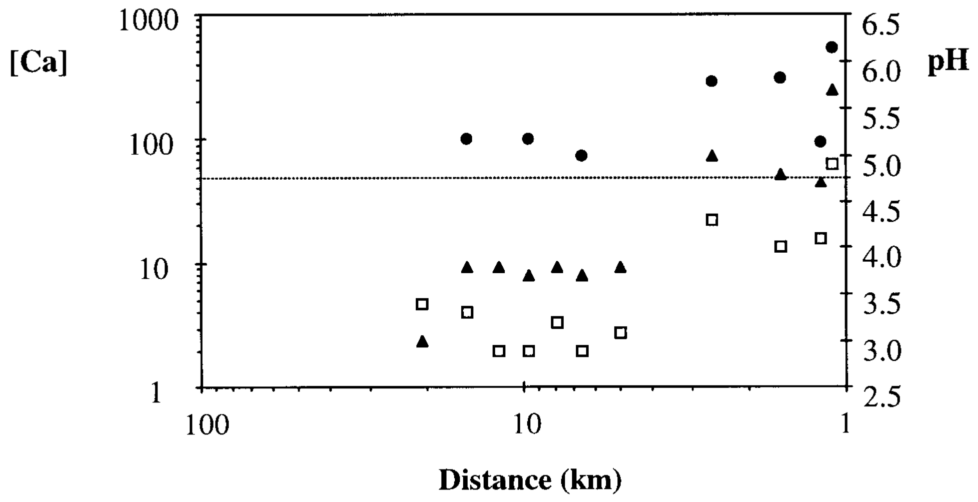

Figure 3.1. Total calcium concentrations (●, mg.kg^{-1} dry wt), pH(KCl) (□) and pH(H_2O) (▲) values of the stored soil samples #11 - 3, P, 2 (from left to right), collected at different distances from the smelter. The dotted line indicates the detection limit for [Ca].

Acidity differed between Budel soils. The pH(KCl)-values after air drying ranged from 2.9 to 5.2. After drying, sieving, homogenisation and storage for ca. 1.5 months, the pH(H_2O) of the stored batches ranged between 3.0 and 5.7, whereas the pH(KCl) varied between 2.9 and 4.9. Close to the smelter the pH(KCl) ranged from 4.0 to 4.9 (locations #2, P, 3 and 4) whereas at larger distance it ranged from 2.9 to 3.4 (for the locations #5 - 11) (Figure 3.1). Both pH(H_2O) and pH(KCl) were significantly negatively correlated to *ln*-transformed distance (r^2=-0.91 and r^2=-0.81 resp., both $P<0.01$). In comparison with artisoil used in earthworm experiments, the pH of the Budel soils was low, in particular at large distances from the smelter. A previous study has demonstrated that *Eisenia andrei* did not reproduce in those soils (Posthuma et al., 1994).

Water retention characteristics also differed among the soils. The maximum water-holding capacity (pF0) of the homogenised stored soils ranged from 7 to 43% (w/w). The field capacity (pF2) ranged from 4 to 23% water (w/w) (Table 3.1). At the sampling date, the water content of the field soils (W1) was much lower than the maximum water holding capacity, except for soil #3. For soils #1, 3, 4, 7, and 8 the water content in the field was higher than field capacity, for #2 and 6 it was more than 7 percent lower, and for #5, 9, 10, 11 field soils were approximately at field capacity. Pore water could be collected from all soils, in amounts of 27 to 45 $ml.kg^{-1}$ field soil. Centrifugation did not result in full extraction of soil water (W2 > 0). This indicates that the water that was collected consisted mainly of pore- and capillary water, and that hygroscopic water is retained in the soil (W2 > W3). In comparison with artisoil used in earthworm experiments, the water holding capacity of Budel soils is low. Toxicity experiments with *E. andrei* in artisoil are often performed at field capacity (pF2), since the animals perform well at this ‘availability’ of water. At this pF-value, artisoil contains ca. 35 percent water (w/w dry wt basis). The optimum soil humidity for *E. crypticus* in artisoil is slightly lower (26% w/w).

The proportion clay of the soils ranged from 1.15 to 1.79% dry wt. The proportions of organic material, as measured by LOI%, ranged from 1.88 to 6.38% dry wt, and total-C% ranged from 1.7 to 4.3% dry wt. LOI% and total-C% were significantly correlated, $r^2 = 0.96$, $P<0.001$). Often, for Dutch soils, the relationship between C% and OM% (measured as LOI%) is given by 1.7*C%=OM% (Locher & De Bakker, 1994; see Table 3.1). For comparison, measurements of artisoil showed 4.44% clay (added was 10% of kaolin clay) and 13.4% organic material (added was 10% *Sphagnum*-peat). For clay%, OM% and total-C%, differences among Budel soils are smaller than between Budel soils and artisoil, while the contents of clay and organic matter are lower in the Budel soils .

3.2 Metal concentrations

The results for pH, LOI%, clay% and water retention characteristics imply that the metals present in the Budel soils may show different partitioning over solid and liquid phase, both between Budel soils and in comparison with OECD artificial soil. This may have consequences for the availability of the metals for the organisms. A large difference in metal sorption properties between Budel soils on the one hand and artisoil on the other hand is expected, due to (1) different proportions of metal sorbing soil constituents, (2) different sorption characteristics of the soil constituents, and (3) different history of metal addition (industrial history *versus* metal-salt addition). To deal with these probable differences in metal behaviour, the metals were extracted by several methods. This enables a more refined characterisation of the metal content of the soils in relation to toxic effects of exposed organisms.

3.2.1 Total metal concentrations

Total metal concentrations in Budel soils generally decreased with increasing distance from the smelter (Table 3.2, Figure 3.2). The pattern of concentration change with increasing distance could be summarized in distance-concentration relationships, following Equation 1; the parameters of the curve are summarized in Table 3.8, where they are compared with the curves for the extractable- and pore water metal concentrations.

#	Ca	Zn	Pb	Cu	Cd	Fe	Cr
				mg.kg^{-1} dry wt			
1	*300.0*	*598.0*	*325.0*	*68.0*	*3.00*	*6550*	*8.00*
2	539.2	1787.3	424.0	250.0	3.92	15686	15.69
P	94.0	347.0	175.0	32.0	3.80	2217	2.00
3	304.3	207.0	147.8	28.7	0.87	6130	40.87
4	288.5	219.2	91.3	17.3	0.77	4740	6.73
5	<50	32.0	40.0	10.0	0.40	1960	3.00
6	74.0	28.2	61.3	5.4	0.29	2935	11.87
7	<50	22.5	20.0	4.0	0.40	1920	4.00
8	100.0	27.0	45.0	3.0	0.20	2820	3.00
9	<50	20.0	25.0	2.0	0.40	1530	4.00
10	100.0	23.0	10.0	2.0	0.30	3360	n.d.
11	<50	10.6	19.3	1.9	0.14	2043	5.87

Table 3.2. Total metal concentrations (mg.kg^{-1} dry wt) of the Budel soils (homogenised, sieved, stored). Measurements were made without prior extraction of pore water or $CaCl_2$ extraction. '<': indicates values below detection limit.

Correlations among *ln*-transformed metal concentrations were significant for most pairs of metals (Table 3.3). This indicates a common source of the metals. Only at location #6, near a motorway, the concentrations of Pb, Cr and Fe showed a local increase of the total metal concentrations compared to the adjacent sites #5 and #7. Total metal concentrations were also correlated to the total Ca-concentration and to pH (whether measured as pH(H_2O) or pH(KCl)). Other correlations between soil factors and metal concentrations are not shown, since they show a similar pattern (the 0.01*M* $CaCl_2$-extractable (mg.kg^{-1} dry wt)- and pore water (mg.l^{-1}) metal concentrations among each other and with pH), or they are not significant (metal concentrations with %clay, pF-characteristics, organic matter content).

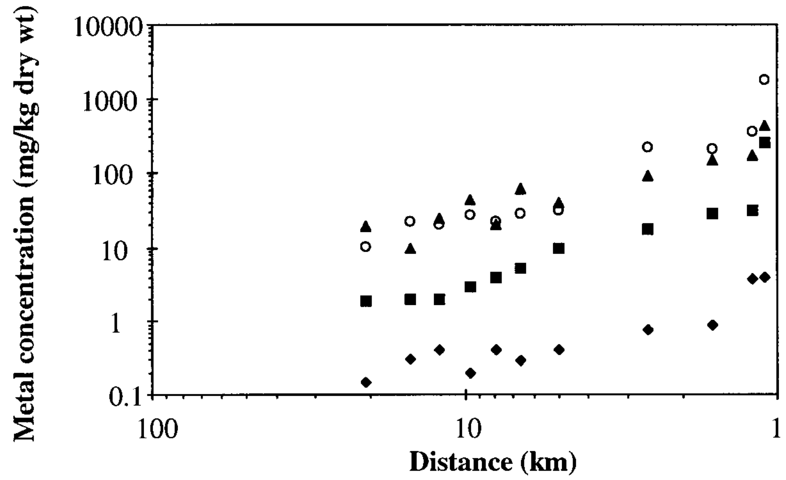

Figure 3.2. Pattern of concentration change (total concentrations) at different distances from the smelter for the metals Zn (○), Cu (■), Pb (▲) and Cd (◆).

	Zn	sign.	Pb	sign	Cu	sign	Cd	sign	Fe	sign	Cr	sign	Ca	sign.
Zn	1													
Pb	0.92	**	1											
Cu	0.97	**	0.94	**	1									
Cd	0.93	**	0.83	**	0.89	**	1							
Fe	0.80	**	0.71	*	0.79	**	0.57	n.s.	1					
Cr	0.38	n.s.	0.45	**	0.43	n.s.	0.16	n.s.	0.70	*	1			
Ca	0.79	**	0.64	*	0.78	**	0.54	n.s.	0.92	**	0.59	n.s.	1	
pH	0.92	**	0.78	**	0.88	**	0.82	**	0.79	**	0.40	n.s.	0.84	**

Table 3.3. Correlations among metals (total concentrations) and between metals and pH(KCl). Sign.=significance level: n.s.: $P > 0.05$; *: $0.01 < P \leq 0.05$; **: $0.001 < P \leq 0.01$.

3.2.2 0.01M $CaCl_2$-extractable metal concentrations

Neutral-salt extractable metal concentrations of type $CaCl_2$-1 generally showed a decrease with increasing distance to the factory for zinc, lead, copper and cadmium, but the pattern of decrease is more variable than for total metal concentrations (Table 3.4). The $CaCl_2$-type 2 extractability of zinc from Budel soils was considerably lower than in $CaCl_2$-type 1, as a consequence of adjusting the pH in the soils for use in earthworm experiments. The distance-concentration curves are summarized below (section 3.2.4).

3.2.3 Pore water concentrations

Pore water metal concentrations in 3 types of pore water are summarized in Table 3.5 for PW1, Table 3.6 for PW2, and Table 3.7 for PW3. The latter soils were also used in the toxicity experiments with worms (see Chapter 4 and 5). Chromium and iron concentrations were not determined.

The absolute amounts of metals extracted from the soil by the collection of pore water was lower than the extraction efficiency of 0.01*M* $CaCl_2$, as demonstrated by the lower proportions (%) of extracted metals (compare Table 3.4 - Table 3.7). With the neutral salt extraction, the extracted proportion reaches up to >50% for cadmium and zinc in some soils, whereas the extracted proportion with pore water is always less than 1 percent.

Pore water metal concentrations generally showed a decrease with increasing distance to the factory for zinc, lead, copper and cadmium. The pattern of decrease is, as also found for the 0.01*M* $CaCl_2$ extractable metal fractions, more variable than in the case of total metal concentrations (Table 3.2).

#	Humidity	Zn	Zn (pH 6)[1]	Pb	Cu	Cd	Fe	Zn	Zn (pH 6)[1]	Pb	Cu	Cd
	(%)	mg.kg^{-1} dry wt						% of total				
1	*2.225*	*224.9*	*n.d.*	*2.0*	*0.6*	*1.02*	*1.4*	37.6	*n.d.*	0.6	0.9	*34.1*
2	2.014	312.8	164.69	0.5	1.0	0.84	1.2	17.5	9.21	0.1	0.4	21.4
P	1.000	164.0	n.d.	3.5	0.2	1.01	9.3	47.3	n.d.	2.0	0.6	26.6
3	2.193	61.2	10.46	3.8	0.7	0.41	35.5	29.6	5.05	2.6	2.5	46.9
4	1.649	80.3	10.13	0.5	0.1	0.30	3.5	36.6	4.62	0.5	0.9	39.1
5	2.153	14.8	0.94	2.0	<0.1	0.25	38.1	46.4	2.93	5.0	n.d.	62.8
6	2.101	10.2	0.72	3.1	<0.1	0.20	77.2	36.1	2.55	5.0	n.d.	69.3
7	2.267	10.7	0.98	<0.5	<0.1	0.26	48.3	47.7	4.34	n.d.	n.d.	63.9
8	2.219	12.0	0.72	2.5	<0.1	0.15	74.0	44.5	2.67	5.6	n.d.	75.2
9	2.108	11.7	1.09	0.5	<0.1	0.25	34.5	58.5	5.45	2.0	n.d.	63.5
10	2.158	14.2	0.35	0.5	<0.1	0.15	62.9	61.9	1.50	5.0	n.d.	50.9
11	2.161	2.3	0.11	0.5	<0.1	0.08	53.8	21.2	1.03	2.6	n.d.	52.1

Table 3.4. Extraction conditions (humidity of the soil, %w/w; 10 g ca. 2% wet soil was extracted in 100 ml extractant) and 0.01*M* $CaCl_2$ extractable metal concentrations (mg.kg^{-1} dry wt, and %) of the Budel soils (homogenised, sieved, stored, pH not adjusted). Measurements were made without prior extraction of pore water. Extractable concentrations expressed in mg.l^{-1} extractant can be obtained by the following calculation: (for example) the 0.01*M* $CaCl_2$ extractable concentration of zinc in soil #2 is 312.8 mg.kg^{-1} dry soil ≡ 307 mg.kg^{-1} ca. 2% humid soil ≡ 30.7 mg.l^{-1}, since 10 g of ca. 2% humid soil in 100 ml extractant was used.

[1] For Zn, additional data were kindly provided by C. E. Smit, for soil batches of which the pH was adjusted to ca. 6.0.

n.d.: not determined; '<' indicates values below detection limit.

The soil treatments may influence the pore water concentrations of metals in a systematic way, e.g. adjusting pH to 5.5 is expected to cause a decreased metal concentration in the pore water. To quantify this effect, the difference in pore water concentrations was calculated per soil, for example as $[Me]_{PW1}$ - $[Me]_{PW2}$, and after that averaged over the soils #2- #11. These calculations show, that the effect of drying, homogenisation, sieving and rewetting caused a decrease of lead and zinc concentrations in the pore water, and a slight increase of zinc concentrations, while cadmium was not affected (comparison of PW1 and PW2). Adjustment of the pH resulted, however, in larger effects, viz. a decreased pore water concentration for all metals (comparison PW2 and PW3). Apparently, the effect of physical treatments (sieving, homogenizing, rewetting) on metal concentrations in the pore water is smaller than the effect of adjusting (increasing) pH.

#	Extr. vol.	Zn	Pb	Cu	Cd	Zn	Pb	Cu	Cd
	ml.kg^{-1}			mg.l^{-1}				%	
1	*44.64*	*47.034*	*0.281*	*0.177*	*0.148*	0.35	0.00	0.01	0.22
2	31.46	61.928	0.083	0.112	0.078	0.11	0.00	0.00	0.06
P	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3	27.02	23.086	0.407	0.070	0.103	0.30	0.01	0.01	0.32
4	27.43	27.819	0.115	0.053	0.089	0.35	0.00	0.01	0.32
5	31.98	3.511	0.222	0.025	0.038	0.35	0.02	0.01	0.30
6	33.48	1.719	0.555	0.058	0.017	0.20	0.03	0.04	0.19
7	36.41	1.295	0.025	0.013	0.020	0.21	0.00	0.01	0.18
8	45.49	2.413	0.180	0.024	0.028	0.41	0.02	0.04	0.64
9	40.06	1.079	0.040	0.017	0.013	0.22	0.01	0.03	0.13
10	23.81	1.857	0.022	0.018	0.025	0.19	0.01	0.02	0.20
11	31.83	0.719	0.146	0.039	0.012	0.22	0.02	0.06	0.26

Table 3.5. Pore water metal concentrations (mg.l^{-1}, or % of total) of Budel soils (PW1). Extr. vol.= extracted volume of pore water per kg field-wet soil. n.d.: not determined.

#	Extr. vol.	Zn	Pb	Cu	Cd	Zn	Pb	Cu	Cd
	ml.kg^{-1}			mg.l^{-1}				%	
1	*59.54*	*30.418*	*0.184*	*0.130*	*0.087*	0.30	0.00	0.01	0.17
2	66.36	35.388	0.075	0.213	0.055	0.13	0.00	0.01	0.09
P	55.77	43.962	0.166	0.090	0.228	0.71	0.01	0.02	0.33
3	67.70	6.095	0.215	0.092	0.024	0.20	0.01	0.02	0.19
4	50.71	12.217	0.197	0.093	0.035	0.28	0.01	0.03	0.23
5	61.73	1.589	0.162	0.039	0.017	0.31	0.02	0.02	0.26
6	63.76	1.118	0.233	0.034	0.015	0.25	0.02	0.04	0.33
7	72.98	1.014	0.050	0.023	0.014	0.33	0.02	0.04	0.25
8	66.53	1.615	0.346	0.064	0.018	0.40	0.05	0.14	0.61
9	59.60	0.948	0.078	0.019	0.010	0.28	0.02	0.06	0.14
10	61.86	0.831	0.025	0.023	0.015	0.22	0.02	0.07	0.30
11	67.85	0.412	0.051	0.022	0.007	0.26	0.02	0.08	0.33

Table 3.6. Pore water metal concentrations (mg.l^{-1}, or % of total) of Budel soils (PW2). n.d.: not determined.

#	Extr. vol.	Zn	Pb	Cu	Cd	Zn	Pb	Cu	Cd
	ml.kg^{-1}			mg.l^{-1}				%	
1	*61.55*	*21.549*	*0.136*	*0.105*	*0.064*	*0.22*	*0.00*	*0.01*	*0.13*
2	58.35	22.504	0.129	0.111	0.066	0.07	0.00	0.00	0.10
P	57.12	4.532	0.061	0.048	0.031	0.07	0.00	0.01	0.05
3	72.32	1.740	0.192	0.112	0.009	0.06	0.01	0.03	0.08
4	70.08	6.586	0.125	0.076	0.021	0.21	0.01	0.03	0.19
5	68.17	0.294	0.075	0.038	0.004	0.06	0.01	0.03	0.07
6	67.08	0.209	0.073	0.033	0.002	0.05	0.01	0.04	0.04
7	69.37	0.124	0.014	0.015	0.002	0.04	0.00	0.03	0.03
8	62.62	0.301	0.183	0.049	0.003	0.07	0.03	0.10	0.08
9	62.75	0.170	0.026	0.020	0.002	0.05	0.01	0.06	0.03
10	68.59	0.098	0.011	0.013	0.002	0.03	0.01	0.04	0.04
11	63.08	0.275	0.050	0.027	0.003	0.16	0.02	0.09	0.11

Table 3.7. Pore water metal concentrations (mg.l^{-1}, or % of total) of Budel soils (PW3). n.d.: not determined.

3.2.4 Distance-concentration relationships

Distance-concentration relationship are summarized in Table 3.8 for the total, the two types of extractable-, and the three types of pore water concentrations. Figure 3.3 shows, as an example, the relationships between distance and the total-, 0.01*M* $CaCl_2$-extractable-, and pore water zinc concentrations measured in the Budel soils. Even though the linear regression model for the total zinc concentration is one of the most accurate models, apparently the data show a large 'noise'.

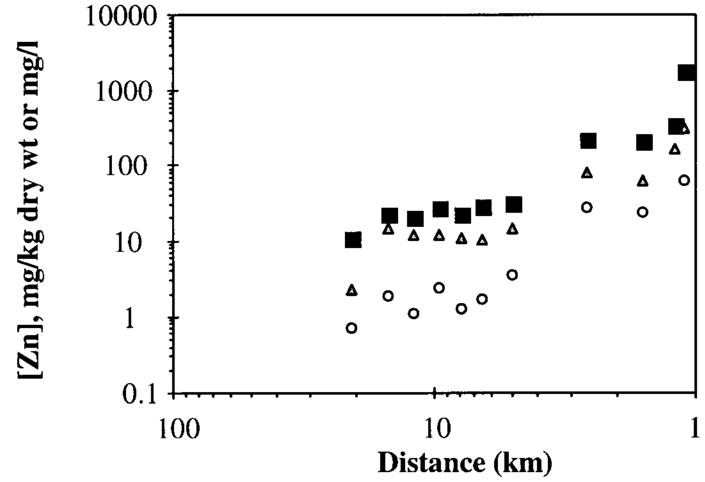

Figure 3.3. Relationship between distance and Zn concentration for three expressions of Zn concentrations, namely total Zn concentrations (■), 0.01M $CaCl_2$-extractable Zn concentrations (type $CaCl_2$-1, ▲), and pore water (type PW1, ○).

The different values of *F* indicate that the proportion of variance in the metal concentrations that is explained by the factor 'distance' differed between metals, and between the different measurement methods applied for each metal (Table 3.8). Next to zinc, it is also obvious for cadmium that local factors cause only slight 'noise' upon the trendlines. The extractable- or pore water concentrations for copper, lead and iron appear to be strongly influenced by local soil factors, except for the pore water concentrations of copper. This is indicated by relatively low *F*-values. For iron the regression of extractable concentrations on distance even showed a significant positive estimate of *A*, which implies that the extractable iron concentration increases with increasing distance. The total chromium concentrations are low, and were not related to distance. Extractable concentrations and pore water concentrations of chromium were below detection limit, or not measured (resp.).

The total calcium concentration is negatively associated with distance. Locations with a total calcium concentration above detection limit showed a stepwise pattern of concentration decrease. The total calcium concentrations of the locations #2, 3 and 4 near the factory varied between 288 and 539 $mg.kg^{-1}$. At more distant sites, but including #P, a maximum value of 100 $mg.kg^{-1}$ was found. This stepwise trend of total calcium concentrations is significantly correlated with pH ($r^2=0.84$, $n=7$, $0.001<P\leq0.01$, see section 3.1 and Figure 3.1).

