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Validation of toxicity data and risk limits for soils: final report

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QUALITY ASSURANCE

This report has been prepared as an overview of the results collected by the three participating institutes, original literature is presented elsewhere (see Project Bibliography). Contributing authors agreed to carry responsibility for the contents of their chapters. An editorial committee, consisting of Dr. ir. C.A.M. van Gestel, Dr. C.E. Smit, Drs D.J Bakker and Dr. L. Posthuma (Chair), with the assistance of Dr. J.W. Vonk, compiled the work into its present form. Prior to release, proof-reading of the full report was executed by Dr. P. van Beelen.

PREFACE

This report presents the main findings of the project 'Validation of toxicity data and risk limits for soils', in short the Validation project. These findings have been compiled from research work that has been performed by research groups at the Vrije Universiteit in Amsterdam, (VU - Department of Ecology and Ecotoxicology), the Netherlands Organization for Applied Scientific Research (TNO - Institute of Environmental Sciences, Energy Research and Process Innovation), and the Dutch National Institute of Public Health and the Environmental (RIVM). The project was co-ordinated by RIVM (Laboratory for Ecotoxicology).

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The results presented could only be obtained due to the help of many organizations and people:

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It has been a fine job to work with all these people, and to be able to present to the readership the resulting summary report on the following pages. It is a pleasure for me to be able to state this in concert with the former project co-ordinators, Patrick van Beelen and Jos Notenboom.

Leo Posthuma

ABSTRACT

Laboratory toxicity tests are important for the derivation of soil quality criteria in the Netherlands. No Observed Effect Concentrations of species or processes are used to derive statistical sensitivity distributions. Hazardous Concentrations (HC5 and HC50) derived from these distributions define the ecotoxicological risk limits. The project 'Validation of Toxicity Data and Risk Limits for Soils' was set up to evaluate the ecological relevance of (1) laboratory toxicity data and (2) ecotoxicological risk limits, and to identify which factors introduce uncertainties. Toxic effects of zinc and other metals were studied on selected species (*Trifolium pratense*, *Folsomia candida*, *Enchytraeus crypticus*, *Eisenia andrei*) and on microbial degradation activities. Differences in bioavailability between soil types were found to be of prime importance in laboratory-to-field extrapolation of toxicity data. Studies with mixtures suggested that each contaminant should be taken into account in risk assessment. Variable exposure conditions appeared to moderately modulate toxicity. Toxic effects of zinc on community endpoints were determined at an experimental field plot and a heavy-metal contaminated field site using enchytraeids, nematodes and micro-organisms. No or weak responses were found at the HC5 level, while measurable effects were present at the HC50 level. This was confirmed by data from the literature. The scientific underpinning of HC values may be improved when toxicity-modulating factors such as bioavailability are taken into account in the interpretation of laboratory toxicity data and when long-term effects of contaminants are focused on. The effect of improved laboratory-to-field extrapolation on the estimated risk limits, however, depends on the relative importance of the correction factors in relation to the variation in species sensitivities. Hazardous Concentrations are valuable as a first-tier risk evaluation system in soil protection policy. In a second-tier (location-specific) evaluation, the most important correction factors for laboratory-to-field extrapolation, named above, should be taken into account for the assessment of the local risk.

Key words:
Validation, Risk Assessment, Laboratory-to-field extrapolation, Statistical extrapolation, Soil, Metals, Zinc, Invertebrates, Microbial functions, PICT, Hazardous Concentrations

SAMENVATTING

Resultaten van laboratorium toxiciteitstoetsen dienen als uitgangspunt bij het afleiden van bodemnormen in Nederland. No Observed Effect Concentrations voor soorten of processen worden gebruikt voor het vaststellen van statistische gevoeligheidsverdelingen. De van deze verdelingen afgeleide Hazardous Concentrations (HC5 en HC50) dienen als ecotoxicologische risicogrenzen. Het project 'Validatie van Toxiciteitsgegevens en Risicogrenzen Bodem' werd opgezet om de ecologische relevantie van laboratorium toxiciteitsgegevens en daarvan afgeleide risicogrenzen te evalueren en om de factoren te identificeren die het meest bijdragen aan de onzekerheden met betrekking tot de risicoschatting. Er is onderzoek gedaan naar de negatieve effecten van zink en andere metalen op enkele geselecteerde toetsorganismen (*Trifolium pratense*, *Folsomia candida*, *Enchytraeus crypticus*, *Eisenia andrei*) en op de microbiele degradatie activiteit. Verschillen in biologische beschikbaarheid tussen bodemtypes bleken van groot belang voor de extrapolatie van laboratoriumgegevens naar de veldsituatie. Uit experimenten met mengsels van metalen kon worden afgeleid dat alle aanwezige contaminanten moeten worden bij de risicobeoordeling. Variabele of sub-optimale blootstellingscondities bleken de toxiciteit in geringe mate te beïnvloeden. De negatieve effecten van zink op levensgemeenschappen van enchytraëen, nematoden en microörganismen werden bepaald in een proefveld en op een verontreinigde veldlocatie. Levensgemeenschapkenmerken werden niet of nauwelijks beïnvloed bij zink concentraties op het HC5-niveau, terwijl meetbare effecten aanwezig waren op het niveau van de HC50. Dit beeld werd bevestigd door literatuurgegevens. De wetenschappelijke onderbouwing van HC-waarden kan worden verbeterd wanneer bij de interpretatie van laboratoriumgegevens rekening wordt gehouden met factoren als biologische beschikbaarheid en wanneer meer aandacht wordt gegeven aan de lange termijn effecten van verontreinigingen. De invloed van een verbeterde extrapolatiemethodiek op de hoogte van de afgeleide risicogrenzen hangt echter af van het relatieve belang van de toegepaste correcties ten opzichte van de variatie in gevoeligheid tussen soorten. De huidige Hazardous Contrentations zijn goed bruikbaar als een eerste generiek beoordelingscriterium in het bodembeschermingsbeleid. In het vervolgtraject moet bij de locatie-specifieke beoordeling van bodemkwaliteit rekening worden gehouden met de hierboven genoemde factoren die het meest van invloed zijn op de extrapolatie van laboratoriumgegevens naar de veldsituatie.

Trefwoorden:
Validatie, Risico Beoordeling, Lab-veld extrapolatie, Statistische extrapolatie, Bodem, Metalen, Zink, Evertebraten, Microbiële functies, PICT, Hazardous Concentrations

EXECUTIVE SUMMARY

This report contains the results of the project 'Validation of Toxicity Data and Risk Limits for Soils', in short the 'Validation project'. The project was initiated by the Dutch Ministry of Housing, Spatial Planning and the Environment in 1992 and has been carried out by the National Institute of Public Health and the Environment (RIVM), the Netherlands Organization for Applied Scientific Research (TNO) and the Vrije Universiteit (VU). The aim of the project was to critically investigate the scientific underpinning of the Dutch soil protection policy and to give recommendations for further improvements of the methods that are used for risk assessment for soils.

Laboratory toxicity data on 'species' and 'microbial processes' are important for the derivation of ecotoxicological risk limits and soil quality standards. These data are used as input for statistical extrapolation models to yield estimated maximum concentrations at which the functioning of ecosystems is assumed to be protected. A previous comparison of different extrapolation methods showed that the estimated risk levels are primarily determined by the input data, the type of extrapolation method is far less important.

The use of relatively simple tests for the derivation of risk limits introduces a number of uncertainties. Differences in sensitivity between species are already accounted for in the risk assessment procedure through the use of statistical sensitivity distributions. So far, the possible modification of toxicity under field conditions is not included in the procedures: it may be questioned to what extent effects observed in laboratory tests indicate effects in the field. Moreover, relatively little attention has been paid to the actual consequences for ecosystems when risk limits are exceeded.

The Validation project focused on two aspects:

1. The relevance of (standardised) laboratory toxicity tests for the prediction of effects in the field.
2. The ecological relevance of risk limits that are derived with statistical extrapolation models.

Different approaches have been used in the project:

- Laboratory research in which test organisms were exposed to polluted field soils and different kinds of experimentally contaminated soils under controlled conditions.
- Experiments in which test organisms were exposed in an experimentally contaminated field plot.
- Observations on communities of micro-organisms, nematodes and enchytraeids in different contaminated field and semi-field soils.

The results have been compared with literature data and results of related projects.

A pollution gradient in the vicinity of a former zinc smelter at Budel, The Netherlands was chosen as field study site. Soil from this area, which is contaminated with zinc, cadmium, lead and copper, was used in laboratory experiments on the earthworm *Eisenia andrei*, the enchrytraeid *Enchytraeus crypticus*, the collembolan *Folsomia candida*, the plant *Trifolium pratense* (red clover), and micro-organisms. From these experiments it appeared that zinc is the

dominant toxic metal in the Budel soil. This metal was therefore used in the laboratory and field experiments with artificially contaminated soils.

For the construction of the experimental field plot, a concentration range of zinc chloride was made in a natural soil, and the contaminated soil was placed in outdoor enclosures at the VU-campus. Experiments with most of the above mentioned organisms were performed in the experimental field plot during three successive years, and the development of the nematode community was followed. In addition, abundance and species diversity of nematodes and enchytraeids were determined in the field gradient near Budel.

Next to these studies, mainly directed at determining effects of zinc on structural characteristics of the soil ecosystem, the effect of zinc on functional parameters was studied. Attention was focused on the effect of zinc on microbial degradation capacity in different soil types and in the experimental field plot. Special attention was paid to the development of microbial tolerance as a result of zinc exposure. This last aspect was also studied at the Budel field site.

Chapter 1 of this report reviews the factors that may influence the extrapolation of laboratory toxicity data to the field situation. These factors involve differences in exposure concentration and conditions between laboratory and field, related to the limited ecological reality and complexity of most laboratory test designs. With respect to the contaminant itself, the most obvious differences are found in the often high bioavailability and homogeneous distribution of contaminants in the soil substrate, combined with the fact that mostly only a single contaminant is tested. Limited ecological reality and complexity involve the choice of test parameters, the use of single-species tests and the exclusion of ecological interactions and processes such as adaptation and tolerance.

Bioavailability appeared to be the most important factor for all types of soil organisms when comparing effects of zinc in different soil types. Differences in bioavailability could be partly corrected for by normalizing effect concentrations on the basis of organic matter and clay content. This normalization, which is used in the Dutch method for deriving risk limits for soils, does not correct for differences in soil pH. Soil pH is a major factor determining metal bioavailability. The relationship between pH and zinc bioavailability is very complicated. An increase in pH causes a decrease of the zinc concentration in the soil pore water and thus leads to a potential decrease in exposure. This was shown in experiments with artificial soil (Chapter 2). A high pH in the soil pore water may at the same time lead to increased sorption of zinc onto cell walls, and to an increased uptake by organisms. This was found in experiments on micro-organisms (Chapter 8).

For some of the test organisms, it was possible to compare the effects of zinc in the experimental field plot soil in several stages of ageing, with the toxicity of zinc in the same soil type immediately after zinc addition. Zinc toxicity for *E. crypticus*, *F. candida* and *T. pratense* in aged soil was a factor of 4 to 10 less than in freshly contaminated soil. This effect was partly caused by the leaching of readily soluble zinc during the first rainfalls following the construction of the experimental field plot. Aside from this, sorption of zinc to the soil solid phase increased with time. This indicates that the concentration in the soil pore water is not only determined by soil characteristics such as organic matter and clay content and pH, but also by ageing processes.

Chemical extraction techniques were applied as an operational measure for bioavailability in the different tests. The results indicate that extraction with a 0.01 M $CaCl_2$ solution is a suitable method to correct for differences in bioavailability between soils for invertebrates and plants. The influence of ageing on bioavailability will be especially important for the risk assessment of existing contaminations. The use of $CaCl_2$ extraction to estimate bioavailable concentrations of metals is therefore especially suitable for location specific evaluation of soil quality, and may be used as a complementary tool for the selection of locations which must be urgently cleaned-up. Soil toxicity tests using freshly contaminated soil form a good basis for the derivation of emission standards, which aim to prevent acute contaminations. In the interpretation of these toxicity tests, attention has to be paid to different uptake routes for various organisms, as uptake route influences bioavailability.

Simultaneous exposure to multiple toxicants is a second factor which distinguishes most field situations from the majority of laboratory toxicity tests. Results of experiments on *F. candida*, *E. andrei* and *E. crypticus* (Chapters 4, 5 and 6) indicate that the effects of mixtures of heavy metals are influenced by sorption interactions in soils (see Chapter 2). The effect of a mixture depends on the toxicity endpoint examined and the exposure level which is taken into consideration. From the results it may be concluded that it is justified to use the concept of concentration addition as a starting point for risk assessment.