Metal	Determination	Parameters of relationship			
		A	B	F	signif. (F)
Zn	Total	-1.4459	6.4944	68.6	***
	CaCl$_2$-1	*-1.3114*	*5.3119*	*64.2*	***
	CaCl$_2$-2	-2.0370	4.1621	63.2	***
	PW1	*-1.5199*	*4.0590*	*71.4*	***
	PW2	*-1.4598*	*3.4106*	*77.0*	***
	PW3	-1.5639	2.1241	30.7	***
Pb	Total	-1.0051	5.6053	58.6	***
	CaCl$_2$-1	*-0.3031*	*0.6655*	*1.1*	*n.s.*
	PW1	*-0.3835*	*1.5270*	*1.0*	*n.s.*
	PW2	*-0.3318*	*-1.6558*	*1.9*	*n.s.*
	PW3	-0.5275	-1.9538	4.2	*
Cu	Total	-1.3919	4.4018	72.2	***
	CaCl$_2$-1	*-1.3567*	*2.7036*	*1.0*	*n.s.*
	PW1	*-0.5361*	*2.4311*	*9.7*	*
	PW2	*-0.6748*	*-1.9493*	*33.4*	***
	PW3	-0.5782	-2.3266	16.2	**
Cd	Total	-0.9574	0.9611	46.0	***
	CaCl$_2$-1	*-0.6622*	*0.2341*	*47.9*	***
	PW1	*-0.7366*	*2.1588*	*33.9*	***
	PW2	*-0.7797*	*-2.5419*	*22.5*	***
	PW3	-1.1397	-3.3645	37.8	***
Fe	Total	-0.4137	8.7307	6.2	*
	CaCl$_2$-1	*0.9888*	*1.5904*	*11.3*	**
Cr	Total	-0.3120	2.2970	1.1	n.s.
Ca	Total	-0.5966	5.6337	3.6	*

Table 3.8. Parameters and significance of the linear regression models relating distance to metal concentrations (both *ln*-transformed). Models with indirect relevance for the experiments with worms are put in *italic* script. Significance of linear regression was tested with an *F*-test according to Sokal and Rohlf (1981). n.s.: $P>0.05$; *: $0.01<P\leq0.05$; **: $0.001 < P \leq 0.01$; ***: $P \leq 0.001$. n.d.: not determined.

3.3 Sorption analyses using total- and pore water concentrations

Based on total metal concentrations and pore water concentrations (PW1 to PW3) to represent the solid and liquid phases of the soil, respectively, average partition coefficients K_p and Freundlich isotherm parameters (K_f and n) were calculated for each metal in Budel soils #2 - #11 (Table 3.9).

3.3.1 K_p - values.

Averaged partition coefficients on the basis of PW1 differed among metals (Table 3.9). For copper and lead (average $K_p > 400$), the sorption to the solid phase was proportionally stronger than for cadmium and zinc. Partition coefficients for these metals showed large standard errors. This may be largely due to the differences in soil pH between Budel sites, but also other differences in soil characteristics may account for this.

			Partition-coefficient approach		Freundlich-isotherm approach		
Metal	Pore water type (Budel)	no.	average K_p $(l.kg^{-1})$	s.e.	K_f $(l.kg^{-1})$	s.e. K_f (lower, upper)	n
Zn	PW1	10	14.6	2.0	13.93	11.5, 16.7	1.023
	PW2	11	24.5	3.3	23.42	19.6, 27.0	1.047
	PW3	11	110.3	17.8	89.12	76, 105	1.215
Cu	PW1	10	417.2	206.1	(2628)	n.d.	(0.576)
	PW2	11	268.0	95.4	(1885)	n.d.	(0.564)
	PW3	11	409.1	190.8	(1962)	n.d	(0.601)
Pb	PW1	10	881.9	476.6	(138.6)	n.d.	(2.064)
	PW2	11	911.5	482.7	(258.3)	n.d.	(1.395)
	PW3	11	1174.9	300.9	(581.9)	n.d.	(1.177)
Cd	PW1	10	17.7	4.3	9.386	3.6, 24.6	1.145
	PW2	11	28.2	5.0	25.557	12.9, 50.6	0.994
	PW3	11	117.8	19.8	26.348	13.8, 50.5	1.350

Table 3.9. Average partition coefficients (K_p) and Freundlich isotherm parameters (K_f and *n*) of Budel soils (32-11) based on total- and pore water metal concentrations (PW1 to PW3).

no. = number of observation points for the average K_p- or for the Freundlich isotherm calculations. Values between brackets indicate that the Freundlich isotherm could not accurately be fitted to the data due to the influence of soil inhomogeneity along the gradient on metal partitioning. Standard errors for *n* are not shown. n.d. = not determined; (lower, upper) gives the lower and upper (backtransformed) values of the standard error.

For zinc and cadmium, averaged partition based on PW2 and PW3 are higher than those based on PW1. For lead the difference was small. For copper the partition coefficients based on PW2 and PW3 tended to be lower than in PW1. This implies that the proportion of metals in the pore water in the homogenised and rewetted soil is lower than in the field soil for both zinc and cadmium, higher for copper, and similar for lead. These effects may be attributable to the lower water content of the field soils (see par. 3.1), to the effects of the soil treatment (drying, homogenisation, storage), or both. With the additional adjustment of pH to ca. 5.5, partition coefficients using PW3 increased compared to PW2 for all metals. This indicates that metals are more strongly bound to the solid phase in the earthworm test system, compared to Budel field soil. Overall, partition coefficients seem to be more sensitive to pH changes than to changes in soil water content in combination with homogenisation.

Next to judging differences between metals on the basis of the average K_p-values, also the local K_p-values can be evaluated, to judge the variability among Budel soils. The local K_p-values differ between sites (Figure 3.4). For lead and copper the highest partition coefficients, i.e. the strongest sorption of metals to the solid phase, were found for soils collected nearest to the factory, for all three pore water types. For zinc and cadmium, however, the highest partition coefficient is found at the location #2 for PW1 and PW2. After adjustment pH to ca. 5.5 the K_p based on PW3 differs between soils, but does not covary with distance for these metals.

Partition coefficients based on pore water concentrations have not been published for artisoil. Data on metal sorption properties of Budel soils can thus not be compared to data for artisoil on the basis of pore water concentrations and K_ps.

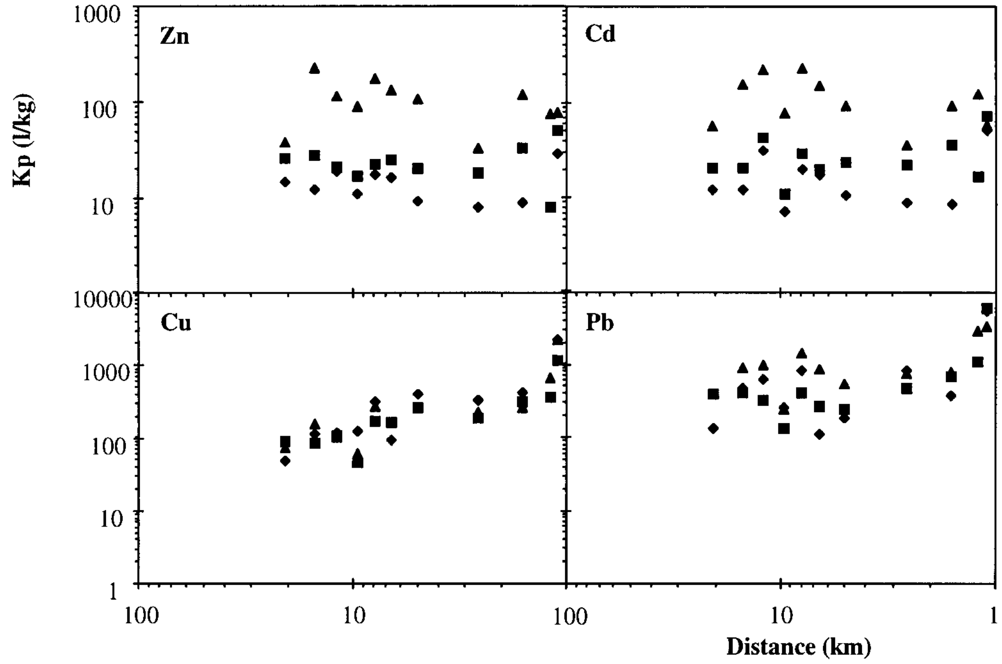

Figure 3.4. Partition coefficients K_p for four metals, calculated as the ratio of total (*Aqua regia*) metal concentrations over pore water concentrations. Pore waters were collected from three differently treated soil batches from each site: ◆ PW1; ■ PW2; ▲ PW3.

Comparison with other Dutch soils is, however, possible on the basis of literature data. Such comparisons show that partition coefficients for zinc and lead in two independently sampled Budel soils are similar (based on pore water similar to PW1), namely 23 and 41 $l.kg^{-1}$ (Zn) and 1326 and 1159 $l.kg^{-1}$ (Cd) (Janssen et al., 1996). For cadmium and copper the values found by these authors were higher resp. lower than our observation, namely 144 and 159 $l.kg^{-1}$ (Cd) and 25 and 38 $l.kg^{-1}$ (Cu). Janssen et al. (1996) showed, furthermore, that field-based partition coefficients of contaminated soils could differ by orders of magnitude between different soil types in the Netherlands.

3.3.2 Freundlich isotherms.

Freundlich isotherms were calculated on the basis of total metal concentrations ($mg.kg^{-1}$ dry wt) and pore water concentrations ($mg.l^{-1}$). The Freundlich shape-parameter *n* is near unity for zinc and cadmium, and deviates strongly from this for copper and lead (Table 3.9, see also: Figure 3.5). For the former metals, the average K_p - value is grossly similar to K_f, with a relatively small standard error for both. For these metals, the Freundlich adsorption isotherm may be used for a gross estimation of metal concentrations in the pore water in a soil from Budel, when the total concentration is known. For copper and lead, however, local factors apparently affect metal partitioning strongly, so that metal partitioning for these metals cannot be accurately predicted from information on total concentrations alone. More complex, multivariate models might be applied to predict metal partitioning of copper and lead more accurately (see, e.g., Janssen et al., 1996; Pretorius et al., 1996).

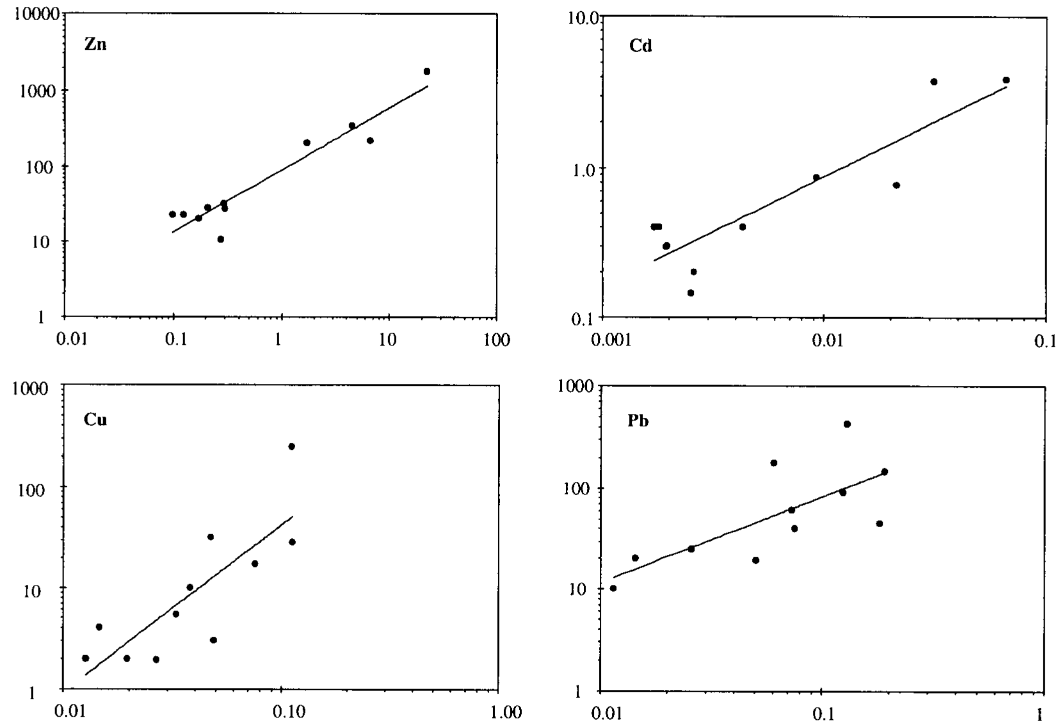

Figure 3.5. Pore water based Freundlich isotherms of the Budel soils in the conditions of the earthworm experiments (based on total- and PW3 metal concentrations). For PW1 and PW2 the curves differed (generally: shifted to the right compared to PW3). Observations are indicated by ●, whereas the fitted lines represent the Freundlich isotherm calculated assuming homogeneity of soil characteristics over the Budel gradient. The isotherm fitted reasonably well for zinc and cadmium, whereas for copper and lead the assumption was apparently violated (see for parameter estimates: Table 3.9). The X-axes give the metal concentrations in the pore water in mg.l^{-1}; the Y-axis gives the total concentration in mg.kg^{-1} dry wt. Note the large differences in X- and Y axes formats, indicating large differences in sorption characteristics between metals.

3.4 Sorption analyses using total- and extractable concentrations

Literature data on Freundlich isotherms for artisoil are not available on the basis of total- and pore water concentrations (as in the previous paragraph). The pore water concentration is only one of the possible ways to address the (potentially bioavailable) metal fraction in soil solution. As an alternative, 0.01M CaCl$_2$-extractable metal fractions have been used to quantify the labile (weakly sorbed) metal fraction for OECD. To enable comparisons between Budel soils and artisoil, the metal concentrations in the 0.01M CaCl$_2$-extracts (Table 3.4) were recalculated to mg.kg^{-1} on the basis of the soil-extractant ratio (ca. 10 g soil of ca. 2% humidity w/w in 100 ml extraction medium[3]) and the data presented in Table 3.4. CaCl$_2$-based Freundlich isotherms

---

[3] The standard procedure is, to make an extract of humid soil, in a soil (dry wt): liquid ratio of 10 g in 100 ml. In the case of artisoil this means making an extract of soil containing 35 or 26% water (for *Eisenia* and *Enchytraeus* experiments, resp.); for Budel soil, the extracts were made from the stored batches (ca. 2% water). In both ases, the original AAS observations were used to express the extractability in mg.l^{-1}.

were fitted to the data from the Budel soils for each metal and compared with similar data for artisoil (Table 3.10).

Metal	Soil type	Exp. code	Experiment description	balance	pH*	K_f ($l.kg^{-1}$)	(s.e.)	*n*	Ref.
Zn	Budel	Ea	$CaCl_2$-1 extract	-	2.9 - 4.9	25.1	(21.7 - 29.0)	0.94	
	Budel	Ea	$CaCl_2$-2 extract	-	ca. 5.5	153	(143 - 162)	1.30	
	OECD	Ea Zn1	Zn only (unbalanced)	N-683/1896	4.8	47.1	(45.8 - 48.5)	1.23	1
	OECD	Ea Zn2	Zn mixed with Cd	N-1486	5.9	59.3	(55.2 - 63.8)	1.24	1
	OECD	Ec Zn1	Zn only	C-824	6.0	195	(181 - 210)	1.91	1
	OECD	Ec Zn2	Zn-only series from Cd+Zn mixture exp.	C-810	6.0	15.6	(13.8 - 17.6	1.02	1
	OECD	Ec Zn2	Zn mixed with Cd from Zn+Cd exp.	C-810	6.0	15.8	(14.3 - 17.7)	1.03	1
	OECD	Ec Zn3	Zn-only series from Cu+Zn mixture exp.	C-1432	6.6	193	(183 -205)	1.29	2
	OECD	Ec Zn3	Zn mixed with Cu from Zn+Cu mixture exp.	C-1432	6.6	71.3	(68.7 - 74.0)	2.00	2
Cu	Budel	Ea Cu1	$CaCl_2$-1	-	2.9 - 4.9	1061	(136 - 8315)	1.04	
	OECD	Ec Cu3	Cu-only series from Cu+Zn mixture exp.	C-1432	6.6	836	(688 - 1017)	1.89	2
	OECD	Ec Cu3	Cu mixed with Zn from Cu+Zn mixture exp.	C-1432	6.6	821	(782 - 862)	2.71	2
Pb	Budel	Ea	$CaCl_2$-1	-	2.9 - 4.9	145	(60 - 351)	2.22	
	OECD	Ea Pb1	Pb only	N-598	4.8	818	(768 - 873)	1.48	1
	OECD	Ea Pb2	Pb only (higher $[Pb]_{max}$)	N-2992	5.3	1480	(1329 - 1651)	2.00	1
Cd	Budel	Ea	$CaCl_2$-1	-	2.9 - 4.9	92.7	(60.4 - 142)	0.71	
	OECD	Ea Cd1	Cd only	N-353	4.8	38.7	(36.6 - 41.0)	1.22	1
	OECD	Ea Cd2	Cd mixed with Zn	N-1486	5.9	44.4	(41.7 - 47.2)	1.27	1
	OECD	Ec Cd1	Cd only	C-126	6.6	12.0	(11.7 - 12.3)	0.98	1
	OECD	Ec Cd2	Cd-only series from Cd+Zn mixture exp.	C-810	6.0	10.0	(9.2 - 10.8)	0.88	1
	OECD	Ec Cd2	Cd mixed with Zn from Cd+Zn mixture exp.	C-810	6.0	13.1	(11.2 - 15.4)	1.01	1

Table 3.10. $CaCl_2$-based Freundlich isotherm parameters (K_f and *n*) of Budel soils and artisoil based on total- and 0.01*M* $CaCl_2$ extractable metal concentrations. No. of observation points for the Budel-Freundlich isotherm calculations as in Table 3.9. Observations in artisoil are recalculated from the data of (1) Weltje et al., 1995, and (2) unpublished data. Recalculation for (1) was necessary to express data in the same units for different soil types, namely $mg.kg^{-1}$ dry wt and $mg.l^{-1}$ for total and extractable concentrations, respectively. Standard errors for *n* are not shown. In experiments in artisoil, the anion concentration was balanced with nitrate (N) or chloride (C) $mg.kg^{-1}$ dry wt. 'Ea' indicates experiments with *Eisenia andrei*; 'Ec' indicates experiments with *Enchytraeus* crypticus; similar numbers following the species code indicate simultaneous experiments, with the same batch of OECD, pH and balance-conditions. The indicated pH is typical for the batch of artisoil that was used for extraction and earthworm exposure, although pH usually rises during the exposure of worms.

Experiments done in artisoil share some characteristics that are relevant for this purpose, namely:

- extracts were made of wet soil, in an amount equivalent to ca. 10 g dry soil in 100 ml extractant; the water content differs between soil used for experiments with *E. andrei* and *E. crypticus* (35% and 26% water on a dry wt basis, respectively);
- anion concentrations were balanced (except for the first experiment with zinc and *E. andrei*), in initial experiments with potassium nitrate and in later experiments with potassium chloride, with the concentration of the balancing anion being equal to the anion concentration in the highest metal treatment;
- soil pH of the soil batch is measured prior to the experiments, but usually rises during earthworm rearing; the 0.01*M* $CaCl_2$ extraction, however, was made prior to exposure;
- soil pH for enchytraeids is usually adjusted to ca. 6, whereas for *Eisenia* it is usually adjusted to ca. 5.5;
- in mixture experiments the proportion of extractable metal *i* may be influenced by the addition of another metal *j*; the isotherm of metal *i* is thus determined in a range where both the concentrations of *i* and *j* are varying. Notice in Table 3.10 that some mixture experiments were done simultaneously (for example a zinc, a cadmium and a zinc+cadmium series), so that the soil batch and balance concentrations of ions were similar: variation in Freundlich parameters is in such cases not influenced by random variation in experimental conditions.

Various comparisons can be made using these data, namely:

- (1) Comparisons of pore water- and $CaCl_2$-based Freundlich isotherms. Both isotherms fitted better to the Budel-data of zinc and cadmium than for lead and copper. Apparently, the local variation causing bad fit for pore waters for these metals (see Table 3.8) also influenced the 0.01*M* $CaCl_2$-extractable concentrations (for further remarks: see paragraph 3.3.2).

*- (2) Comparison among metals with respect to 0.01*M $CaCl_2$*- extractability.* The Freundlich parameter K_f over all measured soils (Budel and OECD) is lower and less variable for zinc and cadmium than for copper and lead. The extractability of cadmium and zinc appears thus to be generally higher than for lead and copper, and better predictable.

- (3) Effect of adjustment pH in Budel soils, and of pH differences between artisoil experiments. The effect of adjustment pH from 2.9-4.9 in Budel soils to ca. 5.5 has only been measured for zinc. The extractability of zinc decreased considerably due to pH adjustment. In artisoil, extracts have been made from soil batches used in various experiments that differed with respect to pH (4.8 to 6.6). The differences result from different pH-adjustment levels between both worm species, and from random variation. Random (unplanned) variation may arise due to differences in: (1) peat batch and peat particle size (1 mm or 0.5 mm) used for preparing the soil, (2) inaccuracies in adjustment soil pH, and (3) addition of the metal itself (metal ions may cause a release of H^+- ions, resulting in low pH at high metal concentrations), or of balance

concentrations of anions. Given this variation, there may also be a tendency that extractability of metals decreases with increasing pH in artisoil of similar kind (for zinc: compare the *E. andrei* experiments EaZn1 with EaZn2, and EaCd1 with EaCd2), although other (unknown) factors appear to have a major influence upon extractability. For example, comparison of the enchytraeid experiments EcZn1 with EcZn2 and EcZn3 show no obvious cause of the relatively low extractability in EcZn2.

- *(4) Comparisons between Budel soils and artisoil.*

(a) Zinc. Only for zinc a comparison can be made between extractability at similar soil pH from both soil types. The extractable zinc concentration of Budel soils is a factor 2 - 3 lower than in artisoil prepared for experiments with *E. andrei* ($K_{f,Budel}$ = 152.7 vs. $K_{f,OECD}$ = 69.3 or 86.9 in the absence and presence of cadmium, resp.). For the experiments with *E. crypticus*, for which finer ground peat (<0.5 mm) is used, both higher and lower extractability was observed in comparison with Budel soils. This variation could not be traced back to erroneous sample handling (Weltje et al., 1995), which implies that the extractability of zinc may be sensitive for uncontrolled experimental conditions. The decreased sorption of zinc in the presence of copper (K_f changed from 244 to 83 $l.kg^{-1}$ in the absence and presence of different copper concentrations in experiment Ec Zn3, resp.) may support this sensitivity hypothesis.

(b) Copper. The extractable concentrations in both soil types are similar, although a difference is to be expected if extracts would have been made from pH-adjusted Budel soils. An increase of the copper K_f for Budel soils upon adjustment of pH would imply that the availability of copper may be higher in artisoil than in Budel soil.

(c) Lead. The extractable lead concentration of Budel soil without pH adjustment ($CaCl_2$-type 1) is high compared to two observations of lead extractability in artisoil (at pH ca. 5.5). It is to be expected that the extractability of lead from Budel soils decrease when the pH rises; whether the extractability will be equal, lower or higher than from artisoil cannot be established from the present data.

(d) Cadmium. Variation in extractability between experiments in artisoil was observed for cadmium. Extractability was considerably lower in experiments with *E. andrei* than in experiments with *E. crypticus* (K_f ca. 60 and ca. 15, resp.). This was not expected, since the soil pH for the latter species is usually adjusted to a higher level- and thus the expected extractability should be lower. The extractability of cadmium from artisoil appeared to be higher than from Budel soils for both species, in particular when it is taken into account that $CaCl_2$-type 1 extraction from Budel soils is more efficient than type-2 extraction.

In the extracts made at the natural pH of the Budel soils (type $CaCl_2$-1), the order of extractability between metals as judged from K_f is Zn > Cd > Pb > Cu, although for the latter two metals the K_f values are highly variable. For artisoil the order appears to be Cd > Zn > Cu > Pb.

3.5 Summary of soil characteristics with respect to biotic responses

The data on abiotic soil characteristics collected for the Budel soils are needed to interpret data on the performance of biota in these soils, and to make the comparisons with metal toxicity in artisoil. The data show that many soil characteristics differ among sites. The following patterns are relevant for the analyses of biological response:

1. In Budel, a concentration gradient exists for various metals, with a decreasing trend of total concentrations at increasing distance from the smelter stack. The total concentrations of all these metals are highly correlated.
2. The low pH of some Budel soils is expected to affect reproduction both in *E. andrei* and *E. crypticus* directly (for optimum curves: see Van Gestel et al., 1992, Dirven-Van Breemen et al., 1994); for *E. andrei* this has been confirmed by observations (Posthuma et al., 1994).
3. Soil pH is correlated to distance to the factory, and thus covaries with the metal concentrations. The pH influences the partitioning of metals over solid and liquid phases, but also the sorption strength of the labile metal fraction to the solid phase.
4. Adjustment of pH of Budel soils to ca. 5.5 is expected to influence both earthworm reproduction directly (improved) and metal availability indirectly (reduced, at least for zinc).
5. Toxic effects of metals in *Eisenia andrei* and *Enchytraeus crypticus* are to be expected when these animals are exposed in the most severely polluted Budel soils. In these soils at least for one metal (zinc), the concentration exceeds the EC50 of these species in artisoil (see Weltje et al., 1995 and Dirven-Van Breemen et al., 1994).
6. Due to the co-occurrence of metals, toxic effects may be caused by their simultaneous action. This requires mixture toxicity data to be used in the interpretation of effect observations.
7. Soil factors other than pH and metal concentrations differed among Budel soils. These factors, e.g. LOI% and %clay, may affect earthworm performance directly or indirectly through their effects on metal partitioning. These effects will, however, result in 'noise' on the concentration-response curves, since these parameters neither co-vary with distance nor with metal concentrations. The influences of factors for which broad optimum curves have been derived for worms during exposure in artisoil (e.g., humidity, OM content), are expected to be low.
8. 0.01*M* $CaCl_2$- extractable- and pore water metal concentrations generally decreased with increasing distance from the factory. The partitioning of the metals over the solid and liquid phase of the soil is affected by local soil factors and works out for the metals in different ways. Lead and copper bind more stronger to Budel soils than cadmium and zinc, and the effect of local soil factors upon sorption is higher for lead and copper than for cadmium and zinc. To assess the available fraction of a metal it is, for this reason, better to use measured rather than interpolated concentration data in the interpretation of reproduction effects in Budel soils.

9. Differences in metal extractability between Budel soils and artisoil were judged by comparison of 0.01*M* $CaCl_2$-extractable fractions. For zinc, extractability from Budel soil appeared 2-3 times lower at similar pH than from artisoil, but other experiments in artisoil, however, showed higher extractability of zinc from Budel soils. For copper and cadmium the comparison between Budel soils at indigenous pH (ranging from 2.9 to 4.9) with artisoil (pH 5.5 or 6) suggests that the extractability is the lowest in artisoil.
10. Body concentrations of exposed organisms integrate the effects of all abiotic factors upon metal availability. Therefore, next to physico-chemical extraction methods as applied in the previous chapters, also the *net* body concentration during and after exposure was determined to judge actual metal exposure of the worms, and the relative importance of the metals for toxicity (see chapter 5.3).

4. PERFORMANCE OF WORMS IN BUDEL SOILS AND ARTISOIL: APPROACH AND METHODS

4.1 Animals

4.1.1 Eisenia andrei

Specimens were obtained from mass cultures kept in climatised conditions (T: 20 ± 2 °C) for many generations. The animals were obtained from a single culture container in which cocoons were produced 21-24 weeks before the start of the experiments. Initial body weights (fresh) were measured prior to starting the experiment, and ranged from 3.442 to 4.074 g per 10 worms. All animals were adults, as assessed from the presence of a well developed clitellum.

4.1.2 Enchytraeus crypticus

Mass cultures of *Enchytraeus crypticus* were kept on agar plates in darkness under temperature controlled conditions (T: 20 ± 2°). Basis of the agar plates was a water extract of peat soil (from Wanneperveen with pH(KCl) = 3.6). Cultures were fed every week with oat meal porridge (consisting of 3 parts rolled oats, 1 part yeast, 1 part cod-liver oil, and 1 part powdered albumen). Each agar plate contained enchytraeids of a single generation. Specimens of the same age with a clearly visible, white coloured egg in the transparent body were selected and transferred into a petri-dish filled with water. Next, these animals were transferred from the water to the test containers.

4.1.3 Enchytraeus albidus

Mass cultures of the larger *Enchytraeus albidus* were kept in OECD-artificial soil in darkness under temperature controlled conditions (T: 20 ± 2°). Cultures were fed every week by putting oat meal porridge (see 4.1.2) in a hole in the centre of the culture jar. Adults with clearly recognisable clitellum were selected for experiments and transferred directly onto the soil in the test containers.

4.2 Preparation of field soils for experiments

4.2.1 Eisenia andrei

In *E. andrei*, the largest influence of metal toxicity on reproduction has previously been observed in soil #2, with a maximum reduction of cocoon production of ca. 40-60 percent in comparison with reference Budel soil (#11) (Posthuma et al., 1994). In order to make a more accurate estimate of a concentration-response curve, an additional concentration point was made by mixing equal weights of soil from sites #2 and P. Some measurements, such as on metal partitioning, have not been made for this mixed soil.

Reproduction of *E. andrei* in Budel soils was studied after ca. 1.5 yr. storage of the soils. The water content of the soils prior to the start of the experiment was 0.96%. Before worms were transferred into the soils, the water content was adjusted to 20% (w/w), to mimic the physiological availability of water in artisoil in all Budel soils.

In a previous experiment (Posthuma et al., 1994) it was shown that the low indigenous pH of some Budel soils strongly affects reproduction of *E. andrei.* This effect also occurs at low pH in artisoil (Van Gestel et al., 1992). To allow for controlling the pH in Budel soils (which is variable, see Table 3.1), titration curves were made for each batch. Titration curves relate added amounts of $CaCO_3$ to pH(KCl) at equilibrium. Based on the titration curves, the amounts of $CaCO_3$ to be added per kg soil were calculated (see Table 5.1). The pH of the $CaCO_3$-treated soil batches was adjusted to ca. 5.5 and measured to check a correct pH-adjustment. For reference, artisoil was prepared following the guidelines of the OECD (OECD, 1984), with a peat fraction sieved over 1 mm. Performance in clean artisoil was monitored to know the reproductive activity during the reproduction experiments. Performance in artisoil with metals was used to know the uptake rate of metals this soil type. Metals were added as chloride salts when metal-contaminated OECD artificial soil was needed.

4.2.2 Enchytraeus crypticus *and* E. albidus

Pilot experiments were conducted in order to check if survival and reproduction of enchytraeids would occur in reference Budel soil (#11) with indigenous pH. The first reproduction experiments with *E. crypticus* and *E. albidus* were executed (July 1993) parallel to experiments with *Eisenia* in Budel soils in which the pH was adjusted to ca. 5.5, and soil humidity was adjusted to ca. 20% (w/w) (see: Posthuma et al., 1994). A second experiment, with *E. crypticus* alone, was started in June 1994, in the same soil batches as used for the experiment with *E. andrei* reported here. For reference, reproduction of the enchytraeids was determined synchronously in artisoil with a 0.5 mm ground peat fraction.

4.3 Studying performance of worms in Budel soils

4.3.1 Eisenia andrei

The experiment design of the reproduction studies in the various soils was adopted from that described by Van Gestel et al. (1989). Toxicity studies with earthworms reported in the literature are often also performed similar to that method. This facilitates comparison with our data. Performance of the worms in artisoil was used as a kind of positive control in the experiments with the Budel soils. Observations were made in quadruplicate. Each experimental unit consisted of a glass jar with ca. 700 g of rewetted soil, to which 10 worms were added.

An experiment consists of 3 subsequent phases, namely:

1. the preconditioning phase (1 week);
2. the exposure phase (3 weeks);

3. the cocoon hatching phase (5 weeks).

Worms were fed each week of the preconditioning and exposure phase by adding ca. 7.8 g food (35 % water content w/w) to each jar. The food consisted of ground cow dung. During the test phases, the jars (phase 1 & 2) or petri-dishes (phase 3) were kept in a climate room (20 ± 2 °C), under constant illumination, to force the animals with their negative phototactic behaviour into the soil.

During the pre-conditioning phase the worms were acclimated to Budel soil from site # 11. For the positive control in OECD-artificial soil, preconditioning took place in that soil type. Site #11 acted as the reference for reproduction in the Budel soils.

After the preconditioning phase, worms were hand-sorted from the soil, counted, rinsed, surface-dried on slightly humid paper tissue, and their fresh weight (per jar) was determined. The number of cocoons produced during the preconditioning phase were counted after separating soil constituents and cocoons through sieving (2 mm mesh) under a shower. The worms were transferred to the soils of the exposure series (soil #2 - #11, #P and the mixture of #2/#P). After the exposure phase, worms were collected and weighed (fresh, per jar). Cocoons were collected by wet sieving, counted, and laid out in OECD artificial medium (ground to 0.5 mm mesh) through which cow dung was mixed. The cocoons were incubated for 5 weeks in climate room conditions. After this period, the contents of the petri-dish was washed over sieves (1 mm and 0.5 mm mesh on top of each other), to count the juveniles hatched from the cocoons. Furthermore, the numbers of infertile cocoons were counted. In contrast to the fertile cocoons, these cocoons do not float in water and contain a white gelly tissue when squeezed.

Body concentrations of the metals zinc, copper, lead and cadmium were determined in *E. andrei* after the three-weeks exposure period. Before preparing the animals for metal analyses, the gut content was voided overnight in a petri-dish, between two layers of humid filter paper. After washing the external body surface, animals were frozen in groups of 5, lyophilised and digested in hot *Aqua regia*. Metal contents were measured with AAS, and are expressed on a dry wt basis.

Observations during this experiment enable establishing of:

- % mortality;
- % body growth;
- number of cocoons per worm per week;
- number of juveniles per worm per week;
- proportion of fertile cocoons;
- body concentrations of metals.

4.3.2 Enchytraeus- species

The fundamental design and backgrounds of toxicity studies with *E. crypticus* and *E. albidus* in soils are described in Dirven-Van Breemen et al. (1994). Some adjustments in the extraction and counting method were applied.

Two experiments were done. In the first experiments (July, 1993) both *E. crypticus* and *E. albidus* were used. In the second experiment (June, 1994) only *E. crypticus* was used. In the first experiments 10- and in the second experiment 15 enchytraeids were transferred to test vials with ca. 10 g soil of the sites #2 - #11. Vials were kept in darkness at a temperature of ca. 17 °C.

Experiments with *E. crypticus* consisted of a single soil exposure phase. Due to the small size of this species, adult worms or cocoons cannot be recovered from the soil, and only the number of juveniles, as the net result of reproduction, could be determined after 4 weeks of exposure. Experiments with *E. albidus* included two phases. After 4 weeks of exposure adult worms were removed from the soil and after another 5 weeks the numbers of cocoons and juveniles were counted.

In the first experiments with *E. crypticus* and *E. albidus*, animals were extracted from the soils at the end of exposure by means of Oostenbrink's eluation technique (originally designed for nematode extraction; see Oostenbrink, 1960). In the experiment with *E. crypticus* of June 1994, soils and animals were fixed at the end of exposure by adding formaline 4% to which Bengal rose was added. Bengal rose stains animals only. Next, simple decantation techniques were used to separate animals from the soil. However, a complete separation of soil particles (organic matter and clay) and animals was not possible. Due to this, counting is more labour-intensive in this method than in Oostenbrink's eluation method, but there is a considerable increase in recovery of animals. When samples contained high numbers of enchytraeids a sample splitter was used for dividing the sample into 8 equal parts. Only a few of these parts were counted and numbers were multiplied by the fraction counted.

Observations during these experiments enable establishing of:

- number of juveniles after 4 weeks of exposure;

and for *E. albidus* in addition:

- number of cocoons per worm per week
- number of juveniles per worm per week

4.4 Exposure assessment

4.4.1 Eisenia andrei

Metal analyses made at the end of the reproduction experiment (see 4.3.1) aimed to reveal the *net* body concentrations of various metals after exposure. The analyses were made to identify the metals that may have contributed to toxic effects. This may neglect the *pattern* of body concentration change over time, which may differ between essential metals (zinc and copper) and xenobiotics (lead and cadmium). Therefore, body concentrations of zinc, copper, lead and cadmium in worms were measured in soil #2, in a time-series. To this purpose, animals were collected before preconditioning (t=0), after 1 week preconditioning (t=7), during exposure (t=8, t=10, t=14, t= 16, t=23, t=37), and after being transferred into clean soil (#11, t=38, t=42, t= 45, t=49, t=56, t=70). Soils #2 was chosen for the study of temporal uptake patterns, because sub-lethal toxic effects in this soil occurred in the reproduction experiment. As control, worms were also exposed in soil #11, but they were less frequently sampled from this soil.

4.4.2 *Enchytraeus crypticus*

The pattern of change of body concentrations over time was tested with *E. crypticus*, in soil #4 with an estimated sub-lethal effect of ca. 50 percent on reproduction. Animals were also exposed in artisoil with similar zinc and cadmium concentrations as Budel #4, which were added as chloride salts. In all soils pH was adjusted to an initial value of ca. 6, which appeared to be an optimum-pH for this species (Dirven-Van Breemen et al., 1994). Furthermore, animals were exposed in Budel soil #11 and in artisoil without added metals, as references. For each soil, 4 vials with ten adults each were used to start the experiment. Animals were kept for 28 days in the exposure soils (accumulation phase), and were then put in clean soil (depuration phase, Budel soil #11 or artisoil, respectively). For metal analyses, animals were sampled in groups of five at various time intervals during both phases, and put overnight on humid filter paper to void the gut content. Fresh weight was measured for various individuals; fresh weight was not determined for all worms since weight loss due to evaporation during weighing prohibited stabile read-out of fresh weights. All animals were frozen, lyophilised, weighed, and prepared for microanalysis of zinc and cadmium body concentrations. Metal concentrations were measured by application of graphite furnace AAS-methods. Furthermore, fresh/dry weight ratio's were estimated for the animals for which both values were obtained.

4.4.3 *Linear compartment analysis of body concentration data*

A first order one-compartment model was fitted to the data on the change of metal concentrations in the body of *Enchytraeus crypticus* during both the uptake and the depuration phase. This model was not applied to the data for *E. andrei*, since the observations indicated that one of the conditions for application of the model, namely a constant exposure, was not met. Parameter estimates of metal kinetics were obtained by a non-linear least squares estimation procedure following Janssen et al. (1991), with:

Equation 5: $$Q_t = Q_0 + \frac{a}{k}(1 - e^{-kt}) \text{ for } 0 < t \leq t_c \text{ (the accumulation phase)}$$

and

Equation 6: $$Q_t = Q_0 + (\frac{a}{k}(1 - e^{-kt}) - (\frac{a}{k}(1 - e^{-k(t-t_c)}) \text{ for } t > t_c \text{ (the depuration phase)}$$

where:

Q_t = concentration of metal in the animal at time t ($\mu g.g^{-1}$ dry wt)

Q_0 = concentration of metal in the animal at time 0 ($\mu g.g^{-1}$ dry wt)

a = assimilation rate constant ($\mu g.g^{-1}$ dry wt.day^{-1})

k = elimination rate constant (day^{-1})

t = time (days)

t_c = time of transfer to clean soil (day)

The equilibrium concentration in an animal can be calculated from the estimated parameters with:

Equation 7: $$Q_{eq} = Q_0 + \frac{a}{k}$$

where:

Q_{eq} = equilibrium body concentration during continuous exposure.

4.5 Effect assessment

Observations on reproductive activity were analysed against distance and concentration of the dominant metal. Reproduction in the reference Budel soil (#11) was adjusted to 100%, and reproduction in other soils was expressed as a fraction of this reference value.

To estimate the ED50 (interpolated Effect Distance where 50 percent reduction of performance is expected due to the mixture of the metals) or EC50 (Effect Concentration for separate metals or metal fractions) a sigmoid curve was fitted to the data. This (logistic) response curve following Haanstra et al. (1985) is given by:

Equation 8: $$y = \frac{C}{1 + e^{-b(\ln(x)-m)}}$$

with:

y = performance in soil with concentration (or distance) x;

C = value for y in reference conditions;

b = slope of the response curve at the E(D)C50-concentration on a logit-log scale;

The E(D)C50 is calculated as e^{-m}.

The concentration-response model was fitted to the observations with a GENSTAT®- or a GraphPad Prism®-programme implemented on a PC, applying non-linear least squares iteration. The model was preferably fitted to the original data, but, if the iteration procedure not converged, fits were made to the average performance of each treatment group.

The obtained parameter estimates were compared to concentration-response curves obtained in a similar way in experiments in artisoil. Comparisons among effect levels in different soils mainly focused on the level of the EC50. In addition, attempts were made to compare the curves over the whole concentration range, with emphasis on the region near the NOEC, since this toxicity parameter is often used for ecotoxicological extrapolation, for example in setting soil quality standards.

4.6 Correlation analyses

Correlations among variables were calculated by Pearson product-moment correlations. Data were logarithmically transformed when necessary, to obtain homogeneity of variances (Sokal & Rohlf, 1981).

5. PERFORMANCE OF WORMS IN BUDEL SOILS AND ARTISOIL: OBSERVATIONS AND ANALYSIS

5.1 Abiotic conditions during experiments

5.1.1 Eisenia andrei

The pH(KCl) of the Budel soil batches was largely similar to the desired value of ca. 5.5 (Table 5.1). The pH slightly increased during the exposure phase. The pH also increased slightly in the simultaneously run observations in artisoil. The acidity of the Budel soils closely mimicked the values and change that occurs in artisoil during reproduction experiments with *E. andrei* (own observations and literature data).

The water content was adjusted to 20% (w/w), and was slightly higher than field capacity (pF2) in most Budel soils (see Table 3.1). The species is relatively indifferent to water contents close to- or higher than the optimum value (Van Gestel et al., 1992). So, the slight differences in water availability are considered insignificant with respect to earthworm reproduction.

Phase	CaCO$_3$	*Eisenia andrei*		*Enchytraeus crypticus* and *E. albidus*		*E. crypticus*
		pH(KCl)		Humidity	pH(KCl)	pH(KCl)
#	(g.kg^{-1})	Begin	End	Exp. 1	Exp. 1, begin	Exp. 2, begin
Pre-condit.						
OECD	-	5.68	-			
# 11	2.28	5.06	-			
Exposure						
OECD-control	-	5.79	6.89			5.11
#2	0.00	5.30	5.66	19.6	5.2	5.21
#P/#2	1.04	5.42	5.44			
# P	2.08	5.46	6.19			
#3	1.10	5.32	6.37	17.6	5.3	5.21
#4	0.38	5.13	6.35	19.4	5.0	4.90
#5	2.42	5.26	6.09	18.9	5.1	5.17
#6	3.60	5.33	5.58	18.6	5.2	5.07
#7	3.15	5.45	5.93	18.1	5.1	4.94
#8	3.10	5.63	6.05	18.2	6.0	5.30
#9	3.60	5.48	5.61	18.1	5.1	5.05
#10	3.00	5.12	5.74	20.9	4.9	5.50
#11	2.28	5.44	6.38	18.4	5.1	5.30

Table 5.1. The amounts of CaCO$_3$ needed to reach an initial pH of ca. 5.5 in the earthworm reproduction experiments (column 2) and the acidity and humidity of the soils during experiments with *Eisenia andrei*, *Enchytraeus crypticus* and *Enchytraeus albidus*, in different phases of the experiments.

5.1.2 Enchytraeus crypticus

- *Experiment 1 (July 1993).* This experiment was run simultaneously with that of *E. andrei* described in Posthuma et al. (1994). At the start, the pH(KCl) of the Budel soils varied between 4.9-6.0 and humidity was between 17.6 and 20.9 % (w/w) (Table 5.1). Since experiments with enchytraeids were performed with small quantities of soil, no samples could be taken for pH measurement at the end of the exposure phase.

The initial pH values and the humidity were similar to the values previously reported for reproduction studies with enchytraeids in artisoil (Dirven-Van Breemen et al., 1994) and were not associated to the distance to the smelter. Therefore, these factors may only cause 'noise' on the concentration-effect relationships for reproduction.

- *Experiment 2 (June 1994).* At the start of experiment 2, pH(KCl) of the Budel soils varied between 4.9-5.5 (Table 5.1). The water content was not measured again. Soil conditions are expectedly similar to the previous experiment, since the same soil batches were used in similar conditions.

5.1.3 Enchytraeus albidus

The experiments with *E. albidus* were performed simultaneously with *E. crypticus* experiment 1 (for observations: see Table 5.1).

5.2 Worm performance in Budel soils: raw data

5.2.1 Eisenia andrei

- *Mortality*. All animals survived the experiment.

- *Body growth*. During the preconditioning phase, average body growth per treatment group ranged from 31 to 43 % .week^{-1} for Budel soils, and 20% .week^{-1} for artisoil (Figure 5.1). During the exposure phase, weight loss was observed in 9 out of 12 groups treated in Budel soils, whereas 1% weight gain was observed in artisoil. Exposure-phase weight loss was smallest in the soils collected nearest to the smelter (#2, P and the mixture #2 and P). It is common that worms that reproduce show reduced growth (see below, and also Van Gestel et al., 1992). Fresh weight data are not further mathematically analysed, due to apparent minimal responses to smelter distance.

- *Reproduction parameters*. The numbers of cocoons (c.w^{-1}.w^{-1}) and juveniles (j.w^{-1}.w^{-1}) produced in Budel soils were generally lower than in artisoil (Figure 5.2 a, b). The proportion of fertile cocoons and the number of juveniles per fertile cocoon, however, were similar in both soil types (Figure 5.2 c, d). In Budel soils collected near the factory, cocoon production, and to a lesser extent juvenile production, was reduced (Figure 5.2 a, b). These effects are not attributable to mortality differences among groups.