Abiotic conditions are a next factor which may differ between laboratory and field. Temperature, soil moisture content and relative humidity, and the availability of food may vary considerably in the field situation and differ from optimal conditions. Although each of these factors influences the functioning of an organism, the alteration of favourable and unfavourable conditions during the experimental field plot experiments resulted in similar effects of zinc in the experimental field plot and the laboratory. This was especially the case in experiments which were performed after some time when the contamination was stabilized. The influence of climatic factors as such on the sensitivity of organisms is relatively small. It must be kept in mind that most experiments were performed when weather conditions were not extreme. Exposure of organisms under unfavourable conditions for a long period of time may eventually enhance effects of zinc. For the Dutch situation, with moderate climatic conditions, it may be assumed that the use of laboratory toxicity tests does not lead to large misinterpretations regarding acute field effects, provided that the exposure concentrations are the same.

The abiotic factors that influence the ecological relevance of laboratory tests mainly involve bioavailability, mixture toxicity and exposure conditions. Considering the differences between laboratory and field, biotic factors are important as well. The limited ecological realism of standardised toxicity tests is reflected by the choice of test parameters, which most of the times deal with short term effects on the individual or population level. Species interactions and processes such as adaptation and tolerance development are usually not incorporated in the test designs. The test organisms are typical 'laboratory species', selected on the basis of the ease of

captive breeding and maintenance in the laboratory. This also holds for the higher organisms used in the Validation project.
Differences in sensitivity between laboratory and field vary, depending on the organism and toxicant considered. There is no reason to assume that laboratory organisms are consistently and decisively more sensitive or insensitive than species in the field, so that it may be assumed that the results of standardized laboratory tests can in this respect be used for the derivation of generic risk limits. However, for long-term risk assessment of contaminants in soils, it is recommended to improve the design of toxicity tests in order to determine ecologically relevant parameters such as population development. Considering site specific risk assessment, characteristics of the local ecosystem should be included into the evaluation. For this purpose, *in situ* soil tests, laboratory bioassays with local species, and field inventories can be considered as possible tools. The results of the Validation project and the experience with various monitoring programs indicate that observations on nematode fauna and microbial communities are suitable in this respect.
The second aspect of the Validation project concerns the ecological relevance of risk limits for soils. The experimental data collected in the project were used to estimate Hazardous Concentrations (HC5 and HC50) for zinc using statistical extrapolation (Chapter 9, see also Chapter 1). It should be explicitly stated that these values do not have any legal status and are derived for the purpose of this project only. The project-specific HC5 and HC50 were derived on the basis of: (1) total zinc concentrations from experiments in which experimental field plot soil was used (aged contamination), (2) zinc concentrations corrected for differences in bioavailability using $CaCl_2$ extractable concentrations and (3) total zinc concentrations from experiments in which freshly contaminated soil was used. The HC5 and HC50 based on data of the experimental field plot soil after ageing (1) are a factor of 2 to 4 higher than those based on freshly contaminated soil (3). This indicates once more that differences in bioavailability between soils influence the estimated ecotoxicological risk limits.
The HC5 and HC50 estimated on the basis of zinc concentrations in freshly contaminated soil have been compared to literature data and to the current Dutch soil quality standards. The project-specific HC5 and HC50 were in reasonable agreement with the Dutch Target and Intervention Values for zinc, differences were a factor of 2 to 3. It must be noted that the Target Value for zinc is based on natural background concentrations in The Netherlands, and thus represents a pragmatic choice instead of an ecotoxicological risk limit. Therefore, a comparison was also made with the HC5s which have been derived recently on the basis of new toxicity data following the 'added risk approach' to take background concentrations into account (project Setting Integrated Environmental Quality Objectives, 'INS' in Dutch). The INS-values for the HC5 (in mg added zinc/kg standard soil) are 132 for 'species' and 16 for 'microbial processes'. In addition, calculations were carried out to derive preliminary HC50s based on the INS-toxicity data set, using the same methodology. Resulting HC50-values (which, similar to the project-specific values, do not have a legal status) were 385 and 207 for 'species' and 'microbial processes', respectively. The difference between the HC5 and HC50 based on the

INS dataset and the project-specific values was less than a factor of 2 when effects on species were considered (Chapter 9).
Structural parameters (abundance and species composition of enchytraeids and nematodes), and development of tolerance of the microbial community were used to investigate the ecological consequences of exceedance of risk limits on the community level (Chapter 6, 7 and 8). Tolerance development was determined by measuring the changes in microbial degradation capacity of the indigenous microbial communities in experimental field plot soil and soil from the Budel gradient. The observations on the nematode fauna and microbial community in the experimental field plot were first compared to the Hazardous Concentrations calculated from the experimental data from the same soil. Besides, $CaCl_2$ extractable concentrations from experiments with the other soil types were used. The species diversity of nematodes and the functioning of the microbial community were affected at concentrations exceeding the HC50s; significant differences from the reference situation were not observed at the HC5 level. This was also found when Hazardous Concentrations were derived on the basis toxicity data from freshly contaminated soils, and when community effects were expressed using total concentrations. For the HC5 and HC50 based on data collected within the framework of the INS-project, the observations also yielded similar conclusions.
Several ecological parameters in the Budel gradient soils were used to validate the ecotoxicological risk limits using true field contaminated soils. The Budel gradient is rather heterogeneous compared to the experimental field plot, where zinc contamination is homogeneous and variation in other (a)biotic factors is relatively small (see also Chapter 2). As a consequence, clear relationships between the level of contamination and the observed changes in the abundance and species diversity of enchytraeids and nematodes along the gradient could not unequivocally be established (Chapter 6 and 7). Organic matter and soil moisture content contributed significantly to observed variability in species composition along the gradient, while the role of metals could not be disentangled from the effects of the covarying factor pH. Heterogeneity could be factored out when tolerance development of the microbial community is regarded, since an internal control is used for every sample (see Chapter 8). From these experiments, it appeared that microbial zinc tolerance was increased with a factor of 100 in the most contaminated soil samples compared to the unpolluted Budel soil. Moreover, increased tolerance was also demonstrated in samples in which zinc concentrations were only slightly above control levels. This indicates that biological effects of pollution are present when the contamination is regarded as negligible on the basis of chemical analyses. Comparison of microbial tolerance at the Budel field site with ecotoxicological risk limits shows that Hazardous Concentrations at the 5 and 50% level give a good indication for the absence or presence of serious effects.
Analyses of literature data from a number of field studies in other pollution gradients indicate that severe field effects occur when the HC50 is exceeded, and that effects are small or absent when concentrations are lower than the HC5 (Chapter 9). It should be noted that the response of some individual species is more sensitive than the community parameters. Although definite conclusions can't be drawn because of the lack of reliable literature data, the results that are

obtained in the Validation project with respect to zinc seem to be applicable to other metals, both essential and non-essential. There is no need to assume that the conclusions are biased by the specific nature of zinc as an essential element, because under the experimental conditions as used in this project, zinc deficiency did not occur.

It is concluded that the ecotoxicological risk limits that are derived according to the methodology currently used in The Netherlands give a plausible indication of the concentrations at which slight or severe effects on community parameters will occur. As such, the methodology is a plausible approach in a first tier of soil ecotoxicological risk assessment. Differences in bioavailability between soils, the local communities, and a number of other factors, cause the field effects of contaminants to differ from place to place. It is therefore unlikely that generic risk assessment methods can exist in which contaminant risks are equally well predicted for all sites and ecosystems. In a second-tier (location-specific) evaluation, the most important factors for laboratory-to-field extrapolation should be taken into account for the assessment of the local risk.

The results of the Validation project provide recommendations for several aspects of soil protection policy. The applicability of laboratory tests will be improved by a proper characterization of exposure concentrations. This involves for instance the determination of exchangeable metal fractions next to total concentrations in soil. Insight into the relationship between external concentrations in soil, internal concentrations in the test organisms and effects will improve our knowledge of the processes that determine bioavailability and toxicity of contaminants. In the development of soil toxicity tests, attention should be paid to the choice of the effect parameter. Toxicity tests with ecologically relevant parameters such as population development are necessary to enable a scientifically justified estimation of long term effects of contaminations.

The results of the Validation project show that bioavailability is an important factor in risk assessment. The current methods for the derivation of generic soil quality standards can be used as a first step in the process of risk assessment. The methods to assess bioavailability as investigated in the Validation project can be applied in location specific risk assessment when soil standards are exceeded locally. A soil type correction that includes pH, next to organic matter and clay content, could be part of this. Chemical extraction methods can be valuable for an practical approximation of contaminant availability, for instance when bioassays are performed. The importance of this type of biological tests for the assessment of soil quality has been demonstrated in this project. Bioassays and field observations may provide information on local effects of contamination. The project has demonstrated the usefulness of an effect parameter based on community tolerance for field studies.

UITGEBREIDE SAMENVATTING

Dit rapport vormt de weerslag van het project 'Validatie van Toxiciteitsgegevens en Risicogrenzen Bodem', kortweg 'Validatieproject'. Het Validatieproject is vanaf 1992 in opdracht van het ministerie van VROM uitgevoerd door het RIVM, TNO en de Vrije Universiteit. Het doel van het Validatieproject is een bijdrage te leveren aan de wetenschappelijke onderbouwing van het Nederlandse bodembeschermingsbeleid en aanbevelingen te doen voor een verdere verbetering van de risicoschattingsmethodiek voor de bodem.

Bij het vaststellen van ecotoxicologische risicogrenzen en daarvan afgeleide bodemnormen spelen laboratorium toxiciteitsgegevens voor 'soorten' en 'microbiele processes' een belangrijke rol. Deze gegevens vormen de invoer voor statistische extrapolatiemodellen, die uiteindelijk leiden tot een schatting van de maximale concentratie van een verontreinigende stof waarbij het normaal functioneren van het bodemecosysteem niet beïnvloed wordt. Uit eerder onderzoek is gebleken dat de hoogte van de afgeleide risicogrenzen niet zozeer wordt bepaald door de gekozen methode van extrapolatie, als wel door de betrouwbaarheid van de toxiciteitsgegevens die als invoer voor de verschillende extrapolatiemethoden worden gebruikt.

Het gebruik van relatief eenvoudige toetsen voor het afleiden van risicogrenzen brengt een aantal onzekerheden met zich mee. Met het optreden van verschillen in gevoeligheid tussen soorten wordt in de risicoschattingsprocedure rekening gehouden via het gebruik van een statistische gevoeligheidsverdelingen. Een aspect dat nog weinig aandacht heeft gekregen is de aanname dat de effecten die in laboratoriumtoetsen worden gemeten een goede indicatie zijn voor effecten in het veld. Een tweede punt dat tot nu toe onderbelicht is gebleven, is de vraag in hoeverre een overschrijding van risicogrenzen inderdaad is gerelateerd aan zichtbare effecten op een ecosysteem of delen daarvan. Het onderzoek binnen het Validatieproject was dan ook gericht op deze twee aspecten:

1 De bruikbaarheid van (standaard) laboratorium toxiciteitstoetsen voor het schatten van effecten in de veldsituatie.
2 De ecologische relevantie van risicogrenzen die met behulp van extrapolatiemethoden zijn afgeleid.

In het project zijn verschillende methoden van onderzoek gebruikt, te onderscheiden in:

- Laboratoriumonderzoek waarbij toetsorganismen onder gecontroleerde omstandigheden zijn blootgesteld aan verontreinigde veldgrond en verschillende soorten kunstmatig verontreinigde grond.
- Onderzoek waarbij toetsorganismen in een proefveld zijn blootgesteld aan een kunstmatig aangebrachte verontreiniging.
- Veld- en proefveldwaarnemingen aan levensgemeenschappen in bodems met een verschillende mate van verontreiniging.

De resultaten worden vergeleken met literatuurgegevens en resultaten van gerelateerde onderzoeksprojecten.