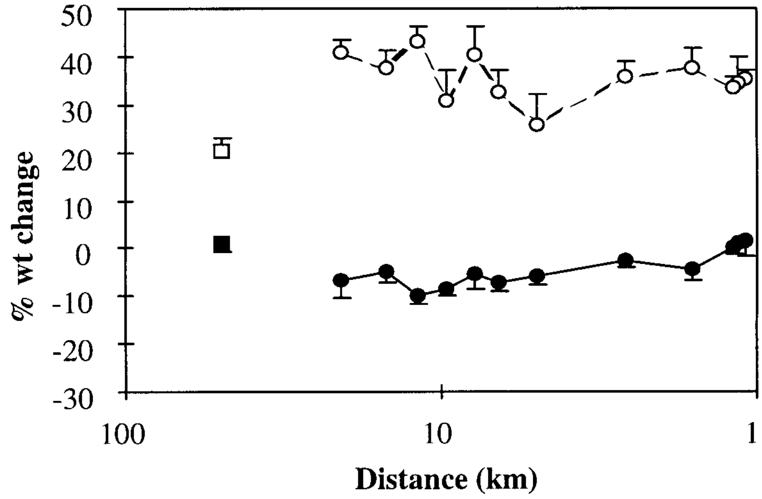

Figure 5.1. Fresh weight changes (average ± s.e., as percentage of initial fresh weight in a phase) in *E. andrei* exposed in Budel soils collected at different distances from the smelter (○, ●) and in OECD-artificial soil (□, ■) during preconditioning (○, □) and exposure (●, ■).

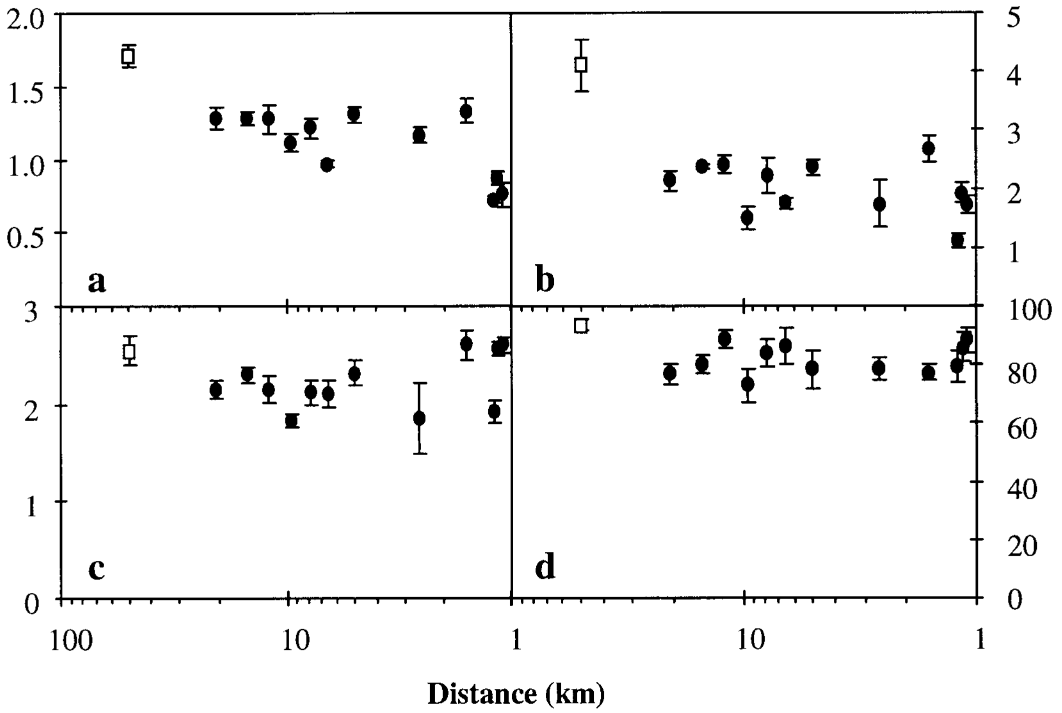

Figure 5.2. Raw data on reproduction parameters in *Eisenia andrei* (average and s.e. per treatment group) for (a) cocoon production ($c.w^{-1}.w^{-1}$), (b) juvenile production ($j.w^{-1}.w^{-1}$), (c) the number of juveniles per fertile cocoon, and (d) the proportion of fertile cocoons. Observations in Budel soils (●) are ordered from clean (left) to contaminated (right), and can be compared to simultaneously collected data in clean artisoil (□).

5.2.2 Enchytraeus albidus.

- Mortality. Most animals survived the exposure phase, except for animals in soil #2 (see Figure 5.3a).

-Reproduction. Juvenile production was expressed as juveniles.worm^{-1}.week^{-1}, assuming that all adults that were present at the start of the experiment contributed to juvenile production. This assumption had to be made in experiments with enchytraeids, since either (1) it is not sure that adults that are not found back died (remnants cannot be found), or (2) offspring may incidentally have reached maturity (as shown by a larger number of adults after exposure than added at the start). In both cases an additional assumption is needed to assess that less- or more adults than the initial number contributed to reproduction. In the present study, with low mortality and low adult offspring, the choice between the possible assumptions had only negligible influence on the outcome of the comparisons made between Budel soils.

The reproduction in Budel soils was low and in contrast to *E. crypticus* not related to the distance to the smelter (compare Figure 5.3b and Figure 5.5b). Only a very few juveniles were produced, with a maximum of ca. 0.1 $j.w^{-1}.w^{-1}$, in soil #4. Apparently, *E. albidus* does not perform well in Budel soils at pH ca. 5.5. Therefore these data are not regarded useful for further analysis and comparison with data collected in artisoil.

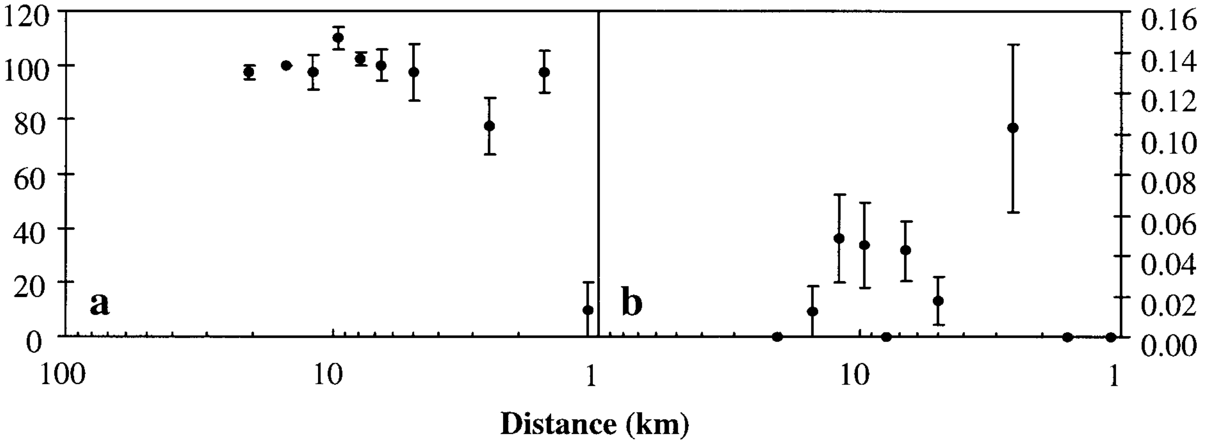

Figure 5.3. Selection of raw data on (a) survival (%) and (b) reproduction characteristics ($j.w^{-1}.w^{-1}$) (average and s.e. per treatment group) for *Enchytraeus albidus* exposed in Budel soils.

5.2.3 Enchytraeus crypticus, experiment 1.

- *Mortality*. All animals survived the exposure phase (not shown in graph).

-*Reproduction*. Juvenile production was expressed in number of juveniles produced per worm per week ($j.w^{-1}.w^{-1}$, same assumption as with *E. albidus*). This parameter tended to be reduced in the soils from sites near the factory (Figure 5.4). In comparison with *E. andrei*, the reproduction in *E. crypticus* was affected more, and effects on reproduction were already strong in soil #4. The highest number of juveniles produced was ca. 4 $j.w^{-1}.w^{-1}$, in soil #8. The strong differences between soils were not attributable to mortality differences among groups.

5.2.4 Enchytraeus crypticus, experiment 2.

- *Mortality*. Most animals survived the exposure phase, except for animals in soil #2 (see Figure 5.5a).

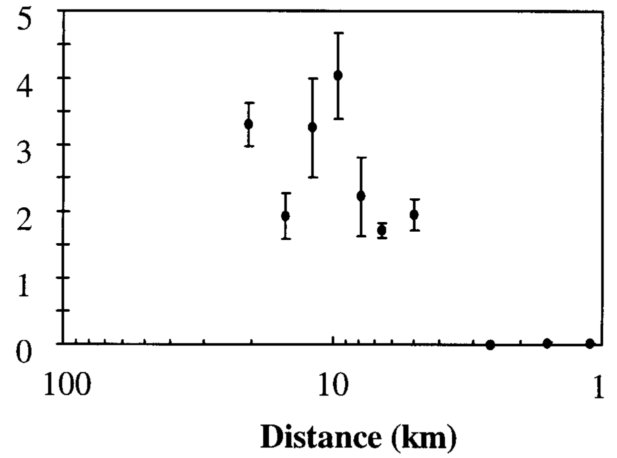

Figure 5.4. Selection of raw data on reproduction characteristics ($j.w^{-1}.w^{-1}$) (average and s.e. per treatment group) for *Enchytraeus crypticus* exposed in Budel soils, experiment 1.

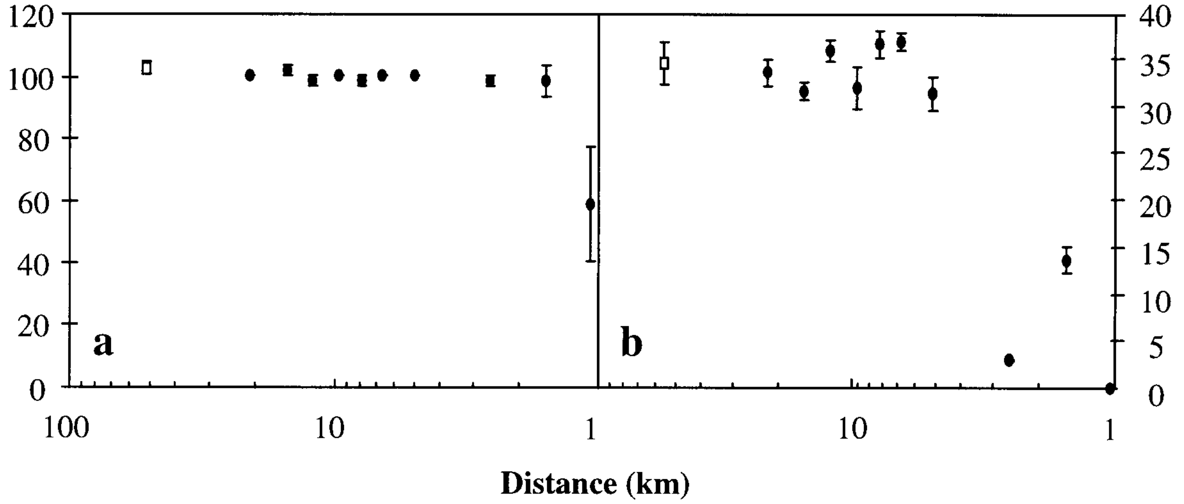

Figure 5.5. Selection of raw data on (a) survival (%) and (b) reproduction characteristics ($j.w^{-1}.w^{-1}$) (average and s.e. per treatment group) for *Enchytraeus crypticus* exposed in Budel soils (●) and artisoil (□) (experiment 2).

-Reproduction. Juvenile production ($j.w^{-1}.w^{-1}$) was higher than in experiment 1, viz. a maximum of ca. 35 $j.w^{-1}.w^{-1}$ (see Figure 5.5b). This can be attributed to the change in recovery and counting method that was adopted due to the use of Budel soils. The method (Oostenbrink's eluation method) applied in experiment 1 seems appropriate for yield comparisons between soils of homogeneous series, like artisoil with different toxicant concentrations (Dirven-Van Breemen et al., 1994). However, the sub-optimal recovery obtained by this method causes bias in case soil composition differs. Clearly, the recovery and counting method used in experiment 2 is much more efficient and probably less sensitive for soil differences.

The maximum number of juveniles that was produced was considerably higher than in the previous experiment. The pattern of reduced reproduction with decreasing distance to the smelter was, however, largely similar to experiment 1. Only for soil #3, a difference between experiments was found, with intermediate numbers of juveniles produced in experiment 2, and no observed reproduction in experiment 1. The data from the second experiment are considered preferable for comparisons with effect levels obtained in artisoil, in particular since reproduction in the reference artisoil was similar to that in Budel soil #11. The low number of juveniles recovered in soil #2 can be partly attributed to adult mortality.

5.2.5 Overview

The data on performance of the three worm species used in the experiments showed that there is a systematic relationship between reproduction and distance to the factory. Body growth, and mortality proved to be less sensitive parameters. Superimposed on the systematic pattern, the variability of the observations is larger than with common laboratory concentration-effect data. Since various soil parameters are correlated to distance (various metals, and pH), results are not unequivocally transferable. This requires a detailed exposure- and effect assessment.

5.3 Exposure assessment

Various metals have elevated concentrations in the Budel soils, and the raw data on mortality, growth and reproduction indicated the existence of possible toxic effects. To identify the contribution of metals to the observed effects, an exposure assessment was made by studying body concentrations in the animals in relation to the characterisation of metal concentrations in the soil (see Chapter 3).

5.3.1 Metal concentrations in Eisenia andrei

Body concentrations of metals after exposure in Budel soils and the dry weight / wet weight ratio are summarized in Table 5.2.

The dry matter content of the worms is not affected by metal exposure or by experimental manipulation (no differences among phases). The metal concentrations in animals reared in mass culture medium (a mixture of horse dung, garden soil and peat) did not change when the animals were transferred and kept in artisoil. Also no differences in body concentrations existed after exposure in clean artisoil or Budel soil #11.

Metal concentrations in worms differed among Budel soils after the exposure phase. Body concentrations generally increased with decreasing distance to the smelter, although variation upon this trend exists, for example for the zinc and copper concentrations in soil #5 and # 6, which are lower than #4 and #7. Comparison of body concentrations of worms exposed in Budel soils #2 and #11 showed an increase with a factor of ca. 3 for zinc, 4 for copper, and 6 for lead. For cadmium the increase was negligible.

The bioconcentration factor is of relevance for comparisons with literature data on metal accumulation in other soil types. Bioconcentration factors were calculated as the ratio of the total metal concentrations in soil and worm (total, mg.kg^{-1} dry wt, Table 5.3). The bioconcentration factor decreased for all metals with increasing total soil concentrations.

Phase	Zn		Cu		Pb		Cd		% dry matter	
medium	AVG	se	AVG	se	AVG	se	AVG	se	mean	s.e.
Preconditoning/Begin										
Mass culture medium	104.4	2.6	11.9	0.4	< 10	n.d.	4.2	0.1	18.0	0.03
Preconditioning/End										
artisoil	106.1	3.3	10.6	0.6	< 10	n.d.	4.3	0.3	15.7	0.78
Budel #11	98.9	0.1	10.9	0.4	n.d.	n.d.	5.2	0.5	16.8	0.84
Exposure phase/End										
Budel #2	331.5	16.2	40.7	1.3	74.4	5.2	9.6	0.3	17.4	0.09
Budel mixture #P/#2	250.5	17.2	28.2	1.2	62.5	3.9	12.1	0.2	17.0	0.31
Budel #P	172.1	4.0	16.0	0.6	49.7	2.3	14.4	0.4	16.5	0.44
Budel #3	143.3	4.5	15.2	0.5	33.7	3.4	12.3	0.7	15.4	0.30
Budel # 4	143.3	4.5	15.7	0.2	32.2	3.2	10.2	0.2	16.9	0.50
Budel # 5	111.0	1.9	11.5	0.5	14.2	0.8	9.3	0.7	16.4	0.37
Budel # 6	125.1	14.8	10.8	0.1	18.6	1.5	8.7	0.2	16.2	0.13
Budel # 7	147.9	4.0	13.5	0.6	13.6	1.4	9.3	0.3	16.2	0.22
Budel # 8	103.1	1.8	11.1	0.4	22.8	2.5	7.6	0.6	15.5	0.54
Budel # 9	106.4	3.1	13.4	1.7	15.1	1.9	8.0	0.4	15.4	0.54
Budel # 10	104.2	3.8	11.1	0.8	n.d.	n.d.	7.8	0.1	16.1	0.81
Budel # 11	101.4	1.5	11.4	0.3	n.d.	n.d.	7.0	0.2	16.2	0.33
artisoil	106.9	1.9	13.4	0.2	<10	n.d.	4.5	0.3	16.2	0.31

Table 5.2. Body concentrations of metals (mg.kg^{-1} dry wt) and proportion dry matter in *Eisenia andrei* from mass culture medium, after exposure in Budel soils and artisoil, in different phases of the reproduction experiment. (AVG=average of 2 - 4 worm batches randomly chosen from the frozen groups of 5 worms, s.e. =standard error, n.d.=not determined, <=below detection limit). All values for Cr were below detection limit (< 4 mg.kg^{-1} dry wt).

	BCFs in Budel soils							
#	Zn	s.e	Pb	s.e.	Cu	s.e.	Cd	s.e.
11	52.4	0.76	1.68	0.60	79.34	2.42	n.d.	n.d.
10	4.5	0.17	0.75	0.47	5.53	0.39	25.6	26.1
9	5.3	0.16	0.61	0.08	6.71	0.85	21.9	20.0
8	3.8	0.07	0.51	0.06	3.69	0.15	45.8	38.0
7	6.6	0.18	0.51	0.18	3.37	0.16	23.9	23.2
6	4.4	0.53	0.30	0.02	2.00	0.03	28.8	29.4
5	3.5	0.06	0.36	0.02	1.15	0.05	24.4	23.2
4	0.7	0.02	0.35	0.04	0.90	0.01	13.1	13.3
3	0.7	0.02	0.23	0.02	0.53	0.02	12.5	14.1
P	0.5	0.01	0.28	0.01	0.50	0.02	3.9	3.8
2	0.2	0.01	0.18	0.01	0.16	0.01	2.6	2.5

Table 5.3. Bioconcentration factors (BCFs) for *E. andrei* calculated as the ratio of the total metal concentration in soil and the concentration in the animal.

5.3.2 The change of metal concentrations in Eisenia andrei *during exposure*

The change of body concentrations over time showed a different pattern in soil #11 and 2, and between zinc, copper, lead and cadmium (see Figure 5.6). Small changes of body concentrations were observed during continuous exposure in soil #11. Large accumulation occurred in soil #2, for zinc, copper and lead. For cadmium it was negligible. The body concentrations of zinc, copper and lead decreased fast after transfer to clean soil (depuration phase), with the major concentration drop visible in the first few days after transfer.

During exposure, body concentrations of zinc, copper and lead showed an initial increase, followed by a systematic decrease after the initial rise. Apparently, the assimilation- or the elimination rates are not constant during the experiment. Therefore, the compartment model could not be fitted to the data, since in the model these rates are assumed to be constant. The variation of uptake rate during exposure can be a consequence of decreased uptake, increased excretion, or a combination of both, which in turn may be an effect of a toxic exposure level. This pattern in usually not found, since toxico-kinetic studies are usually performed at non-toxic levels (see, e.g., Janssen et al., 1991).

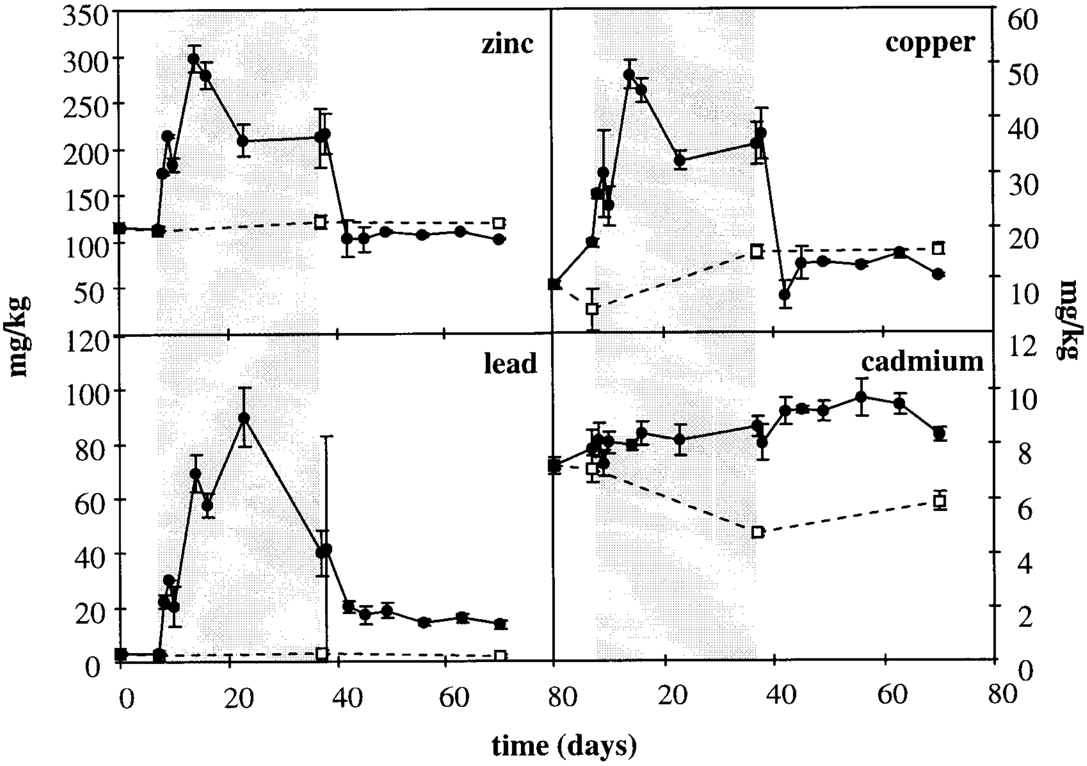

Figure 5.6. Change of body concentration of metals in *E. andrei* (mean and s.e., dry wt basis), for two non-xenobiotic metals (Zn and Cu, top) and two xenobiotic metals (Pb and Cd, bottom) during subsequent preconditioning, exposure and depuration in Budel soils #11-2-11 (●), and continuously in Budel soil #11 (□). The shaded area is the exposure phase. Non-shaded areas are the preconditioning phase (left) and a post-exposure phase (right).

In a similar experiment performed in artisoil, to which the same mixture of metals was added (as metal salts, total concentrations), metal kinetics could not be studied due to large metal-induced mortality. The large difference in mortality between Budel soil #2 and artisoil with the same metal mixture shows that metal availability for worms differed considerably between soil types.

5.3.3 *Indications for the dominant toxic metal in* Eisenia andrei

With the body concentration data two questions were addressed, namely:

- whether various metals, or only one dominant metal, may be responsible for the decreased reproduction observed in Budel soils that were collected near the factory. For this purpose, the body concentration data were compared to literature data in which internal metal concentrations are related to toxic effects on reproduction;
- whether pore water- or 0.01*M* $CaCl_2$-extractable metal concentrations are a better guide to bioavailability than total metal concentrations. For this purpose, correlations between body concentrations and total-, extractable and pore water concentrations were calculated.

- *Internal concentrations*. Literature data on inhibition of reproduction in relation to internal metal concentrations in *E. andrei* are given in Van Gestel et al. (1993). These authors showed that reproduction in artisoil (pH ca. 5.5, pF ca. 2) is significantly affected when internal concentrations exceed 115-150 mg Cd, ca. 32 mg Cr or ca. 150 mg Zn .kg^{-1} dry body wt. In Budel soils the highest body concentrations are for cadmium ca. 14 mg Cd, for chromium less than 4 mg, and for zinc ca. 330 mg.kg^{-1} dry wt (see Table 5.2). Therefore, the contribution of cadmium and chromium to reproduction effects in Budel soils is expected to be low or negligible, assuming that interactions among metals are low or absent. Zinc, however, is expected to be an important toxic metal in the Budel soils since internal concentrations obviously exceed internal NOEC levels. Figure 5.7 clearly shows strong effects on reproduction at internal body concentrations of about 200 Zn .kg^{-1} dry body wt.

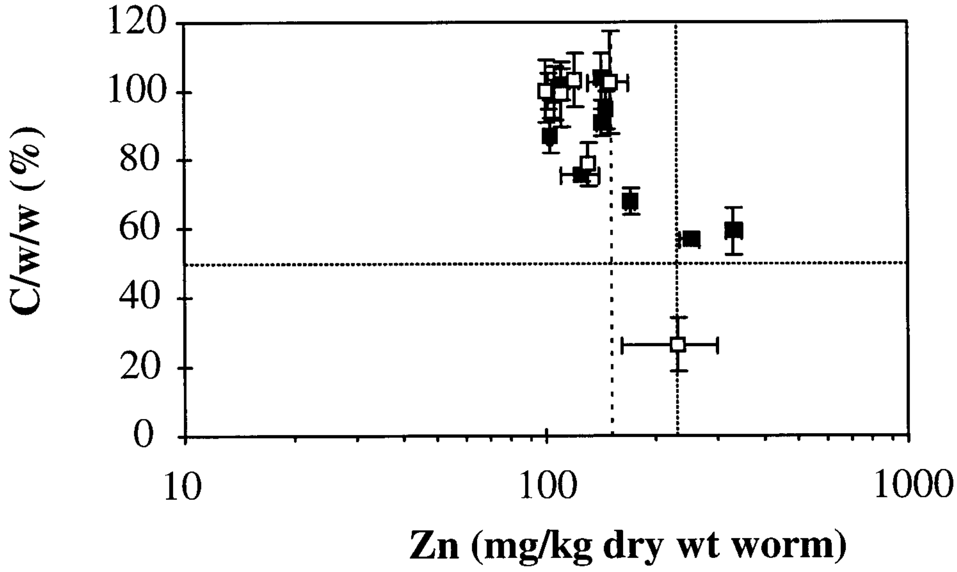

Figure 5.7. Body concentration of Zn in relation to toxic effects in Budel soils (■) and artisoil (□) (pH adjusted to ca. 5.5 in both soils). The dotted lines show the 50%-effect level, and 150 resp. 230 mg Zn .kg^{-1} dry body wt.

Effects on reproduction in artisoil loaded with zinc only, are, however, stronger at the same internal zinc concentration, than in Budel soils loaded with various metals. These observations suggest that zinc may have played an important role as toxicant in the Budel soils collected near the factory, but also that other factors such as the presence of other metals may moderate the toxic effect of zinc present in the body. A slightly less than concentration additive (or antagonistic) effect of the presence of other metals has been observed earlier (Weltje et al., 1995).

The contribution of copper and lead in causing toxic effects in Budel soils can not be evaluated on the basis of internal body concentrations due to lack of data. The role of these metals may be judged by external concentrations only. The (external) EC50 for lead and copper in artisoil are ca. 1500 mg (Mogo, 1993) and 191 mg total metal .kg^{-1} dry wt (Van Gestel et al., 1989), respectively. The highest concentration in Budel soil is for lead 424 mg (total) and for copper 250 mg (total) kg^{-1} dry wt. When judged by total metal concentrations, the EC50 for copper is exceeded in Budel soil #2. For lead, however, the soil concentration is far below the EC50.

Body concentration data suggest that zinc is the dominant toxic metal for *E. andrei* in Budel soils. The contributions of cadmium and chromium seem negligible. Moreover, external effect-concentration data suggest that copper, and to a lesser extent lead, may also have contributed to the reproduction effects. Expression of copper and lead toxicity depends on their availability in Budel soils in comparison with artisoil. Altogether, these observations imply that toxic effects on *E. andrei* in Budel soils may be caused by various metals simultaneously, with as probable order of dominance: zinc >> copper > lead > cadmium/chromium.

- Extracted metal concentrations as a guide towards bioavailability. For zinc, copper and cadmium correlation coefficients (based on *ln*-transformed values) between body- and external concentrations were of similar magnitude for all measurements of external concentration (Table 5.4). This implies that the body concentration of these metals is equally well predictable from total-, 0.01*M* $CaCl_2$-extractable-, or pore water metal concentrations. An example of this similarity is shown for zinc (Figure 5.8). These findings are in accordance with the different measurements of external zinc concentration in Budel soils, since these are highly correlated mutually (Table 3.3). Only for lead, pore water- and total concentrations are better predictors of the internal concentration than the 0.01*M* $CaCl_2$-extractable concentration (type $CaCl_2$-1).

Metal Measure of soil concentrations	Zn		Cu		Pb		Cd	
Soil total	0.823	**	0.744	**	0.874	**	0.700	**
$CaCl_2$-1	0.804	**	0.643	**	0.081	n.s.	0.786	**
$CaCl_2$-2	0.867	**	n.d.		n.d.		n.d.	
PW3	0.793	**	0.592	*	0.541	**	0.637	**

Table 5.4. Correlations between body and external concentrations of metals, for different ways to measure external concentrations. Correlations were determined for *Eisenia andrei* at the end of the exposure period. Correlations are based upon ca. 44 measurements (4 replicates, 11 soils for which data are available, *ln*-transformed), except for Cu, where 0.01*M* $CaCl_2$-extractable concentrations were determined in only 4 soils. n.d.: not determined; **: $0.001 < P \leq 0.01$; *: $0.01 < P \leq 0.05$; n.s: P>0.05.

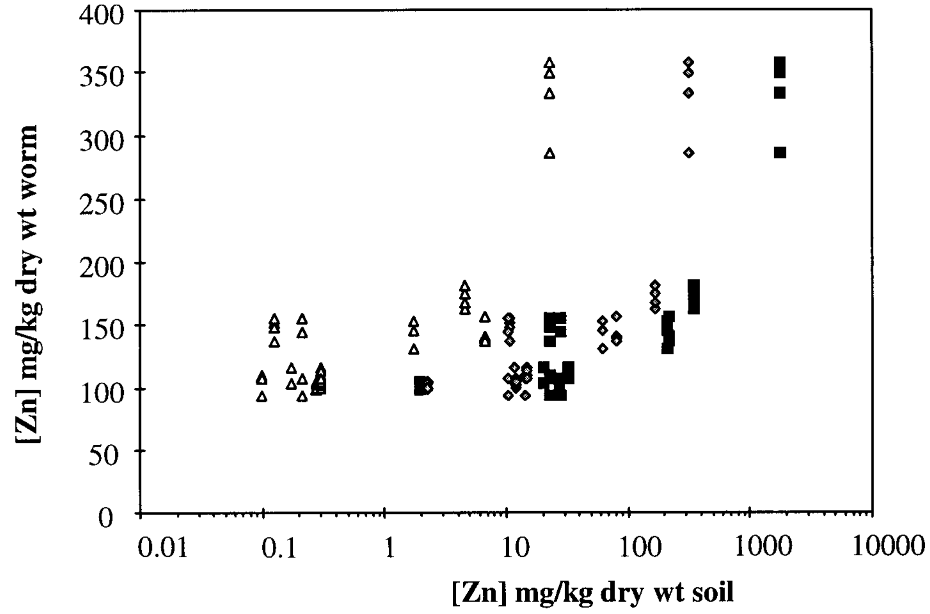

Figure 5.8. Scattergram of internal body concentrations of zinc (Y-axis) in relation to external concentrations of zinc in Budel soils, pH adjusted to ca. 5.5. Note that only the X-axis is depicted logarithmically, for reasons of clearness. The external concentrations are: total (■), 0.01*M*-$CaCl_2$ extractable (type $CaCl_2$-2 ▲) and pore water (type PW3 △).

5.3.4 *Change of metal concentrations in* Enchytraeus crypticus *during exposure*

Unlike *Eisenia andrei*, metals could not be measured at the end of the exposure period in *Enchytraeus crypticus*. Due to the small size of the animals and technical feasibility, only the time-series change of body concentrations was determined for zinc (an essential element) and cadmium (a xenobiotic element), in Budel soils #4 and #11. In soil #4, about 50% reduction in reproduction of *E. crypticus* occurred (see section 5.2.4). For *E. andrei* a similar percentage of reduction was observed in soil #2.

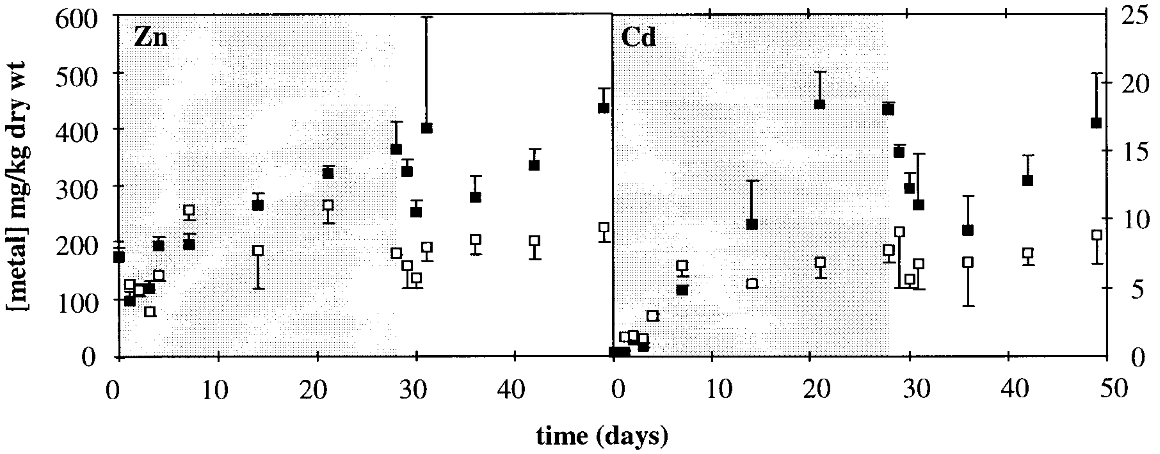

Figure 5.9. Pattern of uptake of zinc and cadmium by *Enchytraeus crypticus* exposed in Budel soil #4 (□) and artisoil with similar metal concentrations (■). The shaded area is the exposure phase, which is followed by depuration in clean soil (Budel #11 or artisoil).

The metal concentrations in *E. crypticus* during the exposure and depuration phases in Budel soil #11 was largely constant (data not shown). In this soil, only the body concentration of cadmium increased slightly during exposure, from ca. 3 to ca. 5 $\mu g.g^{-1}$ dry wt, while the internal zinc concentration was stabile at a level of ca. 150 $\mu g.g^{-1}$ dry wt. In Budel soil #4, and in artisoil with similar amounts of metals, exposure resulted in elevated internal concentrations of zinc and cadmium (see Figure 5.9). The highest concentrations were reached in artisoil for both metals. Apparently, the net bioavailability of metals for *E. crypticus* in the Budel soil #4 is lower than in artisoil with the same metal concentrations.

In the depuration phase, a remarkable increase of the body concentration of zinc and cadmium was observed after a sharp initial drop. This apparent increase could be attributed to a loss of body weight. The weight loss that occurred in the depuration phase was approximately linear with time, from ca 0.7 to ca. 0.3 mg dry wt. individual^{-1} between day 28 and 49. When body metal concentrations were expressed in ng.individual^{-1}, the net body burden of zinc decreased during the depuration period (data not shown). The weight loss may have been caused by a stress related to the manipulation of the animals, to transfer to clean but slightly different soil, to toxic effects in the exposure phase, or to a combination of these reasons. Due to the increase in body concentration, the data are inappropriate to fit a compartment model to the full data series. An estimate of assimilation and depuration constants for both metals in Budel #4 soil and in artisoil was, however, obtained by fitting a one-compartment model only to the uptake data and the data from the first few days of depuration (Table 5.5). During this period only slight weight differences were observed.

The abovementioned difference in apparent equilibrium concentration for both metals between artisoil and the Budel soil was corroborated by the equilibrium body concentrations calculated from the parameter estimates of the compartment model (Equation 7, see Table 5.5). Compared to OECD, in Budel soil #4 a slightly lower assimilation rate constant (a) was determined, while the elimination rate (k) was much larger. This pattern is similar for both metals and means that the elimination rate has a larger influence on the equilibrium than the assimilation rate.

Metal	Cd		Zn	
Soil type	OECD	Budel #4	OECD	Budel #4
Q_0 ($\mu g.g^{-1}$ dry wt)	0.32	0.09	108	120
a ($\mu g.g^{-1}$ dry wt. day^{-1})	1.09	1.05	17	15
k (day^{-1})	0.05	0.14	0.07	0.17
Q_0+ a/k ($\mu g.g^{-1}$ dry wt)	22.2	7.72	358	210

Table 5.5. Parameter estimates of a one-compartment model fitted to uptake and depuration data for Cd and Zn in *Enchytraeus crypticus* in Budel soil #4 and artisoil with similar total metal concentrations.

5.3.5 *Indications for the dominant toxic metal for* Enchytraeus

It is difficult to identify the dominant toxic metal for *E. crypticus* from the available data on metal kinetics. In comparison with *E. andrei*, the increase of the internal cadmium concentration was higher, and that of zinc lower. Apparently, metal uptake kinetics differ not only between metals but also between worm species.

The relative roles that zinc, copper, lead and cadmium play in causing toxic effects in *E. crypticus* in Budel soils can not be evaluated with internal NOECs, since such values are not yet available. Based on external effect concentrations, however, some indications on the relative importance of some metals can also be obtained.

External EC50s for *E. crypticus* in artisoil were for zinc ca. 273 mg and for cadmium ca. 100 mg.kg^{-1} dry wt soil (Weltje et al., 1995). Preliminary data of a concurrent experiment showed an external EC50 for copper of ca. 500 mg Cu .kg^{-1} dry wt soil. Dirven-Van Breemen et al. (1994) reported two additional EC50s for zinc in artisoil, respectively 251 and 186 mg (total) Zn .kg^{-1} dry wt soil. In Budel soil #4 the total concentration zinc, cadmium, and copper concentrations are 219 mg , 0.77 mg, and 17.3 mg Cu .kg^{-1} dry wt soil, respectively. In the soils collected nearer to the factory (#2 and 3) soil metal concentrations were higher. When judged by total metal concentrations in the soil, the EC50 of zinc is exceeded in soils #2, #3, and #4. The external EC50s of cadmium and copper are not reached in the studied Budel soils. For lead and chromium literature data on external EC50s for *E. crypticus* are lacking.

As in *E. andrei*, zinc may be the dominant toxic metal for *E. crypticus*. The relative importance of the other metals, cadmium, lead and copper may, however, differ from the situation in *E. andrei*. This difference may not only bear on the metals when applied singly, but it may also exist due to inter-species differences at the level of mixture interactions.

5.3.6 *Conclusions of the exposure assessment*

In addition to the analyses of the physico-chemical characterisation of the Budel soils (paragraph 3.5), the exposure assessment for both species has shown that:

1. It is likely that zinc is the dominant metal that caused effects on reproduction (and others) in Budel soils in both species, since for this metal the (external) EC50 or NOEC is exceeded most in the Budel soils collected near the factory.
2. It is likely that, next to zinc, other metals may cause toxic effects, since the body concentrations of various metals increased in Budel soils in which effects were found: this implies that mixture effects should be taken into account;
3. The prediction of internal concentrations of zinc for *E. andrei* on the basis of its external concentration can equally be based on total-, extractable-, and pore water metal concentrations in the Budel soils, because of the high correlation between these measurements.

4. The sorption differences between Budel soils and artisoil (see chapter 3) came to expression into the relatively low body concentrations of the studied metals when animals were exposed in Budel soils.

5.4 Effect assessment

5.4.1 From raw data to unravelling causes of reproduction effects: rationale

The raw data showed a trend that reproduction for *E. andrei* and *E. crypticus* decreased with decreasing distance to the smelter (Figure 5.4 and Figure 5.5). It is likely that zinc is a major factor that has caused this trend, but there are also other factors that covary with 'distance to smelter'. Among the measured soil characteristics, known factors are soil acidity and other metals (see section 3.5). It is reasonable to exclude acidity, because this factor was artificially ruled out as determinant factor in the experiments by adjusting pH. The other metals may, however, play a role in causing toxic effects. Correlations of zinc with other measured soil characteristics were not observed.

With pH ruled out, it is possible to express toxic effects on the basis of metal concentrations and to calculate field ECx. This enables comparisons with laboratory derived EC50 or NOEC. In interpreting such comparisons, one should take into account that various factors *simultaneously* contribute to effects in Budel soils, namely:

1. Additional stress factors (next to the metals) may affect the reproduction of worms in Budel soils directly or indirectly. Such factors may only cause 'noise' on the concentration-response curves, since no known factors appear to be correlated to the metal concentrations of the soils.
2. Metal availability may differ, for each metal separately, among Budel soils and between Budel soils as a group and artisoil. The influence of availability differences between soils on toxicity is worked out assuming zinc to be the dominant toxic metal.
3. Budel soils contain a mixture of metals, which may mutually interfere during uptake and intoxication. Despite the apparent dominant role of zinc, other metals may contribute to toxic effects if their concentration is relatively high, or if mutual interferences are strong (see Weltje et al., 1995).

Since these factors operate simultaneously, it is impossible to identify the exact role of all these factors separately in causing toxicity. Only through making simplifying assumptions it is possible to recalculate the raw 'distance' data towards metal concentrations, and to compare the obtained effect levels with data from other soils.

In the next paragraphs, two simplifying assumptions will further be elaborated, following stepwise reasoning for unravelling the causes of the observed effects, viz. subsequently (1) assuming a dominant role of zinc (to study differences in metal availability between soil types), and (2) assuming concentration additive effects of the metal mixtures present in Budel soil.

5.4.2 The possible role of availability differences, focusing on zinc as dominant metal

The difference between the effect levels in different soils is worked out for zinc for both species. These analyses are meant to investigate whether the physico-chemical differences of metal availability between soils (see Chapter 3) are important for differences in toxicity. Large differences in effect levels between soils will be found if the metal fraction used to quantify exposure deviates from the real bioavailable fraction; differences will be absent when the true bioavailable metal fraction is used to express toxic effect levels (or when the extracted fraction perfectly correlates to bioavailability).

- *Eisenia andrei*. The sigmoid response model was used to estimate concentration-response curves for the different measurements of external zinc, despite the 'noise' attributable to variation in local soil factors in Budel soils. With these data, the differences between soils are calculated as the ratio $EC50_{Budel}/EC50_{OECD}$ for zinc The data for artisoil were collected from literature. The comparisons are summarized in Table 5.6 and Figure 5.10.

1. - *Total concentrations*. The difference in EC50 between artisoil and the Budel soils on the basis of total zinc concentrations is 5.9 units. This means that the zinc concentration required for 50% effect is 5.9 times higher in Budel soils than in artisoil (Figure 5.10a). The 95% confidence intervals of both EC50-estimates do not overlap, suggesting a significant difference in EC50s between these soils[4].
2. - *Extractable concentrations*. On the basis of 0.01*M* $CaCl_2$-extractable zinc (type 2), the EC50 in Budel soil was estimated as 183 mg Zn $.kg^{-1}$ dry wt (Figure 5.10b). The 95% confidence intervals of the EC50 could not be calculated. The EC50 in artisoil is 133 mg extractable Zn $.kg^{-1}$ dry wt. The difference in EC50 among artisoil and Budel soils is thus 1.4 units (Table 5.6), which is a reduction compared to the total-concentration comparison. Although the 95% confidence interval could not be calculated for the Budel soils, there appears no significant difference in EC50-level between both soil types. Apparently, the extractable fraction is better correlated to bioavailability of zinc for *E. andrei* than the total zinc concentration. Table 3.10 shows that the extractability of zinc from artisoil at pH ca. 5.5 was 2-3 times higher than from Budel soils at adjusted pH (K_f -values were 153 and 69, resp.). This factor is found back in the reduction of the EC50-ratio of 5.9 to 1.4.
3. - *Other types of concentrations*. The comparison among soil types on the basis of pore water concentrations can not yet be made, due to the lack of data on the pore water concentrations of zinc in artisoil. Furthermore, it was already indicated that differences between both soil types at the level of the NOEC were small when judged by internal concentrations at higher

---

[4] It is important to note already that Budel soil #2, the mixture #P/2 and #3 are widely separated on the X-axis, causing the sigmoid curve to have a bad fit in the area of interest, whereas in the Distance-Effect analysis of Figure 5.12 the responses in these soils are close to the fitted distance-effect relationship. This particular effect causes differences in estimated EC(D)50 values.

body concentrations, however, differences in toxic effects between soils may occur (see Figure 5.7).

To get insight in the difference between soils at different effect levels, we investigated the difference at the EC5-level by a visual data inspection. The EC5-level was chosen rather than the NOEC, since estimation of the latter is hampered by the large 'noise'. The ratio's at the 5%-effect level are calculated as $EC5_{Budel}/EC5_{OECD}$ for zinc, and are respectively (55/95=) 0.58 (total Zn), and (90/47=) 1.9 (extractable, type 2). Visual inspection of the original observations over the whole concentration range indicates that the magnitude of the difference in EC50- and EC5-levels between Budel soils and artisoil (Table 5.6) is typical for the magnitude of differences in toxic effect levels among soils.