Als veldlocatie is gekozen voor een gebied ten noord-oosten van de voormalige zinksmelterij in Budel, waar een vervuilingsgradiënt bestaat van de zware metalen zink, cadmium, lood en koper. Grond uit dit gebied werd gebruikt in laboratorium experimenten met de regenworm *Eisenia andrei*, de potworm *Enchytraeus crypticus*, de springstaart *Folsomia candida*, verschillende plantensoorten, waaronder de klaversoort *Trifolium pratense* en micro-organismen. Uit de experimenten bleek dat in deze grond zink de grootste bijdrage levert aan de waargenomen effecten. In het verdere laboratorium- en proefveldonderzoek met kunstmatig verontreinigde grond is dit metaal dan ook als modelstof gebruikt.
Voor de aanleg van het proefveld is een natuurlijke grond verontreinigd met een concentratiereeks van zinkchloride. Deze grond is op de VU-campus in een serie afzonderlijke, van elkaar gescheiden enclosures gestort. Daarna werden gedurende drie opeenvolgende jaren in dit proefveld experimenten uitgevoerd met de meeste van de hierboven genoemde organismen. Daarnaast werd de ontwikkeling van de nematodenfauna gedurende drie jaar gevolgd. In de vervuilingsgradiënt in Budel werden aantallen en soortsdiversiteit van nematoden en enchytreëen bepaald.
Naast deze studies, die gericht waren op effecten van zink op structurele kenmerken van onderdelen van het bodemecosysteem, werd ook het effect van zink op functionele bodemparameters bestudeerd. De aandacht werd daarbij met name gericht op diverse microbiële afbraakfuncties in verschillende soorten grond en in het proefveld, waarbij speciale aandacht werd gegeven aan het optreden van tolerantie in de microbiële gemeenschap ten gevolge van de aanwezigheid van zink. Ook in de Budelgradiënt werd het optreden van tolerantie in de microbiële gemeenschap onderzocht.
Hoofdstuk 1 van dit rapport geeft een overzicht van de factoren die van invloed kunnen zijn op de extrapolatie van laboratoriumgegevens naar de veldsituatie. Deze factoren hebben onder meer betrekking op verschillen in blootstellingsconcentratie en toetsomstandigheden tussen laboratorium en veld en op de beperkte ecologische realiteit en complexiteit van de meeste laboratoriumtoetsen. Wat betreft de verontreiniging zelf zijn de voornaamste verschillen gelegen in de veelal onnatuurlijk hoge beschikbaarheid en de homogene verdeling van de contaminanten in de laboratoriumtoetsen, waarbij bovendien meestal slechts sprake is van één enkele stof. Wat betreft de ecologische realiteit en complexiteit gaat het om zaken als de keuze van de toetsparameters, het gebruik van single-species toetsen en het uitsluiten van ecologische interacties en processen als adaptatie en tolerantie in de laboratoriumtoetsen.
Biologische beschikbaarheid bleek voor alle organismen de meest bepalende factor te zijn bij de vergelijking van effecten in verschillende bodemtypen. Verschillen in biologische beschikbaarheid bleken slechts ten dele gecorrigeerd te kunnen worden door effectconcentraties te normaliseren op basis van het organische stof- en kleigehalte. Deze methode, die in Nederland gebruikt wordt bij het afleiden van risicogrenzen, houdt echter geen rekening met verschillen in pH, terwijl deze van grote invloed zijn op de biologische beschikbaarheid van metalen. De relatie tussen pH en biologische beschikbaarheid van zink is zeer complex. Een verhoging in pH leidt tot een afname van de concentratie in het poriewater en dus tot een potentiële vermindering in blootstelling. Dit werd duidelijk aangetoond in experimenten met

kunstgrond (Hoofdstuk 2). Tegelijkertijd kan een hogere pH in het poriewater leiden tot een sterkere sorptie van zink aan de celwanden van organismen, waardoor verhoogde opname kan optreden. Dit laatste werd ondermeer aangetoond voor micro-organismen (Hoofdstuk 8).
Voor een aantal toetsorganismen konden de effecten van proefveldgrond in verschillende stadia van veroudering worden vergeleken met de toxiciteit van zink in vers verontreinigde grond van hetzelfde bodemtype. De toxiciteit van zink voor *E. crypticus*, *F. candida* en *T. pratense* nam in verouderde grond af met een factor 4 tot 10 ten opzichte van vers verontreinigde grond. Dit effect werd gedeeltelijk veroorzaakt door het uitspoelen van goed oplosbaar zink tijdens de eerste regenbuien na de aanleg van het proefveld, daarnaast was er sprake van een toenemende sorptie in de tijd. Naast bodemparameters als organische stof, kleigehalte en pH, is dus ook de ouderdom van de zinkverontreiniging bepalend voor de blootstellingsconcentratie in het bodemvocht.
Chemische extractiemethoden werden gebruikt als operationele maat voor biologische beschikbaarheid in de verschillende testen. Uit de resultaten lijkt te kunnen worden geconcludeerd dat extractie met een 0.01 M $CaCl_2$-oplossing een goed werkbare manier is om te corrigeren voor de verschillen in biologische beschikbaarheid tussen gronden voor evertebraten en planten. De verschillen in biologische beschikbaarheid tussen vers verontreinigde grond en verouderde veldgrond spelen met name een rol bij de beoordeling van de risico's van bestaande verontreinigingen. Het schatten van de biologische beschikbaarheid met behulp van $CaCl_2$-extractie leent zich dan ook met name voor het beoordelen van locatie-specifieke bodemkwaliteit en kan bijvoorbeeld als aanvullend instrument worden gebruikt bij het vaststellen van een saneringsurgentie. Voor het afleiden van preventieve emissienormen, die vooral tot doel hebben acute verontreinigingen te voorkomen, vormen de laboratoriumtoetsen met vers verontreinigde grond een goed uitgangspunt.
De gecombineerde blootstelling aan meerdere toxicanten is een tweede factor waarin het merendeel van de laboratoriumtoetsen verschilt van de veldsituatie. De resultaten van experimenten met *F. candida*, *E. andrei* en *E. crypticus* (Hoofdstuk 4, 5 en 6) toonden aan dat metalen elkaars sorptiegedrag in de bodem, en daarmee elkaars beschikbaarheid en het mengseleffect, beinvloeden (zie ook Hoofdstuk 2). Daarnaast blijkt de werking van het mengsel zowel afhankelijk te zijn van de toxiciteitsparameter als van het blootstellingsniveau. Voor de risicobeoordeling van mengsels van metalen lijkt het vooralsnog gerechtvaardigd om uit te gaan van concentratie-additieve werking van de afzonderlijke componenten.
Abiotische omstandigheden zijn een volgende factor van verschil tussen laboratorium en veld. Temperatuur, bodem- en luchtvochtigheid, maar ook de aanwezigheid van voedsel wisselen sterk in de veldsituatie sterk en kunnen afwijken van de optimale omstandigheden. Hoewel elk van deze factoren invloed kan hebben op het functioneren van een organisme, bleek in de proefveldexperimenten de afwisseling van gunstige en ongunstige condities te leiden tot een redelijk vergelijkbare gevoeligheid voor zink ten opzichte van het laboratorium, met name in de experimenten die werden uitgevoerd nadat de verontreiniging in het proefveld enigszins was gestabiliseerd. Dit is een indicatie dat de invloed van klimatologische factoren op de gevoeligheid van organismen redelijk beperkt is. Hierbij moet worden aangetekend dat de

meeste experimenten niet onder extreme weersomstandigheden zijn uitgevoerd. Wanneer een organisme gedurende langere tijd onder zeer ongunstige omstandigheden wordt blootgesteld aan zink, zou dit het uiteindelijke effect wel kunnen versterken. Er mag echter worden aangenomen dat voor de Nederlandse situatie, waar de klimatologische omstandigheden over het algemeen niet extreem zijn, het uitvoeren van toetsen onder gecontroleerde laboratoriumcondities bij gelijk blijvende biologische beschikbaarheid niet leidt tot een grote onder- of overschatting van de toxiciteit onder veldomstandigheden.

De abiotische omstandigheden die de ecologische relevantie van laboratoriumtoetsen beinvloeden hebben met name betrekking op biobeschikbaarheid, mengseltoxiciteit en blootstellingscondities. Ook wat betreft biotische factoren is er een groot aantal verschillen tussen de standaardtoets en de veldsituatie. Het beperkte ecologisch realisme van laboratorium toxiciteitstoetsen komt ondermeer tot uitdrukking in de keuze van toetsparameters, die over het algemeen gericht zijn op het vaststellen van korte termijn effecten op individueel of populatieniveau. Interacties tussen soorten en processen als adaptatie en tolerantie worden in de meeste laboratoriumtoetsen niet meegenomen. De toetsorganismen behoren meestal tot typische 'laboratoriumsoorten', die geselecteerd zijn op kweekbaarheid en hanteerbaarheid en die niet algemeen voorkomen in veldgronden. Dit laatste geldt ook voor de organismen die in het Validatieproject zijn gebruikt.

De gevoeligheidsverschillen tussen laboratorium en veld variëren al naar gelang het toetsorganisme en de toxicant. Er is geen aanleiding te veronderstellen dat laboratorium-organismen systematisch en doorslaggevend gevoeliger of ongevoeliger zijn dan soorten in het veld. Op basis van deze constatering is het in deze zin gerechtvaardigd de resultaten van laboratoriumtesten met deze soorten te gebruiken voor het afleiden van generieke, algemeen geldende bodemnormen. Voor een beoordeling van effecten die op lange termijn kunnen optreden, is het echter wenselijk dat toxiciteitsexperimenten meer worden gericht op het meten van ecologisch relevante parameters zoals populatieontwikkeling. Wanneer het om specifieke locaties gaat, is het wenselijk de karakteristieken van het ecosysteem ter plaatse te betrekken in de beoordeling. In dit geval zou gedacht kunnen worden aan het uitvoeren van in situ bodemtoetsen, laboratorium bioassays met ter plaatse verzamelde organismen en veldinventarisaties. De resultaten van het Validatieproject en de ervaringen die inmiddels zijn opgedaan met monitoringsnetwerken, geven aan dat onder andere de locale nematodenfauna en microbiële gemeenschappen hiervoor zeer geschikt kunnen zijn.

Het tweede aspect van het Validatieproject richt zich op ecologische relevantie van risicogrenzen. Met de in het Validatieproject verzamelde gegevens zijn Hazardous Concentrations (HC5 en HC50) afgeleid voor zink (Hoofdstuk 9, zie ook Hoofdstuk 1). Met nadruk wordt gesteld dat deze project-specifieke waarden geen wettelijke status hebben en uitsluitend voor het doel van dit project zijn afgeleid. De project-specifieke HC5 en HC50 werden afgeleid op basis van de volgende gegevens: (1) totaalconcentraties uit experimenten met proefveldgrond, waarin sprake van een verouderde verontreiniging, (2) concentraties die gecorrigeerd zijn voor verschillen in biologische beschikbaarheid door gebruik te maken van de $CaCl_2$-extraheerbare fractie, en (3) totaalconcentraties uit experimenten met vers verontreinigde

grond. De HC5 en HC50 die werden berekend op basis van de resultaten van experimenten met proefveldgrond zijn een factor 2 tot 4 hoger dan de waarden die werden berekend op basis van gegevens voor vers verontreinigde grond. Dit geeft nogmaals aan dat verschillen in biologische beschikbaarheid tussen gronden doorwerken in de schatting van ecotoxicologische risico-grenzen.
De HC5 en HC50 die werden afgeleid met de gegevens voor vers verontreinigde grond zijn vergeleken met eerder verkregen literatuurgegevens en de momenteel in Nederland geldende bodemnormen. De project-specifieke HC5 en HC50 kwamen redelijk overeen met de huidige streef- en interventiewaarden voor zink, de verschillen bedroegen een factor 2 tot 3. Er moet worden opgemerkt dat de streefwaarde voor zink afgeleid is van de natuurlijke achtergrond-gehalten in Nederland en dus meer een pragmatische keuze is dan een maat voor ecotoxicologische risico's. Daarom is tevens een vergelijking gemaakt met de HC5's die recentelijk zijn afgeleid op basis van nieuw beschikbaar gekomen toxiciteitsgegevens (project Integrale Normstelling Stoffen, INS), waarbij het 'toegevoegd risico-concept' is gebruikt om rekening te houden met achtergrondconcentraties. De INS waarden voor de HC5 (in mg toegevoegd zink/kg standaardgrond) zijn 132 voor 'soorten' en 16 voor 'microbiële processen'. Daarnaast is de INS-dataset gebruikt om volgens dezelfde methodologie voorlopige HC50's af te leiden. Deze waarden (die net als de project-specifieke HC5 en HC50 geen wettelijke status hebben) waren 385 en 207 voor respectievelijk 'soorten' en 'microbiële processen'. Het verschil tussen de op basis van de INS-dataset afgeleide HC5 en HC50 voor soorten en de project-specifieke waarden was kleiner dan een factor 2.
Voor de beoordeling van de gevolgen van het overschrijden van risicogrenzen werd gebruik gemaakt van structurele parameters (abundantie en soortensamenstelling van enchytreëen en nematoden) en van de tolerantieontwikkeling van de microbiële gemeenschap ten aanzien van zink (Hoofdstuk 6, 7 en 8). Deze ontwikkeling van tolerantie werd bepaald aan de hand van de gemeten veranderingen in microbiële afbraakfuncties in gecontamineerde (proef)veldgrond ten opzichte van de controlegrond. Omdat de biologische beschikbaarheid van zink van grote invloed is op de grootte van het effect, zijn de waarnemingen aan nematodenfauna en de microbiële gemeenschap in het proefveld allereerst vergeleken met de Hazardous Concentrations die zijn afgeleid op basis van de gegevens uit experimenten met proefveldgrond, en op basis van de $CaCl_2$-extraheerbare concentraties uit experimenten met diverse soorten grond. Zowel de diversiteit van nematoden als het functioneren van de microbiële gemeenschap bleken aangetast bij concentraties die de aldus afgeleide HC50 overschrijden. Bij de HC5 daarentegen waren er voor beide levensgemeenschapsparameters geen of slechst geringe afwijkingen ten opzichte van de referentiesituatie waarneembaar. De HC5 en HC50 afgeleid uit de experimenten met vers verontreinigde grond bleken eveneens gerelateerd aan de af-, respectievelijk aanwezigheid van effecten op de gekozen levensgemeenschapsparameters. Dit gold ook voor de HC5 en HC50 die werden afgeleid op basis van de in het project INS gebruikte toxiciteitsgegevens.
Naast de waarnemingen in het proefveld, werden verschillende ecologische parameters in de vervuilingsgradiënt te Budel gebruikt voor het valideren van ecotoxicologische risicogrenzen.