	Budel						OECD	unit	Budel/OECD
Parameter	*C*	*b*	*m*	%	EC50	(95% C.I.)	EC50 (95% C.I.)		(ratio EC50s)
total	98.5	-0.67	7.96	37	2553	(856-7616)	429 (336-548)	$mg.kg^{-1}$	5.9
$CaCl_2$-2	94.8	-5.16	5.20	37	183	(n.d.)	133 (117-153)	$mg.kg^{-1}$	1.4
PW3	96.4	-0.76	3.72	35	41	(12-139)	n.d.	$mg.l^{-1}$	-

Table 5.6. Parameters of the concentration-response curves for *Eisenia andrei* exposed in Budel soils, calculated for zinc as the dominant metal. Curves were fitted to zinc concentrations in soil measured with 3 different methods. *C*, *b* and *m* are parameters of the sigmoid concentration-response curve fitted to the original data (see Equation 8). '%' indicates the proportion of variance in the data accounted for by the model. C.I.=confidence interval. OECD EC50s are recalculated from Weltje et al. (1995), and are expressed on a $mg.kg^{-1}$ dry wt basis. The experiment from which the data for the EC50s were obtained is indicated as Ea Zn1 in Table 3.10. All EC50s were recalculated to express them on the basis of $mg.kg^{-1}$ dry wt to allow for comparisons among soils.

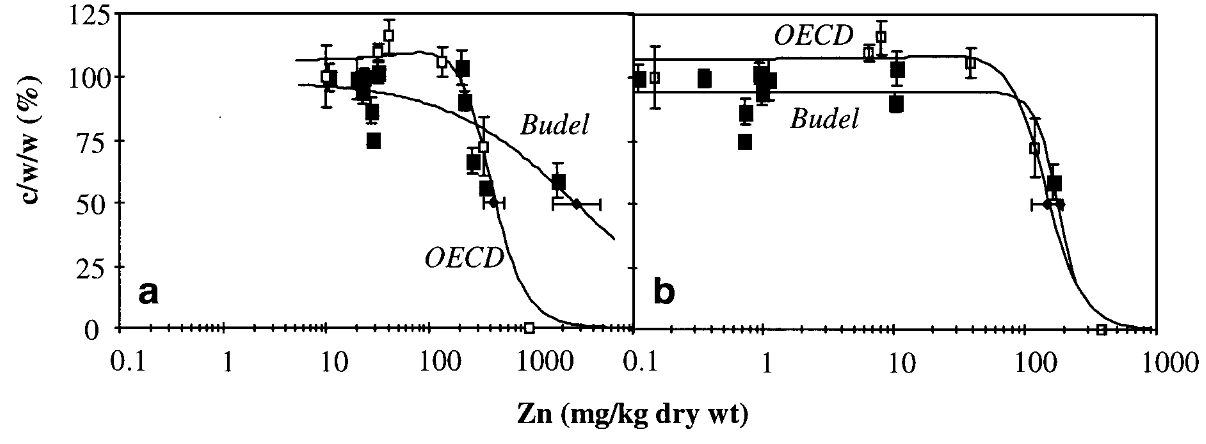

Figure 5.10. Concentration-effect relationships for cocoon production in *Eisenia andrei* in artisoil and in Budel soils (at pH initially adjusted to ca. 5.5), calculated for zinc as the dominant metal. Comparison on the basis of total zinc-concentrations (a) or 0.01*M* $CaCl_2$-extractable concentrations (type $CaCl_2$-2) (b). The average of the observations in artisoil (□) and Budel soils (■) are shown, with their standard errors (vertical bars). The estimated concentration-response curves are shown, following the parameters of Table 5.6, with an indication for the EC50 (◆, with 95% confidence intervals).

- <u>Enchytraeus crypticus</u>. Similar to the approach in *E. andrei* field EC50s were estimated. In contrast to *E. andrei*, however, the effect model was fitted to the means of the treatment groups, since none of the iterations converged when the models were fitted to original data. The findings are summarized in Table 5.7 and Figure 5.11.

1. *Total concentrations*. In *E. crypticus*, the similarity of laboratory and field EC50s was largest when zinc concentrations were expressed on the basis of total concentrations. The EC50 in Budel soil could be compared with laboratory data from literature of three separate experiments. In comparison with artisoil, effects in Budel soil occurred at similar total zinc concentrations (2 experiments, ratio's were 0.80 and 0.97) or at a lower zinc concentration (1 experiment, ratio was 0.57). The shape of the concentration-effect curve for total zinc concentrations is estimated to be steeper for Budel soils than for artisoil (see Figure 5.11a). This may, however, be an artefact due to the lack of partial-effect data between ca. 20 and 200 mg Zn .kg^{-1} dry wt of Budel soil. As a consequence of the large effect observed in Budel soils #2, 3 and 4, the (mathematically symmetrical S-shaped) curve is forced in its angular form in the low concentration range. Comparisons of the whole laboratory- and field concentration-effects curves are not made since they would be numerically biased by this artefact. Visual inspection of the data shows that the difference in effect levels between soils is limited; the range indicated in the table seems to be typical.
2. *-Extractable concentrations*. Effect concentrations were lower in Budel soil than in artisoil when judged by 0.01*M* $CaCl_2$-extractable zinc concentrations. Since extractability differed between the three experiments with artisoil, the difference in effect levels between both soil types depends on the experiment. The ratio $EC50_{Budel}/EC50_{OECD}$ for zinc was 0.38, 0.05 and 0.30, for the experiments Ec Zn1, 2 and 3, respectively. This contrasts with the observations in *E. andrei*. The shape of the effect curves is similar for both soil types when judged by extractable concentrations. This implies that the EC*x*-value for OECD- artificial soil is always higher than the EC*x* of Budel soils, irrespective of *x*.

	Budel					OECD	unit	Budel/OECD
Parameter	*C*	*b*	*m*	%*	EC50 (95% C.I.)	EC50 (95% C.I.)		(ratio of EC50s)
total	101	-33.9	5.32	96	205 (203-206)	254 (n.d.) (a)	mg.kg^{-1}	0.80
						212 (107-416) (b)		0.97
						361 (n.d.) (c)		0.57
$CaCl_2$-2	101	-2.8	1.92	95	6.82 (n.d.)	18.0 (n.d.) (a)	mg.kg^{-1}	0.38
						144 (53 - 386) (b)		0.05
						22.9 (n.d.) (c)		0.30
PW3	108	-1.6	0.24	99	1.27 (0.89 - 1.82)	n.d.	mg.l^{-1}	-

Table 5.7. Parameters of the concentration-response curves for *Enchytraeus crypticus* in Budel soils, calculated for zinc as the dominant metal. Curves were fitted to zinc concentrations in soil measured with 3 different methods. *C*, *b* and m are parameters of the sigmoid concentration-response curve (see Equation 8). C.I.=confidence interval. OECD EC50s are recalculated from Weltje et al., 1995), and are all expressed on mg.kg^{-1} dry wt basis. '*' indicates that the model was fitted to average values per treatment group; fits to original observations did not converge. The artificial exclusion of within-group variance explains the relatively high variance explained by the model (ca. 95% in *E. crypticus* compared to ca. 40% in *E. andrei*, see Table 5.7, Figure 5.11). The EC50s marked with (a), (b) and (c) are derived from the experiments with code Ec Zn1, Ec Zn2 and Ec Zn3, resp., as introduced in Table 3.10, for which the zinc extractability (zinc only, not in mixture) differed considerably. Ec Zn3 data are preliminary estimates from a concurrent experiment.

The increased dissimilarity of effect levels (compared to total zinc concentrations), suggests that the correlation of the 0.01*M* $CaCl_2$-extractable zinc fraction with the bioavailability of zinc for *E. crypticus* is less than when using the total zinc concentration. This conclusion contrasts to the observations that both the extractability of zinc (at similar pH) and the body concentrations were lower in Budel soils than in artisoil.

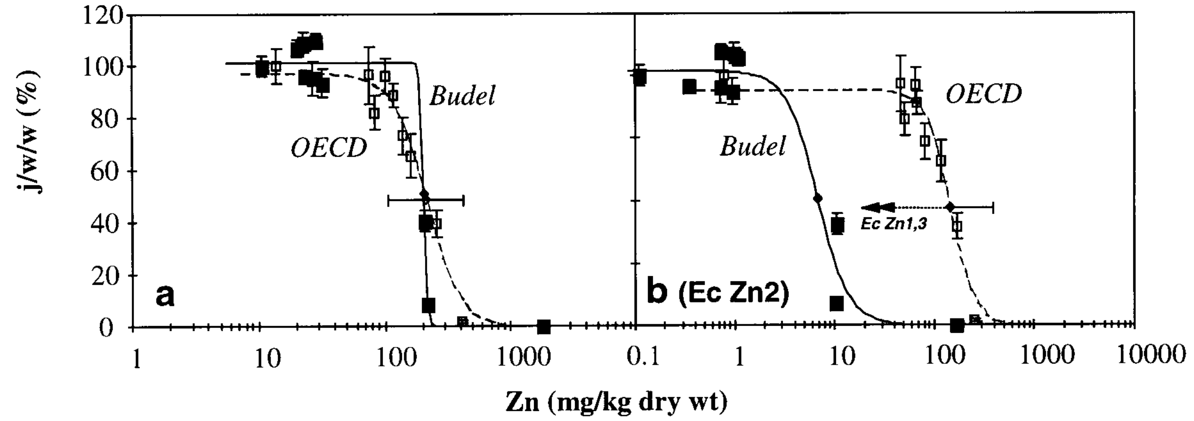

Figure 5.11. Concentration-effect relationships for juvenile production in *Enchytraeus crypticus* in artisoil and in Budel soils (at pH initially adjusted to ca. 5.5), calculated for zinc as the dominant metal. Comparison on the basis of total Zn-concentrations (a) or 0.01 M $CaCl_2$-extractable concentrations at pH ca. 6 (b). In both graphs, the Budel data are compared to the OECD-concentration effect curve of experiment Ec Zn2 (code introduced in Table 3.10). In that experiment, zinc extractability was higher than in the experiments Ec Zn1 and Ec Zn3. Further legend as Figure 5.10. The EC50-error bar of graph (B) for Budel soils is large, but not shown. The arrowheads linked to the curve for OECD, at the EC50-level, indicate the different EC50s found in different experiments.

5.4.3 Conclusions on the possible role of availability differences among soils

When the role of zinc only is regarded, the following conclusions can be drawn on the sensitivity of both species for metals, and on the role of metal availability differences between soil types:

1. - Sensitivity comparison of the species. Comparison of the data for *E. andrei* and *E. crypticus* shows that the species have different sensitivities for zinc. Comparison of the EC50s determined in artisoil demonstrate that *E. crypticus* is more sensitive for zinc than *E. andrei* when judged by total concentrations. Judgement by extractable concentrations proves more difficult, since the extractability of zinc in the batches of artisoil used for the enchytraeids differed strongly. With *E .andrei* having an EC50 of 144 mg extractable zinc .kg^{-1} dry wt, *E. crypticus* may be similarly sensitive (*ibid.* 133 mg .kg^{-1}) or more sensitive (*ibid.* 18 or 23 mg .kg^{-1}). The enchytraeid was also more sensitive than *E. andrei* in the Budel soils, since the EC50s were considerably lower, both for the total- and for the extractable zinc concentration.
2. - Judging availability. For *E. andrei*, the similarity of the concentration-effect curves at the level of extractable concentrations suggests that this particular metal fraction correlates with the bioavailable fraction for this species. This conclusion, based on toxicity data, could not be reached using correlations between external metal concentrations and (equilibrium) body

concentrations using data from Budel soils alone (see Table 5.4). A comparison of body concentrations in artisoil and Budel soils also suggested that the bioavailability of zinc in Budel soils was lower (see paragraph 5.3.2), both for *E. andrei* and *E. crypticus*. The lower apparent bioavailability of zinc in Budel soils is in accordance with its lower extractability (see Table 3.10). In contrast to what is expected from these body concentration data, the difference of EC50s between soils *increased* for *E. crypticus* when using extractable concentrations.

One of the possible reasons for the latter increase of the difference between soils for *E. crypticus* is that the reproduction effects are not only determined by zinc. The contribution of, for example, copper or lead to toxicity may be more relevant than for *E. andrei*. Due to the possible influence of mixture effects it cannot be ruled out that also for *E. crypticus* the extractable concentration is better correlated to bioavailability than the total concentration.

The study of availability differences, for zinc alone, demonstrated that this factor could not give a full explanation for the reproduction effects in Budel soils. Other causes must apparently play a role, although the reasoning did show that bioavailability differences between soil types, and differences in species sensitivity, were important. The next step for explaining the phenomena in Budel soils is therefore the incorporation of mixture toxicity data.

5.4.4 The possible role of mixture effects, judged via distance-effect relationships

- *Principle*. Despite the probably dominant role of zinc, the body concentrations of the other metals were also increased in the soils where reproduction was affected (which was demonstrated for *E. andrei* for Zn, Cu, Pb and Cd, and for *E. crypticus* for Zn and Cd). The simultaneous increase of the concentrations of various metals inside the body (par. 5.3) implies that toxic effects may be attributable to the effect of a mixture. The possible contribution of joint toxicity in Budel soils are judged on the basis of available mixture toxicity data for both species that were collected using artisoil (Weltje et al., 1995, Posthuma et al., 1995). These data show that binary mixtures of metals usually have a slightly less than concentration-additive effect for both species on reproduction, when exposure is expressed through total concentrations. In addition, when using 0.01*M* $CaCl_2$-extractable metal concentrations, the deviation from concentration additivity was shown to be smaller, although still slightly antagonistic.

Before these data can be applied, it is first necessary to calculate the metal concentrations in the mixture related to a certain effect, for example the EC50. To estimate these concentrations, the sigmoid effect model was fitted to the data, with distance to the emission source as explanatory variable. On the basis of the estimated Effect Distance (ED)50, in combination with the distance-concentration relationships of Table 3.8, the concentrations of the metals zinc, copper, lead and cadmium were estimated by interpolation[5]. This was done both at the level of total- and extractable metal concentrations.

- *Calculus*. The following calculation method was further used to take mixture toxicity into account. All metal concentrations are expressed in dimensionless Toxic Units (TU), which are defined from single-metal toxicity experiments with the pertinent species exposed in artisoil. One TU of a metal is defined as the concentration that causes *x%* reduction of reproduction, for example at the EC50-level. The concentration of each metal *i* in Budel soil can be expressed as a fraction of this concentration, given the effect concentration EC*x*, by:

Equation 9:
$$TU_i = \frac{C_i}{EC_{x,i}}$$

with C_i = concentration of metal *i*.
Assuming concentration addition, metal concentrations of different metals can be summed, by:

Equation 10:
$$TU_{mix} = \sum_{i}^{n} TU_i$$

which gives the predicted EC*x* under concentration additivity.

When considering the EC50 level, the calculation of the predicted $TU_{mix,Budel}$ is equivalent to the sum of the ratio's $EC50_{Budel}/EC50_{OECD}$ over all metals (this ratio-method was already applied for zinc alone). If the observed TU_{mix} equals the expected value of TU=1, then the toxic effect levels in both soils are similar; if $TU_{mix}>1$, than toxic effects in Budel soil are less than expected from results in artisoil; if $TU_{mix}<1$, the opposite.

- *Distance-effect relationships for* Eisenia andrei. The ED50-values for cocoon and juvenile production were both estimated about 1 km (Table 5.8). Cocoon production revealed a better fit to the data, since the parameter was less variable than juvenile numbers (see Figure 5.12).
- *Distance-effect relationship for* Enchytraeus crypticus. The ED50-value for juvenile production was estimated as 3.5 km (Table 5.8, Figure 5.13). The 95% confidence interval of

---

[5] In this report we chose to calculate mixture concentrations on the basis of ED50s in combination with the linear-regressions between distance and metal concentration. These may be used accurately when local soil factors do not cause large random variation in extractability or pore water concentrations (see Table 3.8). As equivalent alternatives, we could also have chosen to calculate mixture concentrations on the basis of the EC50s for zinc alone (or starting with one of the other metals, and proceeding following paragraph 5.4.2), in combination with the correlations between *ln*-transformed metal concentrations (see Table 3.3 as an example; not all data have been reported). Both methods suffer from the same uncertainty, namely unexplained variability ('noise') in regression or correlation. The statistical prediction of an (extractable) metal concentration from an interpolated ED50 or EC50 becomes more uncertain when local soil characteristics strongly affect metal partitioning over solid and liquid phase (or: the bioavailability). The outcomes of both methods, in terms of comparisons of toxic effect levels between Budel soils and artisoil, are possibly numerically different, but qualitatively similar conclusions should be reached with respect to differences in effect levels between soils. Both methods were applied here, to judge the influence of statistical noise on the conclusions.

the ED50 is over-estimated due to lack of data on partial effects (i.e., between the sites #4 and 5), and due to the relatively small effects in soil #3. The latter performance may be a result of low metal extractability in this soil compared to soil #4, in which larger effects were found. On the basis of the observations it is reasonable to assume that the ED50 may take values between ca. 1 and 5 km; the data show that it is highly unlikely that 50% effect would occur at distance larger than 6 km.

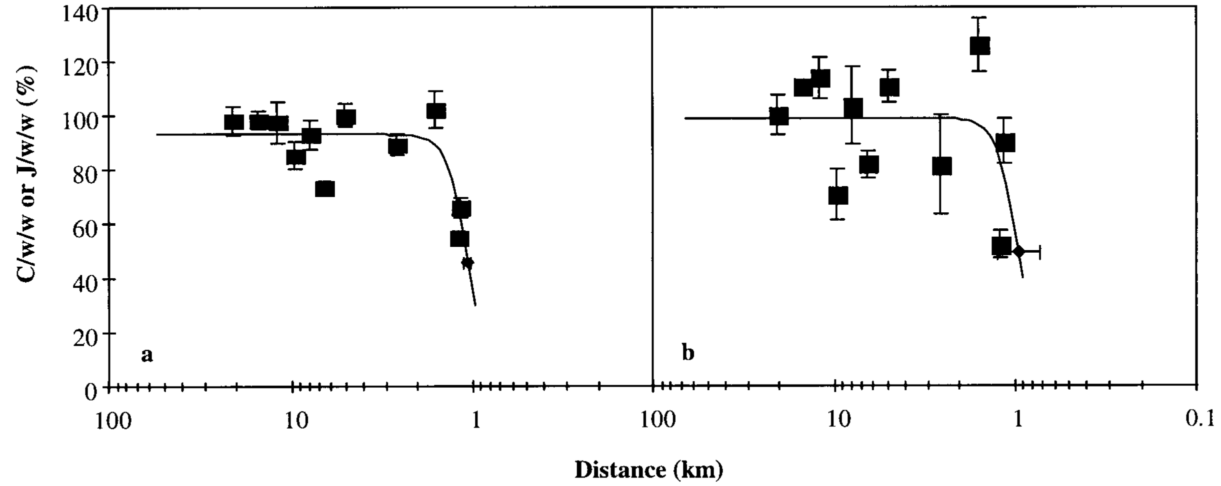

Figure 5.12. Observations (■, with s.e.) and fitted sigmoid distance-effect curves for *Eisenia andrei*, for cocoon (a) and juvenile production (b) in Budel soils. The ED50 is indicated with ◆, with horizontal 95% confidence interval bars for (b).

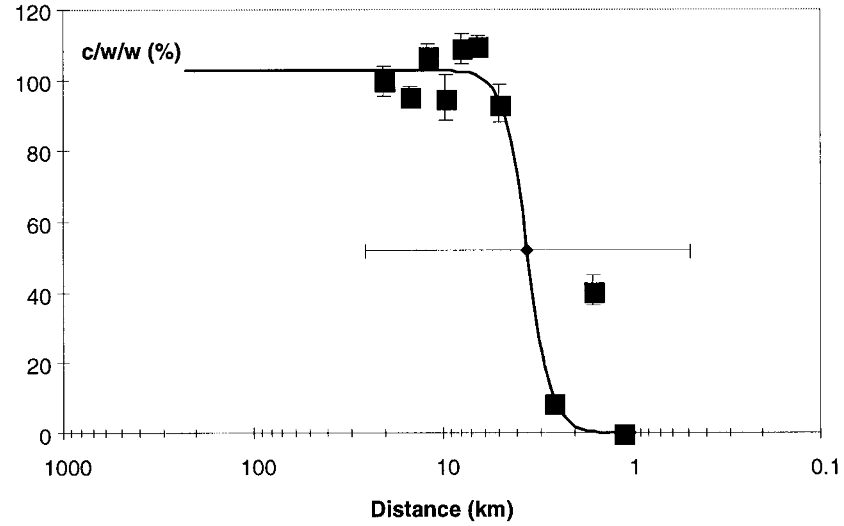

Figure 5.13. Observations (with s.e.) and fitted sigmoid distance-effect curve for juvenile production of *E. crypticus* in Budel soils. The ED50 is indicated (with horizontal 95% confidence interval bars) with ◆.

Species	Parameter	*C*	*b*	*m*	%	ED50(95% C.I.) (km)
Eisenia andrei	cocoon	94.7	7.76	0.053	56	1.05 (1.0 - 1.1)
	juveniles	98.9	6.96	-0.053	12	0.95 (0.7 - 1.2)
Enchytraeus crypticus	juveniles	102.9	6.78	1.262	86	3.53 (0.4 - 22.1)

Table 5.8. Parameter estimates of the logistic distance-effect curve for both species, and the calculated ED50 (ln(ED50)=m) with 95% confidence intervals.

- From ED50 to mixture concentrations: total concentrations. The ED50s were used to estimate the mixture composition on the basis of total metal concentrations. The results are summarized in Table 5.9, together with the EC50s, for separate metals in artisoil, as collected from literature.

Species		Zn	Pb	Cu	Cd	Fe	Cr	**total**
		EC50s in mg (total) metal .kg^{-1} dry wt						**(TU_{mix})**
E. andrei	Budel	576	247	71.5	2.39	5951	9.7	
	OECD (single)	429	1570	191.0	95.1	n.d.	155	
	Reference	1	2	3	1	n.d.	4	
	$TU_i=[Me]_{tot, ED50,Bu}/EC50_{OECD}$	**1.34**	**0.16**	**0.37**	**0.03**		**0.06**	**≥ 1.96**
E. crypticus	Budel	107	76.0	14.1	0.78	3671	6.71	
	OECD (single)	212*	n.d.	500	83.85	n.d.	n.d.	
	Reference	1		5	1			
	$TU_i=[Me]_{tot, ED50,Bu}/EC50_{OECD}$	**0.51**		**0.03**	**0.01**			**≥ 0.55**

Table 5.9. Comparison of ED50-derived total metal concentrations that, in mixture, cause 50 percent reproduction effect in soils from the Budel gradient (column total, TU_{mix}), with the EC50 of single metals in artisoil. The sign '≥' indicates that values may in fact be higher, when artisoil data on missing metals are added. References are: (1) Weltje et al., 1995, (2) Mogo, 1993, (3) Van Gestel et al., 1989, (4) Van Gestel et al., 1992, for Cr III; (5) estimated value from a concurrent experiment (Ec Cu3). * For this value the lowest EC50-estimate was taken, from experiment Ec Zn2. For Ec Zn1 and Ec Zn3 the EC50 was 254 and 361 mg Zn. kg^{-1} dry wt, which shows that the ratio between soils may reduce for zinc, causing the difference between Budel soils and OECD soil to be larger.