Waar in het proefveld nog sprake was van een homogene verdeling van de verontreiniging en een geringe variatie in andere (a)biotische factoren, wordt de Budelgradiënt gekenmerkt door een grote mate van heterogeniteit (zie ook Hoofdstuk 2). Dit heeft ondermeer tot gevolg dat het vaststellen van een verband tussen verontreiniging en waargenomen veranderingen in ecologische parameters uitermate moeilijk is. Voor zowel enchytreëen als nematoden kon het effect van zink op abundantie en diversiteit niet worden losgekoppeld van de effecten van andere parameters (Hoofdstuk 6 en 7). Met name pH, organische stofgehalte en bodemvochtigheid bleken in grote mate mede bepalend voor de waargenomen verschillen in soortensamenstelling langs de gradiënt. Heterogeniteit speelt bij het bepalen van de tolerantie van de microbiële gemeenschap een veel minder verstorende rol omdat de methode per monster gebruik maakt van een interne controle (zie Hoofdstuk 8). Uit de metingen aan micro-organismen bleek dat de tolerantie ten aanzien van zink in de meest gecontamineerde grond met een factor 100 was toegenomen ten opzichte van de niet vervuilde grond in Budel. Opvallend was dat ook in monsters die nauwelijks verschilden in zinkconcentratie ten opzichte van de controle, toch verhoogde tolerantie werd aangetoond. Dit betekent dat de biologische effecten van verontreiniging nog steeds meetbaar zijn wanneer de mate van verontreiniging op basis van chemische analyses verwaarloosbaar wordt geacht. Wanneer de gegevens over het optreden van microbiële tolerantie voor zink in de Budelgradiënt worden vergeleken met de ecotoxicologische risicogrenzen, blijkt dat Hazardous Concentrations op de 5 en 50% niveaus goede indicaties zijn voor het al dan niet optreden van ernstige effecten.

Analyses van literatuurdata over een aantal veldstudies in andere metaalgradiënten suggereren dat er inderdaad ernstige effecten optreden op levensgemeenschapsparameters wanneer de HC50 in het veld wordt overschreden, en dat dergelijke effecten niet meetbaar zijn wanneer de concentraties beneden de HC5 blijven (Hoofdstuk 9). Daarbij moet aangetekend worden dat de respons van enkele afzonderlijke soorten gevoeliger is dan de respons van de levensgemeenschapsparameters. De resultaten die zijn verkregen voor zink lijken ook toepasbaar te zijn op andere metalen, zowel essentiële als niet-essentiële, hoewel het trekken van harde conclusies niet mogelijk is vanwege het gebrek aan bruikbare literatuurgegevens. Er is geen reden om aan te nemen dat de conclusies van het Validatieproject zijn beïnvloed door het speciale karakter van zink als essentieel metaal aangezien onder de heersende experimentele condities zink deficiëntie niet is opgetreden. Er kan geconcludeerd worden dat de generieke risicogrenzen die worden afgeleid met de in Nederland gangbare methodiek een goede indicatie geven van de concentraties waarbij effecten op levensgemeenschapsniveau al dan niet optreden. In deze zin levert de risicoschattingsmethodiek plausibele resultaten voor algemene toepassing. Verschillen in biologische beschikbaarheid en een aantal andere omgevingsfactoren leiden ertoe dat de effecten van verontreinigingen op verschillende locaties zelden vergelijkbaar zijn. Het is dan ook niet te verwachten dat een generieke risicoschattingsmethodiek met even grote precisie de risico's voor verschillende ecosystemen en lokaties voorspelt. Bij een tweede stap, bij een lokatie-specifieke benadering, zouden de factoren die de laboratorium-veld-extrapolatie het sterkst beinvloeden, in de beoordeling van de lokale risico's moeten worden meegenomen.

Op basis van de resultaten van het Validatieproject worden aanbevelingen gedaan ten aanzien van enkele aspecten van het normstellingsbeleid voor de bodem. De bruikbaarheid van laboratoriumtoetsen kan verbeterd worden wanneer de blootstellingsconcentraties beter worden gekarakteriseerd. Dit betekent ondermeer dat niet alleen totaalconcentraties, maar ook de uitwisselbare fractie worden bepaald. Ook de relatie tussen externe concentraties in de grond, interne concentraties in de toetsorganismen en effecten zou inzicht kunnen verschaffen in de processen die van invloed zijn op de biologische beschikbaarheid van contaminanten. Een tweede punt van aandacht bij het verder ontwikkelen van (bodem)toxiciteitstoetsen is de keuze van de effectparameter. Voor een verantwoorde schatting van lange termijn effecten is er behoefte aan toxiciteitstoetsen waarin ecologisch relevante parameters als populatieontwikkeling worden gemeten.
De resultaten van het Validatieproject geven duidelijk aan dat biologische beschikbaarheid een prominente rol speelt bij de risicobeoordeling. De huidige manier van afleiden van generieke bodemnormen is geschikt als eerste stap in het beoordelingsproces. Wanneer er sprake is van overschrijding van generieke bodemnormen, kan vervolgens bij een locatie-specifieke risicobeoordeling gebruik worden gemaakt van de in dit project gebruikte methoden voor het schatten van de biologische beschikbaarheid. Een bodemtypecorrectie waarbij behalve organische stof- en kleigehalte ook de pH wordt betrokken, zou hiervan een onderdeel kunnen zijn. Daarnaast verdient het gebruik van chemische extractiemethoden, bijvoorbeeld bij het uitvoeren van bioassays, meer aandacht. Het belang van dergelijke biologische toetsen voor het beoordelen van de bodemkwaliteit is in dit project duidelijk aangetoond. Bioassays en veldwaarnemingen kunnen informatie verschaffen over de locale effecten van een verontreiniging. Daarnaast is gebleken dat het meten van tolerantieontwikkeling als effectparameter een veelbelovend instrument is voor de beoordeling van effecten van stoffen in complexe veldsituaties.

DEFINITIONS AND ACRONYMS

In the context of this report, the specific terminology of the Dutch risk assessment methodology is frequently used. The terms compiled below are defined for the context of the present report, putting emphasis on the ecotoxicological extrapolation of toxicity data. It should be noted that various definitions have historically changed, as a consequence of the iterative process of the derivation of environmental quality objectives.

Ecotoxicological Risk Limits (ERL)	Limit concentrations in soil that are derived from one of the two chosen cut-off points of an HC_X-curve that is obtained by ecotoxicological extrapolation of toxicity data, viz. the HC_5 (which yield the MPC and the NC as ERL), and the HC_{50} (which yields the ECOTOX-SCC as ERL
ECOTOX-SCC	Ecotoxicological Risk Limit that is derived from an HC_{50}, literally: Ecotoxicological Serious soil Contamination Concentration
Environmental Quality Objective (EQO)	Regulatory chosen quality objective, as determined by a decision based on information from ecotoxicology, human toxicology, and other information (e.g., background concentrations). Also: Environmental Quality Criterion (EQC)
generic risk limit or EQO	risk limit or EQO not specified towards biological species or ecosystem under concern. The value is given for standard soil. Soil-type correction formulae are applied to correct for differences among soil types with respect to clay and organic matter content
Hazardous Concentration - X % (HC_X)	soil concentration that relates to a potential adverse effect for a fraction of X% of the total number of species upon chronic exposure, by definition determined by the statistical extrapolation model
Intervention Value (IV)	Environmental Quality Objective derived on the basis of the serious Soil Contamination Concentration (ECOTOX-SCC)
location-specific risk assessment	risk assessment system in which location-specific data is used beyond the use of the soil-type correction system
Maximum Permissible Concentration (MPC)	Ecotoxicological Risk Limit that is derived from the HC_5
Negligible Concentration (NC)	Ecotoxicological Risk Limit calculated as MPC/100
Soil type correction	The application of compound-specific formulae, to recalculate the TV and IV for standard soil into the TV and IV for the soil type under consideration (based on clay and organic matter content)
Standard soil	Soil type defined by a contents of 10% organic matter and 25% clay
Target Value (TV)	Environmental Quality Objective derived from the Negligible Concentration, or in the case of metals, often by background concentrations

1. GENERAL INTRODUCTION

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1.1 Project overview

1.1.1 Regulatory framework, mission and organisation of the project

The project "Validation of Toxicity Data and Risk Limits for Soil" (in Dutch: "Validatie Toxiciteitsgegevens en Risicogrenzen Bodem"), further referred to as: "Validation project", has been initiated on the basis of questions about the validity of the ecotoxicological risk limits (ERLs).

The current method for the derivation of risk limits was implemented in the Dutch soil protection policy in the late eighties (e.g., VROM, 1989ab, Van De Meent *et al.,* 1990). The method is based upon ecotoxicological extrapolation of toxicity data, and yields so-called potential risk limits. The core questions that initiated this project were related to the problem that ERLs are based on toxicity data obtained in laboratory conditions, for which the field relevance was unknown. Uncertainty on the field relevance of ERLs was frequently encountered as a problem in the application of risk limits in the daily practice of soil protection and soil remediation (see also: Hopkin, 1993; Van Straalen, 1993). Consequently, there was a need to investigate the validity of (1) the laboratory toxicity data for the field, and (2) the ecotoxicological risk limits themselves.

These questions have been re-formulated into a research agenda, during a workshop at the start of the project with participants from regulatory bodies and research institutes (see Notenboom and Van Beelen, 1992). Premises of this research agenda were:

- *standardized laboratory test systems*, since they yield most of the basic toxicity data;
- *generic* ecotoxicological risk limits, since they are an important basis for environmental standards with nationwide application.

It was the mission of this research project to evaluate both aspects critically for soils, using experimental approaches extended with literature data.

This report presents the condensed results of the Validation project, including a summary of the discussions of the evaluation workshop held at the finalisation of the project (February 5, 1998). Original research papers are published elsewhere (see Project Bibliography). The project has been executed as a co-operation of the National Institute of Public Health and the Environment (RIVM), which acted as coordinator, the Vrije Universiteit of Amsterdam (VU) and the Netherlands Organization for Applied Scientific Research (TNO). The project was ordered and financed by the Dutch Ministry of Housing, Spatial Planning and Environment, Directorate for Soil, and was performed during the period 1992-1998.

It is noted that, next to the *generic* risk assessment system addressed in the Validation project, a *location-specific* risk assessment is valuable and necessary once a generic Environmental Quality Objective is exceeded. In this respect, generic risk assessment functions as a 'warning tool' in a two-tiered system of risk assessment. This means that concentrations below generic risk limits should in any case be 'safe' (Denneman, 1998). In the second-tier assessment, the current Dutch 'Intervention approach' (VROM, 1994b), three aspects are considered, viz. actual risks for humans and ecosystems, and for further contaminant distribution. Present research activities aim to refine this system of location-specific risk assessment, by further development of the methods for each of the three risk categories. For example, bioassays will likely play an important role in second-tier assessments (Rutgers and Notenboom, pers. comm.). The results of the Validation project are of importance for both tiers of risk assessment. However, aspects related to location-specific risk assessment could only in part be accommodated in the research agenda, due to conflicting characteristics (e.g., standardized laboratory test systems *versus* tests with locally occurring species, and generic risk limits *versus* location-specific risk assessment). The General Discussion (Chapter 9) presents an evaluation of the project results, and addresses both tiers. This is possible due to the core role of (comparative) bioassay methods in both the Validation project and in proposed methodologies for location-specific risk assessments.

1.1.2 Problem description and validation

In The Netherlands, Environmental Quality Objectives (EQOs) for individual chemicals in soil are expressed by total soil concentrations (VROM, 1994a). Ecotoxicological risk assessment plays an important role in the derivation of EQOs, next to human risk assessment. A simplified scheme for the derivation of EQOs for chemicals, as applied in the Netherlands, is shown in Box 1.1 and Figure 1.1. Ecotoxicological risk assessment presently addresses three aspects, viz. statistical extrapolation of laboratory data to obtain Hazardous Concentrations separately for 'species' and 'microbial processes' and secondary poisoning. The aspects related to secondary poisoning and human risk assessment are not specified, since this reports is concerned with the validity of the statistically extrapolated ecotoxicological risk limits only.

Within this setting, the Validation project focused on two main aspects:

- The representativity of laboratory toxicity data for situations in the field, where exposure and other factors are different from the standardised protocol in a toxicity test.
- The ecological significance of ecotoxicological risk limits for structural or functional community-level endpoints in exposed field communities.

In this report, 'validation' is defined in a restricted way, namely as: *evaluation* of similarity or differences between laboratory-based predictions and true field effects, either at the level of the species (cf. 1) or communities (cf. 2) (Posthuma, 1997). This restriction relates to the problem that risk assessment models usually do not fall in categories like 'proven' or 'false'. According to Suter (1993), a validated risk assessment model should make accurate predictions that resemble reality within specified limits. Consistent with this restricted meaning of 'validation', potential improvements in the risk assessment method should be investigated, and should lead to an improved credibility of the model rather than to true validity. Credibility can be established by experimental testing, peer-review, and use in regulatory practice. For the Validation project, Box 1.1. Simplified terminology of Dutch generic ecotoxicological risk assessment and regulatory objectives

The derivation of Environmental Quality Objectives in the Netherlands covers an ecotoxicological domain and a regulatory domain.

In the ecotoxicological domain, three aspects are studied independently, viz. the risk for secondary poisoning, and the potential risks for 'species' and 'microbial processes', resulting in three types of ecotoxicity data. The risk for secondary poisoning falls outside the scope of the Validation project.

The potential risks for 'species' and 'microbial processes' are calculated using a statistical extrapolation method, but only when enough toxicity data of sufficient quality are available. Otherwise an alternative method is used (procedure not specified here). Prior to calculating the sensitivity distribution, the toxicity data are recalculated to 'standard soil' using the soil-type correction method of VROM (1994a).

The extrapolation procedures yield (continuous) frequency distributions of the sensitivities of 'species' and 'processes' using the methods of Aldenberg and Slob (1993) based on Van Straalen and Denneman (1989). The sensitivity distribution relates ambient soil concentrations (for 'standard soil') to potential effects, which are expressed as fraction of species or functions potentially affected (HC_X, with X = the potentially affected fraction). For example: below the 5% Hazardous Concentration (HC_5) more than 95% of the species or functions is supposed to be protected, and less than 5% may suffer any adverse effect upon chronic exposure.

Two values from the continuous sensitivity distribution have been chosen as limit values, viz. the HC_5 and the HC_{50}. Thus, four hazardous concentrations can become available, viz. the HC_5 and HC_{50} for 'species' and the HC_5 and HC_{50} for 'microbial processes'.

In a final ecotoxicological evaluation (procedure not specified), the three types of ecotoxicity data are weighed. After weighing three ecotoxicological risk limits are quantified, viz. the Maximum Permissible Concentration (MPC), the Negligible Concentration (NC, as MPC/100, with the factor 100 chosen to take uncertainties in laboratory-to-field extrapolation into account, including the potential effects of mixtures of contaminants) and the serious soil contamination concentration (ECOTOX-SCC).

Entering the regulatory domain, weighing of the ecotoxicological risk limits with other information (human risk limits, background concentrations, harmonization between environmental compartments) yields the environmental quality objectives for soil (procedure not specified). Eventually, the following relationships hold if the risk of secondary poisoning is smaller than the potential risks obtained with the extrapolation procedures:

- the HC_5 (of 'species' or 'microbial processes') yields the Negligible Concentration (NC). The NC defines the Target Value (TV) as quality objective. Note that the TV and the NC are not quantitatively associated to a known potential risk of X%, such as the MPC (5%).
- the HC_{50} (of 'species' or 'processes') yields the Intervention Value (IV) as quality objective.This is the concentration triggering further investigations into the urgency of soil remediation.

Since soils may deviate from the 'standard soil' for which the objectives are formulated, the soil-type correction system (VROM, 1994a) is applied again to recalculate the obtained values to the values valid for the soil under investigation.

For various compounds, such as metals, background concentrations are present in field soils, and the TV may be lower than the background concentration. In that case, the 'added risk approach' has recently been proposed (see further Struijs *et al.* 1997). Further, both ecotoxicological risks and human risks are considered; the weighing of both types of data is beyond the scope of this report. For an overview on the derivation of ecotoxicological risk limits, recent data, and the regulatory domain: see Crommentuijn *et al.* (1994), Crommentuijn *et al.* (1997), and VROM (1994ab, 1997), respectively.

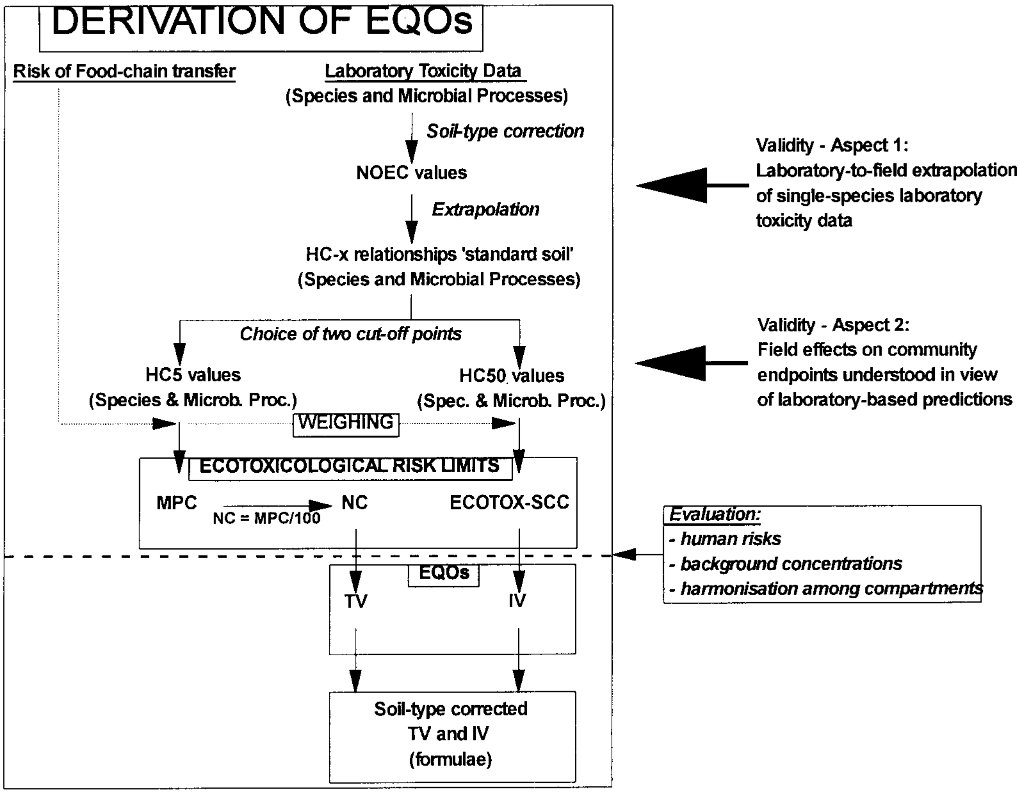

Figure 1.1. Schematic presentation of the derivation of Environmental Quality Objectives for soil from ecotoxicity data and other information. For explanation: see Box 1.1. The dotted horizontal line discriminates between the domain of ecotoxicological extrapolation and weighing of the three types of ecotoxicity data (above the line, executed by RIVM) and the regulatory domain (below the line). The key aspects studied in the Validation project are indicated by bold arrows (right).

experimental approaches are combined with peer-review (e.g., the evaluation workshop, and this and other reports and publications) which fulfills the first two criteria, while evaluation of regulatory practice was beyond the scope of the project. For the Validation project it was deemed crucial that if differences between laboratory and field were found, the evaluation should yield information on the magnitude and probable causes of the difference.

In the Validation project the extrapolation method (statistical extrapolation principle) as such was not discussed. The attention of the project was directed mainly to the quality of the model input data (aspect 1) and the ecological relevance of the risk limits generated by the extrapolation model (aspect 2). Both were addressed using comparative methods, viz. phenomena that occurred in the laboratory were compared with phenomena observed under (semi-)field conditions.
Regarding aspect 1, we have to consider the large difference between laboratory experimental and field conditions, and the resulting representativity of laboratory tests for the field situation. These aspects are further elaborated in section 1.2. It will be clear that certain themes were frequently encountered in these laboratory and field comparisons: effects of soil substrate

(artificial soil or standard soils versus field soils) on toxicant availability, effects of combinations (mixtures) of pollutants, effects of (field) ageing on pollutant toxicity, effects of climatic conditions, differences in population homogeneity and interspecies interactions. Frequently hypothesized differences between the laboratory and the field are listed in Table 1.1.

Table 1.1. Most important and frequently hypothesized differences between field and laboratory conditions.

Level	Field	Laboratory
Abiotic	Low bioavailability	High bioavailability
	Multiple substances	Single substance
	Uncontrolled conditions	Controlled conditions
	High heterogeneity	Low heterogeneity
Population	Population fitness	Single endpoint of lab. population
	Field species	Laboratory species
	Adaptation	No adaptation
	Costs of tolerance	No costs of tolerance
Community	Species interaction	No species interaction
	Community tolerance	No community tolerance

Regarding aspect 2, the ecological significance of risk limits, the extrapolated values should be related to the absence and presence of adverse effects at the HC_5 and HC_{50}, respectively (and at the Target and Intervention Value if these are determined by the HC_5 and HC_{50}). In the Validation project, this was addressed by observations on community endpoints under field and semi-field conditions. These endpoints were compared to the predicted ecotoxicological risks. By virtue of the complexity of this problem, less attention was paid to aspect 2 than aspect 1.

1.1.3 General approaches

Insight into the field relevance of laboratory toxicity data and of risk limits appeared to be lacking in 1992, mostly since the standardised test systems were mainly used for characterizing the relative toxic potential of a chemical. The lack of relevant data made it necessary to obtain experimental data within the project. Throughout the project, the experimental efforts were aimed at:

1. obtaining comparative data, by studying the performance of toxicant-exposed organisms under different conditions;
2. identifying those factors that crucially influence the extrapolation of laboratory toxicity data to the field.

On the basis of the findings, candidate correction factors may be identified, which may help to improve the prediction of field effects from laboratory data.

Heavy metals were chosen as the class of toxicants for the experimental studies, because of their persistence, the relative easy methods for analysis, and the availability of many laboratory data and some field studies. For the field contamination gradient studied in the project, it is plausible that zinc is the dominant metal causing adverse effects to various organisms, hence, this metal was chosen for controlled studies. Further, a selection of laboratory test organisms was made, consisting of two oligochaete species (*Eisenia andrei* and *Enchytraeus crypticus*), a springtail (*Folsomia candida*) and a plant (*Trifolium pratense*). For these groups, standardization of tests systems is finished or in the phase of harmonization. Since tests with microorganisms are also used in the derivation of risk limits (Figure 1.1), the work was extended with two standardized tests on the mineralization rates of various substrates by indigenous soil microbial communities.

To reach the goals of the project, two main lines of research on *aspect 1* were initiated, addressing laboratory-to-field extrapolation:

1. In bioassays, the chosen organisms were exposed to a heavy metal contaminated field soil, collected in a gradient near the zinc factory in Budel, under controlled laboratory conditions. The soils sampled in this gradient appeared to contain a range of zinc concentrations, ranging from approx. 11 - 1750 mg Zn/kg dry soil (total concentrations). Uptake and toxicity of the metals were studied.
2. Toxicity and uptake experiments with the chosen multicellular species, as well as measurements on microbial mineralization activities, were performed under outdoor conditions in experimental field plots, containing a soil type similar to the Budel soils. Soils in these field plots were artificially contaminated with a range of zinc concentrations (nominal 0-3200 mg Zn/kg dry soil). Some experiments were carried out in successive years.

The results of the above experiments were compared to results collected in standardised laboratory tests with the same or other soils which were freshly contaminated with zinc. Although the NOEC is crucial in the derivation of ERLs, comparisons mostly concerned the whole concentration-effect curve, to avoid statistical uncertainties related to low-exposure range measurements.

In addition, three more lines of research were carried out in the framework of the validation of *aspect 2*, *i.e.* the ecological relevance of ecotoxicological risk limits. In these research lines it was investigated whether field effects can be understood in view of the extrapolated risk ('field-to-laboratory understanding'):

1. Observations were made on the total numbers of nematodes and nematode species in the Budel gradient soils, as well as during three consecutive years in the experimental field plots.
2. Observations were made on the total numbers of enchytraeids and enchytraeid species in Budel gradient soils.
3. The occurrence of pollution induced community tolerance (PICT) within the microbial community in the experimental field plots and in the Budel gradient soils was studied.

Since experimental attention was mainly focused on zinc, the general method for EQO-derivation has been reconstructed for this metal. This is necessary to clarify the role of ecotoxicity data, extrapolation, and background concentrations in the derivation of EQOs for this particular metal. The reconstruction is summarized in Box 1.2. Table 1.2 summarizes the numerical data on EQO-derivation. Table 1.3 shows the results of background concentration measurements in field soils in Dutch nature areas.

Box 1.2. Historical overview of the role of ecotoxicity data in the derivation of Hazardous Concentrations and Environmental Quality Objectives for zinc. Crommentuijn *et al.* (1997) recently summarized the relevant data; separate references to the original data are not given here.

- For the *Target Value*, the historical development has been complex. The Target Value of zinc equals 140 mg Zn/kg in standard soil (10% organic matter and 25% clay content). This value is directly related to the background concentration of zinc for standard soil, which has been calculated after measurements of zinc concentrations in Dutch nature areas. The choice to use the background concentration is related to the fact that the pertinent ecotoxicological data yielded values below the background concentration for standard soil, viz. 30-70 mg *added* Zn/kg (Janus, 1992; Cleven *et al.*, 1993). The concept of 'added concentration' (upon the background) was used, to take the natural occurrence of zinc in soils into account. The current Target Value for zinc is thus *not* based on ecotoxicity data.
- The *Intervention Value* of zinc equals 720 mg Zn/kg for standard soil. This value has originally be derived by Denneman and Van Gestel (1990), based on ecotoxicological extrapolation.