A reduction of reproduction of *E. andrei* with 50% apparently occurs in Budel soil at a total metal concentration in the Budel soil mixture of (minimal) 1.96 TU. Metals in Budel soil are apparently half as toxic as in artisoil, under the prepositions that (1) the mixture effect is concentration additive; (2) effects are judged on the basis of total metal concentrations in soil; and (3) the separate or mixture effects of iron is negligible. If data for iron would have been available from an artisoil test, the $TU_{mix,Budel}$ is expected to shift slightly upwards, beyond 1.96.

For *E. crypticus* the calculation based on this assumption shows another picture, with a mixture-EC50 ≥ 0.55 TU. The mixture in Budel soil is apparently twice as toxic for *E. crypticus* as in artisoil, although the EC50-level in artisoil is variable. It is, however, likely that real value is nearer to 1 TU than to 0.55, since the contribution of lead, iron and chromium are not incorporated in this calculation.

- From ED50 to mixture concentrations: Extractable metal concentrations. The mixture results of Weltje et al. (1995) were not only expressed on the basis of total metal concentrations, but also on the basis of 0.01*M* $CaCl_2$-extractable metal concentrations. Due to the use of extractable concentrations, the TUs change both for artisoil and for Budel soil. Only for zinc the extractable concentrations were measured at pH ca. 6. For the other metals extractable metal concentrations were measured without pH-adjustment. This implies that the extractable concentrations for copper, lead and cadmium are overestimated in most Budel soils, since metal solubility is higher at the low field pH than at a pH of ca. 6. The $TU_{mix, CaCl2}$ concentration calculated for

extractable concentrations is therefore biased. Moreover, only for a few metals TUs for artisoil have been calculated. The $TU_{mix,,\ CaCl2}$- calculation for the Budel soil results in mixture-EC50 values of 0.62 TU for *E. andrei* (data for copper, iron and chromium are lacking), and of 0.79 for *E. crypticus* (data for lead, iron and chromium are lacking).

Species		Zn	Pb	Cu	Cd	Fe	Cr	**total**
		EC50s in mg (extractable) metal .kg^{-1} dry wt						(TU_{mix})
E. andrei	Budel	52.9	1.89	13.2	1.2	5.4	n.d.	
	OECD (single)	133	10.5	n.d.	28.0	n.d.	n.d.	
	Reference	1	2		1			
	$TU_i=[Me]_{ext,\ ED50,Bu}/EC50_{OECD}$	**0.40**	**0.18***		**0.04***			**≥ 0.62***
E. crypticus	Budel	38.7	1.3	2.69	0.55	17.09	n.d.	
	OECD (single)	144	n.d.	5.25	61.6	n.d.	n.d.	
	Reference	1		3	1			
	$TU_i=[Me]_{ext,\ ED50,Bu}/EC50_{OECD}$	**0.27**		**0.51***	**0.01***			**≥ 0.79***

Table 5.10. Comparison of ED50-derived 0.01*M* $CaCl_2$-extractable metal concentrations that, in mixture, cause 50 percent reproduction effect in soils from the Budel gradient (column total, TU_{mix}), with the EC50 of single metals in artisoil. The sign '≥' indicates that values may in fact be higher, when artisoil data on missing metals are added. References are: (1) Weltje et al., 1995, (2) Mogo, 1993; (3) unpublished data. '*' indicates that these values were derived from 0.01*M*-$CaCl_2$-extraction type-1, at indigenous Budel soil pH.

The summed ratio's of Table 5.9 and Table 5.10 may shift upwards, since data for some metals are lacking. On the other hand, the value may shift downwards, depending on the shift in extractability of copper and cadmium between the indigenous pH of the Budel soils and a pH adjusted to ca. 5.5. This means that the difference between toxic effect levels between soils cannot be established with certainty.

- Deviation from concentration additivity. The values presented above for both species were based on calculations that assumed concentration additivity. Weltje et al. (1995), however, showed that the mixture effects of various binary metal mixtures in artisoil were less than concentration additive (antagonistic) for both species. Mixture-EC50s in artisoil ranged from ca. 1.4 TU for *E. crypticus* exposed to a mixture of cadmium and zinc, to 2.1 TU for *E. andrei* exposed to a mixture of zinc and copper. The tendency that a higher concentration of metals was needed in a binary mixture to cause a certain effect was found at the levels of both the total- and the extractable metal concentrations.

Antagonism will shift the summed ratio-values for both species from 1.96 resp. 0.55, found for the level of total soil concentrations, downwards. When judged by extractable concentrations, antagonism will also shift the values of 0.62 and 0.79 downwards.

5.4.5 *Conclusions about the possible role of mixture toxicity*

Difference in toxic effect levels between Budel soils and artisoil are summarized in Table 5.11 in TU-values. Values of 1 TU indicate that toxic effect levels in both soils are similar, values larger than 1 TU indicate that toxic effects in Budel soils are less than expected, and values less than 1 TU indicate the opposite.

	Zn only		Mixture	
	Total	Extractable	Total	Extractable
E. andrei	5.9	1.4	1.96	0.62
E. crypticus	0.57 - 0.97	0.05 - 0.38	0.55	0.79

Table 5.11. Comparison of toxic effect levels for two worm species in Budel soil and artisoil, assuming zinc as the only metal causing toxic effects in Budel soil (left), or assuming mixture effects to be concentration additive (right). Comparisons are made on the basis of total-, and 0.01*M* $CaCl_2$-extractable metal concentrations. Values larger than unity indicate that a metal (mixture) concentration that causes 50% effect in Budel soil would cause stronger effects in artisoil. Values below unity indicate the opposite. For all mixture data uncertainty exists due to lack of data: upwards shifts from additional artisoil toxicity data or downwards shifts from additional availability data or from antagonistic effects may results in *net* higher, equal or lower values for these entries.

Before making a final evaluation of the differences between effect levels in both soil types, it is useful to study the possible bias of the values. Bias may namely be present for several reasons, namely as a result of (1) unknown data and (2) statistical uncertainties.

1. The tabulated values may show both upwards and downwards shifts, as a consequence of adding further toxicity-, availability or mixture data. The magnitude of both shifts cannot be established with certainty from the present data. Inspection of the missing OECD-data, however, shows that the magnitude of the upward shift will be limited. This may hold in particular for *E. andrei* for which most data are available. Also for *E. crypticus* the upward shift may be limited, since the metals with the highest soil concentrations in Budel soil have already been incorporated. Inspection of the magnitude of antagonism, furthermore, suggests that the downward shift factor will lie in a range of ca. 1-2. Although the balance of these counteracting shifts cannot be calculated, incorporation of mixture toxicity reduced the difference in toxic effect levels between soil types that were calculated on the basis of zinc alone. Assuming concentration additivity, all estimated values for the difference between artisoil and Budel soils were near unity, with a lowest estimate of 0.55 and a highest estimate of 1.96.
2. The summary table shows that there is a large influence of random factors on the magnitude of difference between the effect levels of soil types. This follows from a comparison of two independently derived EC50-values, namely those derived for zinc. The analyses showed a considerable difference between the concentration of zinc related to 50 % effect when calculated following the two methods that were applied (see also footnote 5 on page 60). Ideally, the EC50 for zinc alone under the assumption of zinc dominance should be exactly similar as for zinc alone under the assumption of additive effects in the mixture (viz. 2553 and 576 mg total Zn .kg^{-1} dry soil): only the statistical method used to estimate these values

differ (resp. the zinc concentration used directly to estimate the EC50, and the zinc concentration derived indirectly from a distance-concentration relationship and the ED50). There is no principal objection against one of both ways of analysis, and the difference is apparently following from the facts that: (a) the random variation is handled in different ways, and is introduced at different stages of the calculation, (b) non-linear curves are involved with a low number of data points where the largest changes of response occur, in particular for *E. andrei*. The influence of random factors makes it only possible to qualify differences between soil types by trend or degree, rather than by absolute values or by a certain analysis of causes.

In view of the uncertainties, and on the basis of the available data from artisoil, the toxic effects of metals in Budel soils tend to be less than expected for *E. andrei*, and stronger for *E. crypticus*, when using total metal concentrations. However, toxic effect levels differ by apparently less than a factor two, when availability differences and mixtures are both taken into account through $0.01M$ $CaCl_2$-extractable concentrations. Although not evident from the toxicity observations for *E. crypticus*, it is reasonable to use the extractable concentration also for this species, in view of the body concentration data.

In conclusion, toxic effect levels of metals were grossly similar, in soils as different as Budel soil and artisoil and with considerably different metal sources and ages of contamination. For a prediction of metal effects on worms in Budel soils in the field it should be kept in mind that the study was made with Budel soils at adjusted pH. The indigenous pH of Budel soils is generally lower than used in the experiments, which usually implies that the extractability (and probably the bioavailability) of metals in the original field soils is higher. In this sense, the summarised values would underestimate the effects of Budel soils on earthworm reproduction in the field, since the metal availability is higher at the indigenous soil pH of Budel soils.

6. DISCUSSION AND CONCLUSIONS

6.1 Approach

The present study focused on the differences in toxic effect levels between soils, and on the explanation of the (dis)similarities. The major conclusion, with emphasis on availability differences and mixture effects in the studies, is that the difference between toxic effect levels in soils differing with respect to composition, origin and metal source, may be relatively small, at adjusted pH. This is: the difference is smaller than an order of magnitude when using total metal concentrations, and probably only a factor of ca. 1-2 in an upward or downward direction when taking bioavailability and mixture toxicity into account.

On the one hand, the observed difference is small in view of the number of uncertainties that may play a role when toxic effect levels are compared between soil types. This conclusion was reached on the basis of effect-studies for the two worm species that could successfully be reared in both the artisoil and the smelter soils. Regarding the differences in sensitivity between both species, the similarity of results strengthens the opinion that the conclusion is robust.

On the other hand, the adjustment of pH required for the study is an important soil modification that has affected both the availability of the metals for the animals by interfering with sorption, and the performance of the worms directly. This may hamper the straightforward use of the obtained data for extrapolation to other soils, and to predict earthworm performance at metal-contaminated field sites. Support for our conclusions is, however, present in a closely related study. The difference between laboratory and field soils has namely also been addressed by Spurgeon & Hopkin (1995). In that study, toxic effects of metals on mortality, growth and reproduction of *E. fetida* were established in artisoil, and the data were compared to toxic effects in metal contaminated soil collected in the surroundings of a zinc smelter at Avonmouth, UK. Based on total metal concentrations, toxic effects in the field soil were overestimated when predicted from artisoil experiments, similarly as in our data. Comparisons with data on the distribution of various earthworm species in the smelter area supported this conclusion, since earthworm species could be found at sites that would cause full mortality when judged by total metal concentrations. The pH adjustment of the smelter soil, required in our work, was not necessary with the Avonmouth soil, since this soil has a pH of ca. 6. The results from the Avonmouth-artisoil comparisons, without pH adjustment, grossly corroborated our results. Therefore, our findings may be valid for other soils too.

Although Spurgeon at al. (1995), also demonstrated a dissimilarity of toxic effect levels between artisoil and field soils, the assessments of exposure and of mixture effects were made in less detail than in the present paper. The present work focused specifically on:

- physico-chemical extractability of metals in relation to bioavailability
- mixture effects.

These factors could be studied in detail, since other factors (such as pH) were factored out, or good arguments exist to consider these insignificant (such as humidity). By studying toxic effects in pH- and humidity adjusted smelter soil with a mixture of metals under laboratory conditions, an answer was sought to the question whether earthworm species respond similarly at similar exposure, or not. Stated otherwise, how should exposure be defined in a way that it becomes insensitive for differences between soils. Below, the availability- and mixture aspects, which were treated in the separate chapters on exposure and effects, will be integrated.

6.2 Extractability of metals, (bio)availability and exposure assessment

6.2.1 General aspects

The physico-chemical characterisation of the Budel soils demonstrated that the soils differed in many aspects form artisoil. Quantitatively, the Budel soils generally had a lower (indigenous) pH, a lower proportion of clay and organic matter, and a lower (absolute) water content. For other factors only a qualitative assessment can be made due to the lack of quantitative data. With respect to quality aspects of the metal source, soil contamination in the Budel area resulted from a secondary smelting process, which usually results in the emission of metal oxides (Buchauer, 1973), whereas in artisoil the metals are often added as a solution of the metal-salts. In general, the chemical form in which the metals are emitted differs from that of most laboratory toxicity tests. Furthermore, the quality of the soil constituents to which metals may adsorb should be taken into account. This holds both for the form of organic matter and for the clay (which are *Sphagnum*-peat and kaolin in artisoil).

The abovementioned quantitative and qualitative aspects related to the physico-chemical behaviour of the metal in the soils should be covered routinely when studying soils for their ‘toxic potency’, to get insight in the (potentially) (bio)available metal concentrations. Routine coverage can presently only be accomplished with operationally defined extraction techniques (Ure & Davidson, 1995), in combination with multivariate techniques to take into account the influence of important factor as pH and organic matter on metal extractability (see, e.g., Janssen et al., 1996, Janssen & Posthuma, in prep.). It is evident, that such operationally and functionally defined techniques do not aim to give a causal-analytical description of how and why metal speciation equilibria respond to soil factors, and do not identify which metal species can actually be taken up by exposed organisms. However, an approach as followed in the Janssen-papers may give a compromise between what one wants to know about (bio)availability, and what is feasible in daily practice. By using the operationally defined techniques, and by using easily measured soil variables, a relatively accurate prediction of the (bio)available concentration of earthworms can be given[6].

Taken these considerations into account, the approach followed in the present research started from the viewpoints that (1) the extractable fractions of the metals are better correlated to (bio)availability than the total metal concentration (this doesn't imply that they are *equal* to it), assuming that exposure is mediated by pore water transport, and that (2) sorption characteristics depend on various important factors such as pH, organic matter content, clay content and cation exchange capacity (Ritchie & Sposito, 1995), which may affect the sorption of different metals in different ways.

6.2.2 Peculiarities of artisoil

The sorption characteristics of metals to artisoil are of importance when mutual comparisons are made with toxic effects in other soils. An analysis of metal sorption in artisoil reveals some phenomena worth to consider thoroughly.

Artisoil is composed of four basic constituents, viz. sand, peat, clay, $CaCO_3$ and water. With respect to sorption, the following comments can be made.

Sorption to sand is negligible, compared to peat and clay.

Sorption to the typical peat fraction of artisoil separately have not been published, to our knowledge. Addition of organic material, and the quality of organic material, is known to induce a range of changes in chemical equilibria due to associated changes in pH and cation exchange capacity (He & Singh, 1993). Organic material, in general, influences the uptake of metals by soil organisms and this differs per metal (e.g., Ma et al., 1983, Beyer et al., 1987).

Sorption data for the clay fraction show that kaolin ($Al_2Si_2O_5(OH)_4$) generally has a lower sorbing capacity than more common European clay minerals such as illite, vermiculite and montmorillonite (see Spurgeon & Hopkin, 1995). The sorption of metals to clay is influenced by pH. Sorption-edge experiments, i.e. studying sorption in kaolin suspensions at a constant metal concentration but at different pHs, showed that the sorption of metals to the clay was non-linear (Holm & Zhu, 1994). In an essentially carbonate-free medium, a steep increase of sorption of metals occurred at various pH-values, namely for Cd^{2+} beyond pH 6-7, for Pb^{2+} beyond pH 4, and for Cu^{2+} beyond pH 5. Below the mentioned pH-values, sorption was low or negligible. The pH-dependence indicates that sorption to kaolin consists of surface complexation. Sorption of Cu^{2+} was, however, strongly affected by the presence of dissolved organic matter (DOM), for which this ion has a high complexation affinity. As a further complexing factor, the calcium carbonate that is added to the artisoil to adjust the pH, may strongly affect metal sorption to kaolin through the formation of slightly soluble salts, such as cerussite ($PbCO_3$) or malachite ($Cu(OH)_2CO_3$).

---

[6] It is stressed that bioavailability is *not* covered routinely by the Janssen-approach, since this phenomenon has a species-specific component (which causes *bio*availability to be ill-defined when not recognised).

These data illustrate that the sorption strength of metals in artisoil can not directly be estimated from separate data on the soil constituents that were used. The dependence of metal sorption, e.g. on pH and on the carbonate additions used for pH adjustment, is of particular importance for the pH range that is commonly used in the experiments. In this pH range, usually ca. 5.5 for *E. andrei*, and ca. 6.0 for *E. crypticus*, small changes of pH or carbonate level may lead to large changes in metal sorption to, e.g., the clay fraction, which in turn may give rise to apparently 'random' variation of extractability. As a consequence, variation in toxic effect levels may occur even between two identical experiments in artisoil that differed only with respect to the batches of the soil constituents used.

These phenomena may explain the differences between replicated experiments that were found in our laboratory (Weltje et al., 1995, see also Table 3.10). These differences may have occurred in experiments with *E. crypticus,* where large difference in metal extractability occurred. It was also shown, however, that the largest effects occurred in the soil with the highest extractabilility (see Table 5.7 and Table 3.10). The EC50, expressed as total concentration, was lowest in experiment Ec Zn2, where the extractability was highest. The observations suggest that 'random' variation of extractability may occur between artisoil batches, but that toxicity in artisoil covaries accordingly.

This example illustrates that it is useful to make a detailed study on the metal sorption characteristics of artisoil. A metal-specific characterisation of the sorption characteristics of the artisoil can then be made, and the sensitivity of metal sorption for differences in pH, carbonate content and peat batch can be studied. Such a characterisation is of particular use since artisoil is an internationally accepted and frequently applied standard substrate for laboratory toxicity experiments with soil organisms (this also holds for other 'standard' soils too when they are used for the same purpose). If the sorption strength then appears to be strongly influenced by the quality of the soil constituents, then this should be taken into account in the design of experiments, in particular with reference to pH control. The use of a single clay type carries the risk of steep changes in sorption at small changes of pH or other relevant factors. It may be that the peculiarity of artisoil is not so much its artificiality itself, but rather its homogeneity, causing non-linear effects in sorption to be potentially strong.

6.2.3 Comparison of artisoil with Budel soils

In the present work, sorption of metals was studied following a functionally and operationally defined approach, that has not been developed to identify the equilibrium processes and the metal speciation in detail (Ure & Davidson, 1995). The extraction methods used are considered to give a more refined analysis of potential metal availability for organisms than total concentrations. Comparisons of the extractions of OECD soil only, in different experiments with the same metal, showed some large differences, that may have resulted from non-linear sorption processes. For the present purpose, however, it is interesting to judge the difference between artisoil with added metals, and the Budel soils. These comparisons showed that the extraction methods were sensitive enough to demonstrate relevant differences in metal sorption. Metals in Budel soils with adjusted pH tended to be bound stronger to the soil matrix than in artisoil (although the OECD-extractability varied); without pH adjustment, some metals were equally extractable in Budel soils and artisoil.

The differences in (bio)availability, as assessed by the differences in extraction efficiencies between artisoil and Budel soils at adjusted pH, seems to have consequences for metal uptake in the animals. In artisoil, at the same total concentration of a metal, the uptake form artisoil is higher, and the metal mixture of soil #2 caused 100% mortality. Although the bioavailability of the metals in the soil cannot be exactly quantified from such data, the body concentrations, which can be interpreted as the *net* effects of all factors that influence bioavailability, were lower in the Budel soil than in the laboratory systems.

Some of the present day work on the bioavailability of metals for soil organisms is characterised as a (multiple) correlations-approach. Various authors try to find (causal) correlations between various measures of external concentrations (total, various extractable fractions), via soil characteristics, with internal (body) concentrations. A recent example of this approach is the paper of Gonzalez et al. (1994), who studied metal accumulation in worms (*Lumbricus terrestris*) in relation to five subsequently extracted metal fractions (with increasing extractive strength) from 12 sites along a contaminated river. On the bases of the highest value for the correlation coefficient between a metal fraction and the earthworm body concentration, the authors identified a certain fraction for a metal as bioavailable. Such analyses carry the risk of faulty interpretation (e.g., autocorrelation, multiple colinearity, or small variability in an important variable which is therefore not recognised as being important), which are particularly tricky when studying relatively homogenous areas. This may have been the cause for the observation that the body concentration in worms, when judged by Budel soils alone, was equally predictable from total- and extractable concentrations (see Table 5.4), whereas incorporation of the artisoil results showed a large difference in predictability.

Janssen and Posthuma (in prep.) follow a similar approach, but they used a wide variety of Dutch soil types. The physico-chemical partitioning of metals is correlated to equilibrium body concentrations of earthworms via a multivariate analysis. It should, however, be taken into account that judgement of *net* bioavailability from body concentrations at equilibrium alone may be limited. In our experiments, the evolution of the body concentration showed time dependent uptake rates. At a constant exposure rate, the equilibrium is expected to reach its value after a constantly decreasing rate of concentration increase. In our observations, in soils causing ca. 50% reproduction effects, such a decrease was observed. This may be attributable to: (1) toxic effects, causing lower crawling activity of the worms, resulting in lower exposure, (2) increasing soil pH in the range with strong non-linear sorption and desorption processes, or (3) an increased efficiency of a metal-excretion mechanism. The decrease is not obvious when animals are sampled at a single moment in time, but it may occur. This phenomena may be an (up till now) neglected source of variation in body concentrations. It suggests that an approach of bioavailability *via* body concentrations should look at the instantaneous uptake rate of a toxicant from the exposure medium, viz. fitting parameter '*a*', the accumulation rate constant of Equation 5, from a short time-series observation on body concentrations. The results of Janssen and Posthuma's study will be reported elsewehere.

6.3 Effect assessment

6.3.1 Effects in artisoil

Results of toxicity experiments performed in our laboratory showed that there may be slight differences between experiments with respect to the obtained EC50 (see Table 5.7). Various causes may contribute to these differences:

- differences in worm performance, that can be judged by comparisons of reproductive activity among the control treatments between experiments (e.g., the comparison of control reproduction in 9 experiments by Van Gestel et al. (1992), shows variation between 0.5 and 1.4 cocoon .worm^{-1} .week^{-1}),
- different batches of peat to prepare the soil,
- slightly different pH, resulting from peat-specific and experiment-specific addition of calcium carbonate,
- general differences in experimental set-up.

Some of these factors may also influence toxicity through influencing bioavailability (see previous paragraph). For *E. crypticus*, it can also be added that the recovery methods to collect the juveniles from the soil differed between experiments (see Weltje et al., 1995). The gross similarity of the concentration-effect curves for the experiments 1 and 2 in Budel soil with *E. crypticus*, however, suggests that only the recovery differed between both methods.