It should be noted that the Target and Intervention Values are given for standard soil. In daily application, the data are recalculated to soil-specific values by application of the soil-type correction method (VROM, 1994a).

It should further be noted that, since the establishment of the EQOs by VROM (1994a), additional ecotoxicity data have been obtained in the 'ecotoxicological domain'. These data have been summarized recently (Crommentuijn *et al.*, 1997). They allow for statistical extrapolation of HC_5 and HC_{50} data, independently for 'species' and 'microbial processes' as indicated in Figure 1.1. The data on 'species' concern 4 plant species, one oligochaete, one mollusc, and one crustacean, and a total of 44 test data. The data on 'microbial processes' concern 10 microbial processes, and a total of 27 test data.

Table 1.2. Summary of the data underpinning the current Dutch Environmental Quality Objectives for zinc in soil. All values refer to standard soil (10% organic matter, 25% clay). Data were taken from the Tables compiled by Crommentuijn *et al.* (1997, Table numbers in that report are given) and from VROM (1994ab).

Criterion *Based on* (Not based on)	Domain	Zn (total) mg/kg dry wt	Soil type *(Remark)*	Table # or Reference
Target Value	EQO	140	standard soil	Table 5.1
Background Conc.	Background	140	standard soil	Table 5.1
	Ecotox. data, '90	7.3*	*(Value not used for TV, <<140)*	Table 2.1
	Ecotox. data '92	30-70**	*(Value not used for TV, <<140)*	Table 2.1
(HC_5-species)	Ecotox. extrapol. '97	132**	standard soil	Table 7.1
(HC_5-micr. proc.)	Ecotox. extrapol. '97	16**	standard soil	Table 7.1
Intervention Value	EQO	720	standard soil	VROM (1994a)
HC_{50}	Ecotox. extrapol.	720	standard soil	VROM (1994b)
(HC_{50}-species)	Ecotox. extrapol. '97	385**/***	standard soil	Table 6.8.1
(HC_{50}-micr. proc.)	Ecotox. extrapol. '97	207**/***	standard soil	Table 6.8.2

*Lowest NOEC of few ecotoxicity data; **added concentration; ***preliminary calculations for General Discussion

Table 1.3. Some selected data on measured background concentrations in Dutch nature areas.

Soil type	Zn (mg/kg dry soil)
peat	62-150
peaty clay/clayey peat	62-150
clay	81-153
sandy loam	28 - 189
sand loam	6.4 - 62

The reconstruction shows that (1) the Target Value is *not* based on a statistically extrapolated HC_5 value, and (2) the Intervention Value is based on (and is equal to) an extrapolated HC_{50}. Consequently, the studies on Aspect 2 focused mainly on comparison of observed field effects to the Target Value in the low exposure range, and to the HC_{50} (=IV) in the high exposure range (see further Chapter 2, 6, 7 and 8). During the preparation of the General Discussion, a reconsideration of zinc ecotoxicity data by Crommentuijn *et al.* (1997) yielded new data. Using the new data, HC_5 values for zinc were derived, independently for 'species' and 'microbial processes'. The extrapolation of HC_{50} values for these groups was, however, not (yet) made. This will be done in a forthcoming reconsideration of the Intervention Values, in which the human toxicity and ecotoxicity data will be reconsidered simultaneously (pers. comm. Crommentuijn). Therefore, the data of Crommentuijn *et al.* (1997) were used to derive preliminary estimates of HC_{50} values for this report, independently for 'species' and 'microbial processes' (see Table 1.2). The new HC_5 and HC_{50} values were used in the General Discussion (Chapter 9), but not in the chapters prepared earlier (Chapters 6, 7 and 8).

1.2 Aspects of laboratory to field extrapolation

The extrapolation of laboratory toxicity data to sensitive species is the key issue in the derivation of environmental quality objectives in The Netherlands. So far, the modification of toxicity under field conditions is not included in the procedures. Van Straalen and Denneman (1989) have pointed out that toxicity data obtained from laboratory tests may not be valid under field conditions. Responses measured in laboratory tests may differ from field effects due to factors that are related to (soil) chemical, biological or exposure conditions (Römbke and Moltmann, 1995; Notenboom and Van Beelen, 1992).

In Table 1.1 the characteristics of laboratory tests are summarised which may cause differences in abiotic conditions and ecological reality and complexity in comparison with the field situation. Most laboratory tests involve the exposure of one selected test species to a single contaminant, which is mixed homogeneously in with a well defined (artificial) test substrate. Chemicals are added to the soil only shortly before the experiments start, resulting in high bioavailability of the toxicants. Tests are performed under constant, more or less optimal conditions and exposure time is relatively short. In the field, organisms are exposed to mixtures of chemicals under fluctuating conditions. In the case of persistent chemicals, exposure can be

extended over a long period of time. The toxicants are dispersed heterogeneously through and over the soil, and in many cases, the contamination has developed to a stable chemical form. Processes of avoidance, adaptation and ecological compensation, which can occur in field situations, are not incorporated in the laboratory test designs. From this, it is clear that some characteristics of laboratory tests can result in an overestimation of toxicity, whereas others may lead to an underestimation of toxicity in the field situations.
The importance of each of the characteristics mentioned in Table 1.1 is illustrated below, by giving a short introduction into the processes involved. The experimental approaches which were used within the framework of the Validation project to address these issues are outlined in section 1.3.

1.2.1 Bioavailability

Several authors have identified differences in exposure concentrations as one of the most important complications with regard to the extrapolation of laboratory derived toxicity data to field situations (Crossland, 1994; Spurgeon *et al.*, 1994; Van Gestel, 1992; Van Straalen and Denneman, 1989). Unless these differences can be compensated for, a comparison of toxicity data obtained under different environmental conditions will be hard to make. The proper assessment of bioavailability, which is related to the amount of chemical which can be taken up by biota, is therefore a key issue when the predictive value of laboratory toxicity data is considered.
It is assumed that for soil fauna, the bioavailability of contaminants will depend mainly on the sorption equilibrium between soil and pore water. Van Gestel and Ma (1990) have shown that the acute toxicity of organic contaminants (chlorobenzenes and chlorophenols) for earthworms is determined by concentrations in the soil pore water, which can be calculated using sorption data. The type and content of the soil organic matter and the lipophilicity of the compounds are the main factors determining the concentration of organic chemicals in the soil pore water fraction.
The situation is complex for heavy metals. Looking at 'suply' as a result of physico-chemical equilibria, sorption processes to solid and liquid phase constituents are crucial. The adsorption of heavy metals onto soil is dependent on many factors such as metal type, type and content of organic matter and clay, pH, cation exchange capacity and ionic strength of the pore water (Van Riemsdijk and Hiemstra, 1993). Other factors like the concentration of aluminium, manganese, and iron oxide or hydroxide content, and calcium concentration are also important (Kiekens, 1990; Temminghoff *et al.*, 1995). In case of metal uptake by plants, the situation is further complicated by the fact that plants and their associated mycorrhiza can influence metal speciation in the rhizosphere by root exudation of various organic and inorganic substances (Van Straalen and Verkleij, 1993).
The situation is also complicated by differences in 'biological demand'. The influence of soil type on bioavailability of heavy metals has been the subject of many studies. One of the approaches has been to describe heavy metal accumulation by organisms as a function of various soil characteristics. Van Gestel *et al.* (1995) summarised relationships obtained from the

literature on earthworms and have shown that various soil factors, such as soil pH, organic matter and clay content, influence heavy metal uptake. Experimental data on other organisms are needed to determine whether the relationships obtained for earthworms are also valid for species with different morphological and ecological characteristics (Posthuma *et al.*, in press.).

A second approach is to focus on ion activities in the soil solution as a measure of bioavailability. It is assumed that from the metal species present in the soil pore water, the free metal ions are taken up most easily by biota. Although a number of models have been developed to predict the distribution and speciation of metals over the various soil fractions (Hesterberg *et al.*, 1993; Wolt, 1994), the applicability of these models to predict heavy metal uptake by living organisms is capable of addressing all equilibria between solid-liquid and biotic phases in soil, including replenishment fluxes, once metals are taken up from the pore water (De Rooij and Smits, 1997).

So far, the most frequently applied method for the assessment of bioavailability is the use of relatively simple extraction techniques (Houba *et al.*, 1996; Lebourg *et al.*, 1996). These techniques initially applied to assess soil fertility, consist of shaking a small amount of soil in water or in a weak salt solution. Using this method, Novozamsky *et al.* (1993) have shown that bioaccumulation of cadmium by maize can be predicted by the amount of metal which was extracted from the soil by shaking with a 0.01 M $CaCl_2$ solution. When this method is generally applicable and uptake and toxicity of metals by soil organisms can be estimated in a comparable way, the predictive value of laboratory toxicity tests and the assessment of soil quality might be improved.

1.2.2 Multiple substances vs. single contaminants

In case of multiple substances, toxicological interactions can cause an increase or a reduction of toxic effects compared to the toxicity caused by the single substances. In addition, the ecotoxicity of mixtures is influenced by non-toxicological interactions such as sorption interactions and interactions during uptake. Since sorption of chemicals to soil is usually strongly influenced by soil characteristics, both aspects need to be considered in mixture toxicity studies in soil. This linkage has been elaborated by Van Gestel and Hensbergen (1997) and Posthuma *et al.* (1997), and is summarized below.

Considering toxicological models only, joint effects of metal mixtures are commonly established by application of the relative concentration addition (RCA) model (Sprague, 1970, De March, 1987). According to RCA, EC_{50}s for a species are first determined per substance. These EC_{50}s are then defined as one Toxic Unit (TU, dimensionless). All other metal concentrations are expressed as fractions of these TU. The property of dimensionlessness allows for summing the TUs for each constituent of a mixture to express the mixture concentration. In a mixture experiment, the observed toxicity (EC_{50} expressed as TU) can be evaluated versus an expected value. The expected value is usually generated with the concentration additivity as null-model (De March, 1987). This null-model applies to mixtures of chemicals that show no interaction, and that have the same mode of action. The expected EC_{50} of any mixture of such chemicals is 1 TU, but this only holds true if only toxicological interactions are addressed. In ecotoxicity studies, the value of 1 TU functions as benchmark, which has lost the mechanistic interpretation linked to the purely toxicological concepts of concentration additivity. In the studies performed in the project, observed mixture effects were therefore characterized only as more or less than concentration additive.

Considering interaction levels, it is well known that interactions may occur at different levels (Calamari and Alabaster, 1980). First of all, (physico)chemical interactions in the environment determine the sorption and bioavailability of metals. Second, interactions at the physiological level affect the uptake and internal transport and determine the effective concentration at the target site. A third level of interaction is formed by the intoxication processes at the target site inside an organism, including the binding at receptors.

The effect of interactions outside the organism depends largely on metal specific differences in affinity for adsorption sites and on the selectivity of adsorbents for different metals (Alloway, 1990). The adsorption processes are directly related to competitive binding at the cell walls or cell membranes of the organism, and influence the active uptake of metals by carrier proteins or through ion channels. Inside the organism, the affinity of metal ions for transport proteins, receptors or other binding sites determines the level of effect.

Interactions are metal specific and do not necessarily have to be the same at the different levels: within plants, zinc and cadmium act less than concentration additive (antagonistically), whereas they stimulate each other's sorption to the root surface (Alloway, 1990). As a result, mixture effects can have different aspects, depending on the metals, the effect parameter, the incubation time and exposure concentration and on the level of effect examined. Only a limited number of mixture toxicity studies have been performed on terrestrial animals and plants, probably as a result of this complexity.

The above implies that the combined characteristics of the environment, the organism and the chemical characteristics of the individual substances determine whether the impact of a heavy metal mixture differs from that of the individual components. Insight into the most important processes at all levels of interaction seems to be indispensable to develop general rules with respect to mixture effects of heavy metals in comparison with toxicity of single substances. Especially, in compartments with a high sorption potential, such as many soils, joint effects can not be explained without proper analysis of the bioavailability (Van Gestel and Hensbergen, 1997, Posthuma *et al.*, 1997)

1.2.3 Uncontrolled conditions vs. controlled conditions

It is generally assumed that a good performance of the control group is indicative for good health and implies the ability to withstand a certain degree of toxic stress (Cowgill, 1987). As a result, the test conditions prescribed in most test protocols are based on an optimal performance of the control group and toxicity parameters from standardised tests always pertain to optimal conditions. The variability of conditions encountered in the field can, however, influence the functioning of organisms and thereby induce changes in susceptibility for toxicant stress. Unfavourable conditions may form an additional stress, which may change sensitivity to toxicants.

Little data are available about the influence of confounding factors on toxicity for plants. Nutrient availability, temperature, radiation, air humidity and soil moisture content are likely to be important. Some evidence exists that heavy metal toxicity is reduced when nutrient availability is high (Wang, 1991). On the contrary, bioavailability and uptake of metals may increase when plants use root exudation to increase availability of essential elements. In addition, it may be assumed that passive uptake of toxicants will be increased in case of high evaporation.

For fauna, temperature is an important factor modifying toxicity (Cairns *et al.*, 1975). An increase in temperature generally leads to increased activity, which in most cases will be accompanied by changes in uptake and elimination of toxicants. As a result, tissue levels of heavy metals may be affected. This has been shown for some terrestrial organisms like earthworms (Bengtsson and Rundgren, 1992), springtails and mites (Janssen and Bergema, 1991), but consequences for toxicity are as yet not clear.

Like temperature, relative humidity and soil moisture content can show considerable fluctuations in the field. Literature data indicate that the uptake and toxicity of pesticides is enhanced under wet conditions because of an increased activity of animals (Jagers op Akkerhuis and Hamers, 1992). On the other hand, Demon and Eijsackers (1985) have demonstrated that drought stress can also lead to increased sensitivity to toxicants.

Literature data show that food limitation is another important factor which may modify toxicity in field situations compared to the laboratory. From studies with aquatic crustaceans, it appears that toxicity is dependent on food quantity (Chandini, 1988, 1989; Enserink *et al.*, 1995), and on food type and quality (Stephenson and Watts, 1984; Winner *et al.*, 1977). For terrestrial organisms, it has also been shown that heavy metal toxicity is dependent on the availability of food (Bengtsson *et al.*, 1985; Van Gestel *et al.*, 1991). The toxicity of cadmium to the bacterium *Klebsiella aerogenes* was increased 150 fold under sulphate growth limitation (Van Beelen and Doelman, 1997). In general, heavy metal toxicity is enhanced when organisms are exposed under suboptimal nutritional conditions.

The importance of confounding factors for the extrapolation of laboratory toxicity data to the field will depend on whether negative and positive effects will compensate each other under the fluctuating conditions which are encountered in nature.

1.2.4 Heterogeneity

Heterogeneity is closely linked to both spatial and temporal variations in bioavailability and exposure conditions. Depending on the mobility of the organism, spatial variability of contaminant concentrations in soil can lead to considerable differences in exposure (Marinussen and Van der Zee, 1996). Model calculations on the uptake of cadmium by earthworms showed that larger mobility on average leads to lower internal cadmium concentrations; however, the probability of exposure to high contaminant concentrations increases with increasing mobility (Marinussen and Van der Zee, 1996). In a study on springtails, Krogh (1995) has shown that toxicity may be significantly decreased if the animals are able to actively avoid exposure by migration to an uncontaminated area.

Soil temperature and humidity may show time-dependent differences between sites, resulting in different microhabitats through time. Long-term temporal changes in bioavailability of heavy metals may occur when environmental parameters such as pH and organic matter change through time. Seasonal differences in decomposition rate may be important with this respect, since the binding capacity of organic matter for contaminants changes over the different stages of decomposition (Bakker and Notenboom, 1994). Temporal changes in land use can induce changes in environmental conditions, which in turn can affect the storage and buffering capacity of the soil. This is the case when agricultural land is used for forestry and increased acidification results in an increased mobility of accumulated heavy metals (Salomons, 1993).
In the field, spatial and temporal heterogeneity may result in differences in the distribution of organisms between sites over a certain period of time. The extent of spatial and temporal variation should therefore be considered, not only when sampling schemes are designed, but also when the abundance of certain organisms is used as an ecological indicator of soil quality.

1.2.5 Population fitness

Populations in the field have an internal structure, determined by the variation in the life-history characteristics of the individuals. Factors like age distribution, different stages of development, distribution of weight, sex ratios, etc. are important in determining the performance or "fitness" of a population in the field. In standard laboratory toxicity tests only one or a few life-cycle components are taken as end-points of effect assessments, like survival, reproduction and growth. The tests are usually performed using individuals selected from one or a restricted number of stages of the life-cycle, instead of using a fully developed population. However, effects of chemicals on the mentioned toxicity end-points do not provide sufficient information to judge population fitness under toxicant stress.
To extrapolate toxicity data obtained with individuals to field populations, it is essential to understand the mechanisms that exist in a population to compensate or to magnify toxic effects on individuals. Life-cycle toxicity experiments have been carried out to find population performance indices which may serve as effect criteria to predict population fitness.
Several performance indices have been used to measure population fitness. Kammenga and Riksen (1996) used the intrinsic rate of population increase of a population with a stable age structure as an index of fitness. Carrying capacity and biomass turnover rate have also been used (Van Straalen and De Goede, 1987). From work of Van Straalen *et al.* (1989) it appears that differences in the life-history between species may have a profound effect on the responses of populations to a toxicant. Two species were chosen with very different life-histories, viz. the springtail *Orchesella cincta* and the mite *Platynothrus peltifer*. Although these two species appeared to be equally sensitive to cadmium if judged by the most sensitive individual sublethal criteria (reproduction, growth), they differed in sensitivity when judged by population growth. With increased cadmium stress *O. cincta* died before *P. peltifer*, but *P. peltifer* was affected before *O. cincta* with regard to population growth. This concept was further elaborated by Crommentuijn *et al.* (1995). A Sublethal Sensitivity Index (SSI, the ratio between the LC_{50} value for survival, and the EC_{10} (or NOEC) for a sublethal toxicity end-point, like reproduction)

was proposed as a parameter to express maintenance of sublethal functions under toxicant stress. It was concluded that the SSI could be a valuable parameter for evaluating the likelihood of population-level effects of toxicants.

It will be clear that better insight into the relationship between the impact of toxicants on life-history characteristics and population increase will improve the predictive value of single species tests.

1.2.6 Test species

Laboratory test species are generally selected on the basis of ease of captive breeding and good maintenance in the laboratory, enabling experimental manipulation, or ready collection in the field. Less attention is paid to the question as to what extent the sensitivity of laboratory species is representative for related species in the field. For example, the oligochaete compost worm *Eisenia* is often used in standardised laboratory tests, but actually we do not know whether it may be taken as representative for the Lumbricidae, let it be for the larger taxum of oligochaete worms, or the Annelide worms in general. However, it is remarkable to note that it has in common with a number of species of the non-related nematodes its sensitivity for benomyl and related substances (Driscoll *et al.*, 1989).

There are a number of aspects in which laboratory species may differ from species in the field.

1. The test species does not occur in ecologically relevant ecosystems. This is the case for *Eisenia* spec. which occurs in compost but not in soil.
2. Their behaviour in soil might differ from field species. Earthworm species may have different mobilities in soil (Heimbach, 1992). It is well known that three groups of earthworms can be distinguished, viz. epigeic, anecic and endogeic, each with their specific habitat depth in soil. Such differences in mobility and the rate of migration through heterogeneously contaminated soil may lead to different exposure and uptake (Marinussen, 1997).
3. The sensitivity towards pollutants may be different. Heimbach (1992) showed 1/10 EC_{50} of pesticides to be a good predictor for the effect on the total number of earthworms in the field. This does not exclude that there may be differences in effects on individual species, since Kula (1995) and Ma and Bodt (1993) showed an up to 80-fold difference in sensitivity among earthworm species.

Focusing on heavy metals, there are additional data available for the third aspect. Spurgeon and Hopkin (1995) found that in a polluted soil near a zinc smelter, *Lumbricus castaneus* and *L. rubellus* were more sensitive to Zn than four other worm species. On the other hand, the NOEC (Zn) for *Eisenia* reproduction after exposure to the polluted field soil was closely associated with the critical concentration for extinction of earthworms in the field surrounding the smelter (Spurgeon and Hopkin, 1995).

Among Collembola it appeared that *Folsomia candida* (which is used as test organism in a standardised test but also occurs in the field) had about equal sensitivity to dimethoate as *F. fimetaria* (Krogh, 1995), but that *F. candida* was much less sensitive to cadmium than *Orchesella cincta* (Crommentuijn *et al.*, 1995).

The recommended choice of test plants in the OECD toxicity test guideline (OECD, 1984b) covers a wide variety of taxa, but most species concern cultivars of crops, not typical wild species. *Trifolium pratense* also occurs as wild flower. Sensitivity for cadmium did not differ more than a factor 10 between three plant species, including *Avena sativa* (oats) as representative for mono-cotyledons (Adema and Henzen, 1986).
From the point of view of experimental validation studies it is practical importance that a test species has properties which enable it to be tested in field soils. This severely limits the potential choice of test species, since considerable effort and time are spent between starting work on potential test species and international acceptance of in standardised test protocols.

1.2.7 Adaptation and cost of tolerance

Adaptation (i.e. the acquisition of tolerance) of laboratory test organisms to toxicants generally does not take place during the short time period of a standard laboratory test. However, in the field, where natural populations are exposed for decades, adaptation may occur, especially in the case of persistent heavy metal pollution. Also, evolution of resistance to pesticides is a well-known problem. Genetic selection may play a role in this process. The occurrence of heavy metal adapted plant and microbial communities has long been demonstrated, but until recently terrestrial invertebrates obtained less attention.
Adaptation to metals in some terrestrial invertebrate species can be achieved within a few generations (Posthuma and Van Straalen, 1993). However, metal tolerance may also occur on prolonged individual exposure without genetic adaptation. It can be the result of increased detoxification mechanisms or compartmentalisation of the toxicant through physical acclimation.
For the following reasons adaptation is relevant for laboratory-to-field extrapolations:

1. The use of a tolerant base population in laboratory tests would lead to an underestimation of ecological risks.
2. Adaptation is the process by which risk, based on prediction from laboratory stocks, is reduced: true effects in the field might be compensated by adaptation. Adapting species have an increased chance of population persistence compared to non-adapting species.

Posthuma and Van Straalen (1993) reviewed metal adaptation in populations of terrestrial invertebrates. By applying five criteria, they confirmed the evidence for its occurrence in a restricted number of terrestrial species. Genetic variation for tolerance and life-history characteristics appeared to allow for the development of tolerance in *O. cincta* populations. In many cases adaptation occurs by modification and intensification of existing mechanisms of metal assimilation, excretion, immobilisation and compartmentalisation.
An altered life-history is often part of the complex adaptation syndrome. Donker *et al.* (1992) found that near the Budel factory isopods had a smaller body size and the percentage of gravid females was lower than at a reference site. In relation to body size the isopods at the factory site carried larger broods. They concluded from life-history patterns that the isopods were sublethally affected, although they were able to maintain their presence.

Genetic adaptation within populations occurs by loss of sensitive individuals which do not possess or cannot maintain a tolerance mechanism to cope with toxicants. The maintenance of a tolerance mechanism may be physiologically or energetically costly, so that allocation of nutrients or energy to other functions is reduced. Such "costs of tolerance" are frequently suggested as an inevitable consequence of being tolerant. Posthuma and Van Straalen (1993) distinguished two types of costs: the increased expenditures as a consequence of individual exposure, which is a plastic response often expressed in physiological terms, and expenditures associated with the maintenance of a tolerance mechanism evolved by genetic adaptation mechanisms. The latter costs are the real costs of tolerance, whereas the former are in fact "costs of individual exposure". The fitness of an individual at a contaminated site is affected by both types of costs.

Donker (1992) found that energy reserves in isopods living in a zinc smelter contaminated field were lower at exposure to higher metal concentrations. The low energy reserves did not result in shorter survival times upon starvation. There have been reports of dependence on Zn of isopods from Zn contaminated sites, probably due to the high sequestration capacity of these animals (Van Capelleveen, 1987). They performed less on normal reference substrate. This may explain the often observed absence of adapted populations in nearby non-polluted sites, because of reduced fitness of the tolerant animals to cope with normal situations (Van Capelleveen, 1987).

On a community level, the loss of sensitive individuals and species may result in a shift in the structure of the ecosystem. In this case the average increase of tolerance of the remaining species is exhibited as an increase of tolerance, referred to as Pollution Induced Community Tolerance (PICT). This concept, which was used in the present project, is further dealt with in section 1.2.9.

1.2.8 Species interaction

Many of the methods used to assess the toxicity of chemicals to soil organisms are single-species tests. Such tests do not assess the effects on interactions between populations or on community- and system interactions of toxicants within ecosystems (Parmelee *et al.* 1993). Ecosystems are characterised by structural characteristics (e. g. density of populations and diversity) and functional characteristics, like nutrient cycles and energy flows. The functional properties are often considered to be less sensitive to toxic substances than the structural ones (Van Straalen and Verkleij, 1993).

Important system properties to be considered in evaluating the effects of toxicants on ecosystems are (Van Straalen and Verkleij, 1993):

1. Connectivity, i.e. the number of interconnections in the food web. In an ecosystem with high connectivity, toxic effects do not express themselves quickly because of functional redundancy, i.e. changes in ecosystem structure may occur without loss of function, because other species will take over the function of the lost ones.
2. Resilience. Ecosystems may restore themselves after a disturbance. Degradation of the toxicant, adaptation of populations and recolonisation ability may play a role.