For *E. andrei*, variation in NOECs or EC50s between experiments was observed, using both *E. andrei* and *E. fetida*, and looking at results from different laboratories (see Van Gestel et al., 1989, Van Gestel et al., 1991, Van Gestel et al., 1992, Spurgeon et al., 1994, Spurgeon & Hopkin, 1995). For zinc, the largest difference between experiments was a factor of ca. 1.6 (the ratio of maximum/minimum-value) at the EC50-level, with differences in the duration of the experiment and feeding condition. For copper, lead and cadmium, these factors were ca. 3.6, 1.2, and 2. At the level of the NOEC the differences are larger, since the NOEC is determined not only by earthworm performance, but also by the exact exposure concentrations applied in the experiment and by signal-noise ratio (Hoekstra & Van Ewijk, 1993). For *E. crypticus*, we only have data from own research, which show a similar level of variability of EC50-levels (see Table 5.7).

	Species	Characteristic	NOEC	EC50	pH	specifications	
Zn	*Eisenia fetida*	Cocoons	199	276	6.3	no food, 56 days	Spurgeon et al., 1994
	Eisenia fetida	Cocoons	237	-357	6.1	food, 21 days	Spurgeon & Hopkin, 1995
	Eisenia andrei	Cocoon	n.d.	429	4.8	food, 21 days	own data
Cu	*Eisenia fetida*	Cocoons	32	53.3	6.3	no food, 56 days	Spurgeon et al., 1994
	Eisenia fetida	Cocoons	29	-716	6.1	food, 21 days	Spurgeon & Hopkin, 1995
	Eisenia andrei	Cocoons	n.d.	191	6.3-7.1	food, 21 days	Van Gestel et al., 1989
	Eisenia andrei	Cocoons	56	>100	6.2	food, 21 days	Van Gestel et al., 1991
Pb	*Eisenia andrei*	Cocoons	n.d.	1570	5.3	food, 21 days	Mogo, 1993
	Eisenia fetida	Cocoons	1810	1940	6.3	no food, 56 days	Spurgeon et al., 1994
	Eisenia fetida	Cocoons	608	1629	6.1	food, 21 days	Spurgeon & Hopkin, 1995
Cd	*Eisenia fetida*	Cocoons	39.2	46.3	6.3	no food, 56 days	Spurgeon et al., 1994
	Eisenia fetida	Cocoons	152	-295	6.1	food, 21 days	Spurgeon & Hopkin, 1995
	Eisenia andrei	Cocoons	10	>100	6.0	food, 21 days	Van Gestel et al., 1992
	Eisenia andrei	Cocoons	18-32	33-96	6.7-6.8	food, 21 days	Van Gestel et al., 1991
	Eisenia andrei	Cocoon	n.d.	95.1	4.8	food, 21 days	own data
Cr III	*Eisenia andrei*	Cocoons	n.d.	155	6.0	food, 21 days	Van Gestel et al., 1992

Table 6.1. Toxic effect levels collected during studies with two species of *Eisenia*, in slightly different conditions. Some EC50-values of Spurgeon & Hopkin, 1995, obtained by logit-transformation, were estimated to be negative. In such cases, the NOEC was used for the comparisons, considering that EC50>NOEC.

These data suggest that comparison of toxic effect levels between soils should take (some) variation of effect levels in the artificial soil tests into account. The variation may, in part, be caused by differences in metal availability between experiments. A comparison of artisoil experiments can, however, not be made at the level of extracted- or even bioavailable metal fractions due to lack of data. Only our enchytraeid data with zinc and cadmium have indicated that such differences may be large.

6.3.2 Comparison of effects in Budel soil with artisoil

The experiments described in this report focused on the question whether effect levels in different soils would be similar given similar exposure. A comparison like this can only be made successfully, when the collected data are of sufficient quality. In the present Budel observations, there was no indication of bad performance of worms due to the characteristics of Budel soil *per se*. Reproduction in reference Budel soil (#11) was only slightly lower than the reproductive activity in artisoil for both species. This implies that pH adjusted Budel soil did not pose specific problems in studying dose-response relationships. At indigenous pH of the Budel soils, however, Posthuma et al. (1994) showed that reproductive activity of *E. andrei* was strongly reduced.

Comparison of the responses in Budel soils and artisoil suggests that the former were more variable. Superimposed on the metal concentrations, local soil factors apparently influenced the reproductive performance. Although this variation at adjusted pH is not caused by a known factor that covaries with distance, it has caused statistical bias, in particular for *E. andrei*. In this species, a maximum of ca. 50% effect was observed in only one of the soils studied. This causes the outcome of EC50 estimation-methods to be highly dependent on the properties of this particular soil. It may have caused the divergence of the EC50-concentrations for zinc alone, which were estimated as ca 2550 $mg.kg^{-1}$ (total Zn, using concentration-effect relationships for zinc alone) and as ca. 575 $mg.kg^{-1}$ (total Zn, from distance-effect relationships). When judging the pattern of the original observations, it is clear that this soil (#2) caused less effects then may be expected from the response pattern expected from the series of soils collected at larger distances. For *E. crypticus*, this type of bias was lower, since the EC50-estimate was based on large response in several soils, namely almost full inhibition of reproduction in 3 Budel soils.

Given the information on variability in effect levels within a single soil type (artisoil data), and the bias in effect-level estimation, it can still be concluded that the effect levels differ between soils by less then an order of magnitude.

6.3.3 Observations on other species exposed in Budel soils

Next to worms, the gradient soil samples from Budel were also used to study toxic effects in other species groups. Detailed reports of these studies have appeared for plants and springtails, and some findings are summarized below.

- *Plants*. Various studies were done with plants in the same series of Budel soils as used here (with soil #1 as an additional observation compared to the earthworm experiments). Van Der Hoeven & Henzen (1994b) found that the EC50 of zinc (total concentration) for growth of *Trifolium pratense* as estimated from the gradient approach differed by more than a factor two from the value that was obtained by studying growth in clean Budel soil (#11) to which metal salts were added. When expressing the difference on the basis of the 0.01*M* $CaCl_2$-extractable fraction (measured at indigenous pH), the difference between EC50s was less than 25%. In this comparison, the difference of EC50s was demonstrated between laboratory-added zinc and zinc (in a mixture) originating from the zinc smelter, which grossly factors out the soil type as a cause of the difference. The explanations for the difference in effect level can thus be, that added zinc is more available and more toxic compared to smelter-zinc, while mixture effects may also play a role (see below). For *Sinapis alba*, similar observations were made in the Budel soils from the gradient, but in that study slight pH differences between the soils caused larger effects then the metals (Van Der Hoeven & Henzen, 1994c)

Mixture effects for *Trifolium* were analysed in the reference Budel soil (#11), by addition of cadmium and zinc (Van Der Hoeven & Henzen, 1994d). It appeared that the response of growth to the binary mixture was slightly less then concentration additive (antagonistic).

-*Springtails*. Smit & Van Gestel, in prep. studied the survival, individual growth and reproduction of the springtail *Folsomia candida* in the Budel soils, adjusted to pH ca. 6. No relationship between total zinc concentration and body growth or reproduction was found, whereas this relationship could be demonstrated in two artificially contaminated soils with similar total zinc concentration ranges, namely Budel soil #11 and LUFA-soil. The lack of response in the Budel soil gradient could partly be explained when metal extractability differences (water and 0.01*M* $CaCl_2$-type 2) were taken into account, although there was also evidence that the soil properties directly influenced the sensitivity of the species for zinc.

The detailed reports for plants and springtails, thus, also suggest that differences in metal availability are an important source of variation of toxic effect differences between soils. In addition, less than concentration additive effects with mixtures of metals were also found to be relevant for a plant species.

6.3.4 Laboratory toxicity experiments, outdoor mesocosm studies and real field studies

The findings of the present study were obtained in laboratory conditions. In addition to the present studies, environmental variability, e.g. in temperature and humidity conditions, may be relevant in the field. A study on the toxic effects of zinc on the performance of *E. andrei* has recently been performed in enclosures within an outdoor experimental field artificially contaminated with different zinc concentrations (mesocosm study). For a preliminary report: see Van Beelen and Notenboom (1996).

Despite the usefulness of mesocosm studies as an intermediate step between laboratory and field, real field observations are needed to complete the investigations into the factors that are important in predicting toxic effects of chemicals in the field. By performing field studies in a way comparable to the observations made here, taking mixture effects and bioavailability into account, it can be investigated whether cocoon production, growth or mortality, are associated to population viability. This was recognized by Kammenga (1996) as a crucial factor in laboratory-field extrapolation, since different fitness characteristics may be differently associated with population viability. For population studies, it should however be taken into account that control of exposure conditions, for example through adjustment of pH, is impossible in field conditions. This implies that the ratio of 'signal' (toxic effect) over 'noise' in such studies will be worse than in the present analyses. 'Noise' caused by the variation in indigenous pH was so large that the toxic effects of metals on cocoon production in *E. andrei* could not be distinguished in a previous study (Posthuma et al., 1994).

Observations in laboratory-, semi-field- and field conditions are all useful in their own right. It can be anticipated, however, that false positives (effect in the laboratory systems, but not at contaminated field sites) and false negatives (effect in the field, but not in laboratory conditions) may all occur due to the complexity of the systems under investigation. On the basis of this consideration, Chapman (1995ab) concluded that 'validation' in the sense of being able to make exact predictions is not feasible. Rather, validation should be considered as the careful scrutiny of toxic effects of chemicals in different conditions, so as to identify the factors that may modify toxic effects, and to know how strong their influence is. Following this reasoning, the results of the present studies with earthworms have shown that incorporation of both metal availability differences between soils and mixture toxicity significantly improves the accuracy of laboratory-field extrapolation of toxicity data.

7. REFERENCES

Beyer, W. N., Hensler, G., & Moore, J. (1987) Relation of pH and other soil variables to concentrations of Pb, Cu, Zn, Cd, and Se in earthworms. *Pedobiologia* 167-172.

Boekhold, A. E. (1992) *Field scale behaviour of cadmium in soil.* Ph. D. Thesis, Landbouwuniversiteit, Wageningen, The Netherlands.

Buchauer, M. J. (1973) Contamination of soil and vegetation near a zinc smelter by zinc, cadmium, copper and lead. *Environ. Sci. Technol. 7,* 131-135.

Chapman, P. M. (1995a) Extrapolating laboratory toxicity results to the field. *Environ. Toxicol. Chem. 14,* 927-930.

Chapman, P. M. (1995b) Do sediment toxicity tests require field validation? *Environ. Toxicol. Chem. 14,* 1451-1453.

Chardon, W. J. (1984) *Mobiliteit van cadmium in de bodem.* Staatsuitgeverij, Den Haag, The Netherlands.

Dirven-Van Breemen, E. M., Baerselman, R., & Notenboom, J. (1994) *Onderzoek naar de geschiktheid van de potwormsoorten Enchytraeus albidus en E. crypticus (Oligochaeta, Annelida) in bodemecotoxicologisch onderzoek.* RIVM-report no. 719102025, Bilthoven, The Netherlands.

Gonzalez, M. J., Ramos, I., & Hernandez, L. M. (1994) Distribution of trace metals in sediments and the relationship with their accumulation in earthworms. *Intern. J. Environ. Anal. Chem. 57,* 135-150.

Gooddy, D. C., Shand, P., Kinniburgh, D. G., & Van Riemsdijk, W. H. (1995) Field-based partition coefficients for trace elements in soil solutions. *Europ. J. Soil Sci. 46,* 265-285.

Gupta, S. K., & Aten, C. (1993) Comparison and evaluation of extraction media and their suitability in a simple model to predict the biologival relevance of heavy metal concentrations in contaminated soils. *Int. J. Environ. Anal. Chem. 51,* 25-46.

Haanstra, L., Doelman, P., & Oude Voshaar, J. M. (1985) The use of sigmoidal dose response curves in soil ecotoxicological research. *Plant Soil 84,* 293-297.

He, Q. B., & Singh, B. R. (1993) Effect of organic matter on the distribution, extractability and uptake of cadmium from soils. *J. Soil Sci. 44,* 641-650.

Hoekstra, J. A., & Van Ewijk, P. H. (1993) Alternatives for the no-observed-effect level. *Environ. Toxicol. Chem. 12,* 187-194.

Holm, T. R., & Zhu, X.-F. (1994) Sorption by kaolinite of Cd2+, Pb2+ and Cu2+ from landfill leachate-contaminated groundwater. *J. Contamin. Hydrol. 16,* 271-287.

Janssen, M. P. M., Bruins, A., De Vries, Th., & Van Straalen, N. M. (1991) Comparison of cadmium kinetics in four soil arthropod species. *Arch. Environ. Contam. Toxicol. 20,* 305-312.

Janssen, R. P. T., Pretorius, P. J., Peijnenburg, W. J. G. M., & Van Den Hoop, M. A. G. T. (1996) *Determination of field-based partition coefficients for heavy metals in Dutch soils and their relationships with soil characteristics.* RIVM-report no. 719101023, Bilthoven, The Netherlands.

Janssen, R.P.T. & Posthuma, L. (in prep.) Assessment of the exposure of earthworms *Eisenia andrei* by heavy metals in contaminated soils.

Kammenga, J. E. (1995) *Phenotypic plasticity and fitness consequences in nematodes exposed to toxicants.* Ph. D. Thesis, Landbouw Universiteit, Wageningen, The Netherlands.

Locher, W. P., & De Bakker, H. (1994) *Bodemkunde Van Nederland. Deel 1. Algemene Bodemkunde*, Malmberg, Den Bosch.

Ma, W.-C., Edelman, Th., Van Beersum, I., & Jans, Th. (1983) Uptake of cadmium, zinc, lead and copper by earthworms near a zinc-smelting complex: influence of soil pH and organic matter. *Bull. Environ. Contam. Toxicol. 30*, 424-427.

Marinussen, M. P. J. C., & Van Der Zee, S. E. A. T. M. (1994) Spatial variability, risk and extent of soil pollution: conceptual approach of estimating the exposure of organisms to soil contamination. *Groundwater Quality Management. Proc. of the GQM 93 Conference.* pp. 245-255, IAHS Publishers.

Mogo, F. C. (1993) *Combination toxicity of heavy metals for earthworms (Eisenia andrei).* M. Sc. Thesis, E.E. 101, I.H.E., Delft, The Netherlands.

Notenboom, J., & Posthuma, L. (1994) *Validatie toxiciteitsgegevens en risicogrenzen bodem: voortgangsrapportage 1993.* RIVM reportno. 719102029, Bilthoven, The Netherlands.

Notenboom, J., & Posthuma, L. (1995) *Validatie toxiciteitsgegevens en risicogrenzen bodem: voortgangsrapportage 1994.* RIVM-report no. 719102045, Bilthoven, The Netherlands.

Notenboom, J., & Van Beelen, P. (1992) *Validatie van risicogrenzen voor de bodem. Verslag van een workshop gericht op de opzet van een onderzoeksprogramma.* RIVM-reportno. 719102020, RIVM, Bilthoven, The Netherlands.

Notenboom, J., Vaal, M.A. & Hoekstra, J.A. (1995) Using comparative ecotoxicology to develop Quantitative Species Sensitivity Relationships (QSSR). *Environ. Sci. & Pollut. Res. 2*: 242-243.

OECD (1984) OECD-Guideline for Testing of Chemicals No. 207. Earthworm Acute Toxicity Tests, Organization for Economic Cooperation and Development, Paris.

Oostenbrink, M. (1960) Estimating nematode populations by some selected methods, In: *Nematology* (Sasser, J. N., & Jenkins, W. R., Eds.) pp. 85-102.

Pepels, A., & Lagas, P. (1993) *Biologische beschikbaarheid van zware metalen in de grond: selectie van bepalingsmethode en beoordeling van toepassingsmogelijkheden (een literatuurstudie).* RIVM report no. 715701001, Bilthoven, The Netherlands.

Posthuma, L. (in press) Effects of toxicants on population and community parameters in field conditions, and their potential use in the validation of risk assessment methods, In:

Ecological Principles for Risk Assessment of Contaminants in Soil (Van Straalen, N. M., & Lokke, H. E., Eds.).

Posthuma, L. (1992) *Genetic ecology of metal tolerance in Collembola.* Ph. D. Thesis, Vrije Universiteit, Amsterdam, The Netherlands.

Posthuma, L., Boonman, H., Mogo, F. C., & Baerselman, R. (1994) *Heavy metal toxicity in Eisenia andrei exposed in soils from a gradient around a zinc smelter (Budel), and comparison with toxic effects in OECD-artificial soil.* RIVM-reportno. 719102033, Bilthoven, The Netherlands.

Posthuma, L., Weltje, L., & Notenboom, J. (1995) Mixtures of toxicants and their effects on soil animals. In: *RIVM Annual Scientific Report 1994* pp. 137-138, Bilthoven, The Netherlands.

Pretorius, P. T., Janssen, R. P. T., Peijnenburg, W. J. G. M., & Van Den Hoop, M. A. G. T. (1996) *Chemical equilibrium modelling of metal partitioning in soils.* RIVM report no. 719101024, Bilthoven, The Netherlands.

Ritchie, G. S. P., & Sposito, G. (1995) Speciation in soils, In: *Chemical Speciation in the Environment.* (Ure, A. M., & Davidson, C. M., Eds.) , Chapman and Hall, London.

Smit, C. E., & Van Gestel, C. A. M. (in prep.) Comparison of the toxicity of zinc for the springtail *Folsomia candida* in artificially contaminated and polluted soils.

Sokal, R. R., & Rohlf, F. J. (1981) *Biometry. The Principles and Practice of Statistics in Biological Research. 2nd Edition*, W.H. Freeman and Company, New York.

Spurgeon, D. J., & Hopkin, S. P. (1995) Extrapolation of the laboratory-based OECD earthworm toxicity test to metal-contaminated field sites. *Ecotoxicology 4*, 190-205.

Spurgeon, D. J., Hopkin, S. P., & Jones D.T. (1994) Effects of cadmium, copper, lead and zinc on growth, reproduction and survival of the earthworm Eisenia fetida (Savigny): assessing the environmental impact of point-source metal contamination in terrestrial ecosystems. *Environ. Pollut. 84*, 123-130.

Steur, G. G. L., & Heijink, W. (1991) *Bodemkaart Van Nederland Schaal 1:50000. Algemene Begrippen En Indelingen*, Staring Centrum, The Netherlands, Wageningen.

Ure, A. M., & Davidson, C. M. (1995) Introduction to speciation, In: *Chemical Speciation in the Environment* (Ure, A. M., & Davidson, C. M., Eds) pp. 2-5, Chapman and Hall, London.

Vaal, M.A, Van Der Wal, J.T., Hoekstra, J.A. & Hermens, J. (in prep) Variation in the sensitivity of aquatic species in relation to the classification of environmental pollutants.

Van Beelen, P. & Notenboom, J. (1996) *Validatie toxiciteitsgegevens en risicogrenzen bodem: voortgangsrapportage 1995.* RIVM reportno. 607505002, Bilthoven, The Netherlands. (*in prep.*)

Van Der Hoeven, N., & Henzen, L. (1994a) *Groei van de plantensoorten Lolium perenne, Vicia sativa en Trifolium pratense op grond uit Budel en effecten van zink en cadmium op de groei.* IMW-R 94/004, Delft, The Netherlands.

Van Der Hoeven, N., & Henzen, L. (1994b) *Groei van de plantensoort Trifolium pratense op elf gronden uit Budel gemonsterd in een gradient.* IMW-R 94/041, Delft, The Netherlands.

Van Der Hoeven, N., & Henzen, L. (1994c) *Groei van de plantensoort Sinapis alba op elf gronden uit Budel gemonsterd in een gradient.* IMW-R 94/045, TNO, Delft.

Van Der Hoeven, N., & Henzen, L. (1994d) *Combinatietoxicologie van de metalen zink en cadmium en de plantensoort Trifolium pratense op relatief schone grond uit Budel.* report nr. IMW-R 94/130, TNO (IMW), Delft, The Netherlands.

Van Gestel, C. A. M. (1992) Validation of earthworm toxicity tests by comparison with field studies: a review of benomyl, carbendazim, carbofuran, and carbaryl. *Ecotox. Eviron. Saf. 23*, 221-236.

Van Gestel, C. A. M., Dirven-Van Breemen, E. M., & Baerselman, R. (1992) Influence of environmental conditions on the growth and reproduction of the earthworm *Eisenia andrei* in artificial soil substrate. *Pedobiologia 36*, 109-120.

Van Gestel, C. A. M., Dirven-Van Breemen, E. M., & Baerselman, R. (1993) Accumulation and elimination of cadmium, chromium and zinc and effects on growth and reproduction in *Eisenia andrei* (Oligochaeta, Annelida). *Sci. Tot. Environ. Suppl. 1993*, 585-597.

Van Gestel, C. A. M., Dirven-van Breemen, E. M., Baerselman, R., Emans, H. J. B., Janssen, J. A. M., Postuma, R., & Van Vliet, P. J. M. (1992) Comparison of sublethal and lethal criteria for nine different chemicals in standardized toxicity testst using the earthworm *Eisenia andrei. Ecotox. Environ. Saf. 23*, 206-220.

Van Gestel, C. A. M., Van Dis, W. A., Dirven-Van Breemen, E. M., Sparenburg, P. M., & Baerselman, R. (1991) Influence of cadmium, copper, and pentachlorophenol on growth and sexual development of Eisenia andrei (Oligochaeta: Annelida). *Biol. Fertil. Soils 12*, 117-121.

Van Gestel, C. A. M., Van Dis, W. A., Van Breemen, E. M., & Sparenburg, P. M. (1989) Development of a standardized reproduction toxicity test with the earthworm species *Eisenia fetida andrei* using copper, pentachlorophenol, and 2,4-dichloroaniline. *Ecotox. Environ. Saf. 18*, 305-312.

Van Loon, J. C., & Barefoot, R. R. (1995)Trends and developments, In: *Chemical Speciation in the Environment* (Ure, A. M., & Davidson, C. M., Eds) pp. 335-388, Chapman and Hall, London.

Van Straalen, N. M., & Denneman, C. A. J. (1989) Ecotoxicological evaluation of soil quality criteria. *Ecotoxicol. Environ. Saf. 18*, 241-251.

Van Straalen, N. M., Leeuwangh, P., & Stoltelder, P. B. M. (1994)Progressing limits for soil ecotoxicological risk assessment, In: *Ecotoxicology of Soil Organisms.* (Donker, M. H., Eijsackers, H., & Heimbach, F., (Eds.)) pp. 397-409, Lewis Publishers, Boca Raton, Florida, USA.

Weltje, L., Posthuma, L., Mogo, F. C., Dirven-Van Breemen, E. M., & Van Veen, R. P. M. (1995) *Toxische effecten van combinaties van cadmium, zink en koper op terrestrische oligochaeten in relatie tot bodem-chemische interacties.* RIVM-report nr. 719102043, Bilthoven, The Netherlands.