Generally all these aspects of community interactions are lacking in standardised laboratory tests. To get some insight into the role of species interactions, experiments with more than one species in microcosms kept in the laboratory have been carried out. Parmelee *et al.* (1993) exposed microcosms containing a.o. nematodes of different trophic levels, Acarina and Collembola to different toxicants and could observe toxicant-specific disturbances in trophic structure. Bogomolov *et al.* (1996) studied the effect of copper on some structural and functional characteristics in soil microcosms. Substrate induced respiration, a measure for microbial biomass, proved to be the most sensitive parameter. Multi-species experiments, are, however, often considered to be costly, and have not been standardized.
Predator-prey interactions play an important role in foodweb interconnections. They can be studied in two-species toxicity tests, which are easier to standardise. It has been shown that the sensitivity of the Collembolan *Folsomia fimetaria* to the insecticide dimethoate was changed by the presence of a predating Acarina species (Kirk and Krogh, 1995).
A special place in the species interconnectivity deserve the mutualistic interactions. Such critical interactions are important and disturbance may have serious implications. However, no standardized toxicity tests are available to evaluate the impact of toxicants on such interactions.

1.2.9 Community tolerance

In section 1.2.7, the adaptation of field populations to heavy metals has been discussed. Differences in the degree and rate of adaptation between species living together in a community and the disappearance of sensitive species may result in shifts in the structure of the ecosystem. By these mechanisms the average tolerance in a community increases, which can be measured in artificial exposure experiments. Crucial is that the occurrence of community tolerance can grossly be considered as strong evidence for the occurrence of toxic effects in the field on an ecological significant level. This approach is called Pollution Induced Community Tolerance (PICT), originally developed on the basis of responses in aquatic ecosystems (Blanck, *et al.*, 1988). It can be used as a parameter for effects of toxicants on field communities, including terrestrial systems (Posthuma, 1997). The increase of average tolerance indicates that the exposure to pollution has been sufficient to initiate a response. The principle and approach of this technique are summarised in Chapter 8.
The PICT approach is in particular suitable to measure the development of tolerance in microbial communities in soil (Doelman and Haansta, 1979, Díaz-Raviña *et al.*, 1994), although it has also been successfully applied to estuarine nematode communities (Millward and Grant, 1995).

1.3 Experimental approaches

It will be clear that it was not possible to equally pay attention to all of the above mentioned aspects of laboratory to field extrapolation for all investigated species. Depending on the species and the type of toxicity tests involved, different issues were addressed. The first item, bioavailability, played a key role in all subprojects since this involves the proper assessment of exposure. For several organisms it was tried to gain insight into the processes which determine bioavailability of zinc by linking zinc toxicity in different soils to concentrations in various soil fractions and by using extractable zinc concentrations to express effects. Interactions between toxicants were investigated for earthworms, enchytraeids and springtails. The influence of uncontrolled conditions was implicitly studied using the experimental field plot and was covered in laboratory experiments on springtails. Heterogeneity was not investigated as such, but played a role in the interpretation of the field data collected for micro-organism, nematodes and enchytraeids at the Budel field site. For the latter group, the field observations were used to compare the sensitivity of field species with that of laboratory-cultured organisms. Where possible, effects of zinc were evaluated on the population level using reproduction or population growth as the toxicity endpoints. Effects of zinc on the community level were investigated for nematodes and micro-organisms. Adaptation and tolerance, was not addressed in the experimental work at the level of species, only at the level of communities. In the general discussion (Chapter 9), the information which was obtained from the experimental work is combined with literature data to arrive at conclusions on the field relevance of laboratory toxicity data, and the ecological relevance of ecotoxicological risk limits based on these data.

1.4 Outline of this report

This report summarises the more detailed reports which are listed in the project bibliography, and gives an overview of the results of the project.

Chapter 2 gives the characteristics of the three soils types used for most species, including sorption of metals to these soils. This chapter also deals with the availability of the metals in these soils through their potential to release metals for uptake.

Chapters 3, 4, 5 and 6 address experimental results concerning toxicity data of zinc for single species (*Trifolium pratense, Folsomia candida, Eisenia andrei and Enchytraeus crypticus,* i.e.: laboratory-to-field extrapolation, aspect 1 of the project).

Chapter 6, 7 and 8 address the observational results concerning ecological relevance of ecotoxicological risk limits of zinc for field communities of enchytraeids, nematodes and micro-organisms (i.e.: understanding of field responses in view of predicted risks, aspect 2 of the project).

Chapter 9 presents the general discussion on both aspects of the project, extending the experimental results with literature data, also on other metals than zinc.

Chapter 10 presents the conclusions and recommendations.

Chapter 11 presents a report of the workshop held in February, 1998.

Finally, references are summarized, and a project bibliography is added to this report to refer to experimental data from the Validation project (to be) published elswhere.

2. HEAVY METAL BEHAVIOUR IN OECD ARTIFICIAL SOIL, BUDEL GRADIENT SOILS AND EXPERIMENTAL FIELD PLOT SOIL - METHODOLOGICAL ASPECTS AND SOIL CHEMICAL CHARACTERISATION OF CONTAMINATION

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2.1 Introduction

As was outlined in Chapter 1, the central aim of the Validation project was to identify the factors that may contribute most to the differences between laboratory toxicity data and effects in the field situation. Two lines of research were followed to reach this goal (Chapter 1). First, the performance of several test organisms was determined after incubation in heavy metal contaminated field soils under laboratory conditions and compared to effects of zinc observed in (standardised) toxicity tests using different artificially contaminated soils. Field soils were collected along a pollution gradient in the vicinity of a zinc factory in Budel, the Netherlands. Second, the toxicity of zinc for several test organisms was determined under outdoor exposure conditions in an experimentally contaminated field plot in which zinc was the only added contaminant. The plot was constructed in June 1994 at the university campus of the Vrije Universiteit in Amsterdam. Zinc concentrations in the soil were monitored regularly to gain insight into the development of the contamination through time and to investigate the influence of ageing of contamination on the effects of zinc.
This chapter summarizes some important characteristics of the commonly used standardized substrate (OECD artificial soil), and it describes the sampling and chemical analyses of the Budel gradient soils and the construction and monitoring of the experimental field plot. The partitioning of heavy metals over the various soil fractions in the three substrates and the development of the zinc contamination in the experimental field plot are described. To evaluate the (dis)similarity of the different soils, including a 'natural' zinc contamination, zinc partitioning in the OECD artificial soil is compared to that in the test field soil, in the Budel gradient soils and with literature data. Furthermore, site-specific environmental quality objectives for zinc are calculated according to the Dutch Soil Protection Act, to allow for an interpretation of the toxicity data in terms of ecosystem risk.

2.2 Methods

2.2.1 OECD artificial soil

OECD artificial soil (OECD, 1984a) is composed of various constituents (sand, peat, clay, calcium carbonate and water), together defining a standardized culturing and exposure substrate for various soil organisms. The use of such a well-defined test substrate allows for a standardisation between laboratories or a comparison of toxicity data obtained with different test species.

The preparation of the soil has been standardized in an OECD test guideline. The soil is made by mixing 20% kaolin clay and 10% ground *Sphagnum*-peat (< 1 mm) with 70% silver sand (all on dry weight basis), to which mixture an appropriate amount of calcium carbonate is added The latter addition is made to set the acidity of the soil to a predefined value, for example: 6.0± 0.5 for experiments with the earthworm *Eisenia* spec.. Demineralized water is added to set the humidity level of the soil to a predefined value, for example 55% water is added in the case of experiments with *Eisenia* spec.. For other species the soil preparation may be slightly modified, e.g. for *Enchytraeus crypticus* peat is ground to a size smaller than 0.5 mm, predefined acidity is 0.5 units higher, and only 35% water is added. Contaminants can be added as stock solutions in the demineralized water (e.g., metals), or can be mixed through the soil in other ways (e.g., poorly dissolving substances).

Although OECD artificial soil is well defined in comparison to field soils, even relatively small variations within the limits of the OECD guideline can have considerable impact on the behaviour of contaminants. The purpose of this part of the research was to determine to what extent metal sorption to OECD artificial soil is sensitive for variation in soil make-up procedures. We investigated (1) the effect of different acidity levels upon metal sorption, (2) the effect of different batches of basic constituents (both 1 mm and 0.5 mm ground), and (3) interactions among metals. These effects were studied by determination of the distribution over solid phase and pore water.

2.2.2 Sampling, treatment and analysis of Budel gradient soils

Soil samples were collected along a heavy metal contaminated gradient in the vicinity of the Budelco zinc factory near Budel on March 11, 1993. Sample sites were located at 11 different distances, from 0.4 to 21 km Northeast of the factory stack. The most distant sampling location was used as a reference site. At each distance, soil was collected at 5-7 randomly selected subsites to reduce variation in soil conditions other than metal pollution. The litter layer at the sites was removed, and 40 to 60 kg of soil was collected from the top 10 cm of the soil. The subsamples collected at each distance were transported to the laboratory and air-dried to a water content between 1 and 1.8 % (w/w). Thereafter, the subsamples were mixed and homogenised in a concrete mill and sieved (4 mm mesh). During homogenisation, the water content was adjusted to 2 % by addition of deionised water. The resulting 11 soil batches, further referred to as soil #1 to #11, were stored at 5 °C until use.

Soil pH, organic matter and clay content were measured and total metal concentrations of the soil batches were determined after *aqua regia* digestion. To study the distribution of zinc, copper, lead and cadmium over the solid and aqueous soil phase, 0.01 M $CaCl_2$ exchangeable, water-soluble and pore water metal concentrations were also determined following standardised methods. The reader is referred to Posthuma and Notenboom (1996) for further details.

The original pH of soils #1 to #11 was too low for the culturing of some of the test organisms, and it was therefore decided to adjust the soil pH for various experiments to a value of 5 to 6 using $CaCO_3$. In addition to the data that are presented on the heavy metal partitioning in the original soils #1 to #11, the distribution of zinc over the pore water fraction in a selected series of pH adjusted samples (indicated as #1a to #11a) is also described. The latter data are used to give an indication of the effect of pH increase on zinc partitioning. The effect of $CaCO_3$ addition on sorption and partitioning of zinc depends on the extent of pH increase. Since pH adjustment was done by each participant prior to the own experiments, soil pH differed between the experiments. As a result, the actual zinc concentrations in the pore water, $CaCl_2$ exchangeable or water-soluble fractions on which effect concentrations are based may differ between the experiments carried out by the respective participants.

2.2.3 Construction and monitoring of the experimental field plot

Six plots were constructed in July 1994 based on Faber and Verhoef (1991). The plots, indicated A to F, consisted of 10 equal compartments, separated by stainless steel plates and a layer of sharp sand (Figure 2.1), isolated from the subsoil by a waterproof foil. Uncontaminated soil was collected at the exploitation works of the Panheel Group near Heel, Limburg, The Netherlands in February 1994. The soil was sieved, homogenised and contaminated with $ZnCl_2$ to achieve a nominal concentration range of 0, 32, 56, 100, 180, 320, 560, 1000, 1800 and 3200 mg Zn/kg dry soil. To improve the soil structure, sharp sand was added to the soil (9 % on a dry weight basis). Zinc concentrations were assigned to each of the compartments following a completely randomised block design (Sokal and Rohlf, 1995), leading to six replicated series of the complete concentration range. A 30 cm deep soil layer was placed in each compartment. A drainage system was installed to control soil moisture content. After preparation, organic matter and clay content of the soil were 2.0 and 2.9 %, based on dry weight.

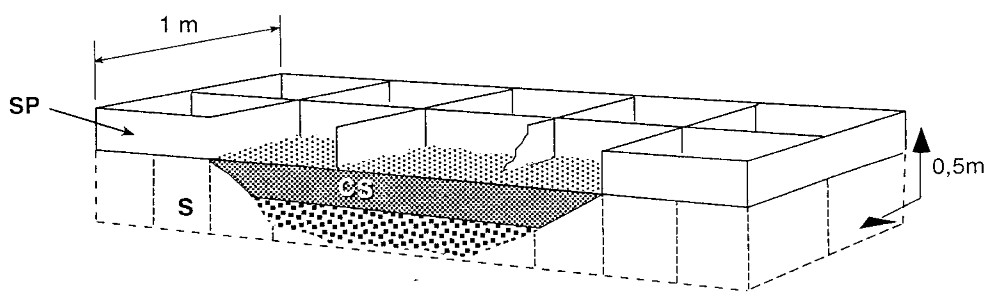


Figure 2.1. Enclosure plot (1 x 5 x 0.5 m) divided into 10 compartments by stainless steel plates (SP). CS = contaminated soil, S = sand.

Soil samples were collected at regular time intervals to determine soil pH and total zinc concentrations of the soil in each compartment. To study the distribution of zinc over the solid and aqueous soil phase, 0.01 M $CaCl_2$ exchangeable and water-soluble zinc concentrations were also determined following standardised methods. The first sampling of plots A-F took place directly after construction of the test field in July 1994. Thereafter, soil samples were taken from plots B-F in February and October 1995 and May 1996. In October 1995, an additional sampling was carried out to determine the vertical distribution of zinc in the compartments with the highest nominal zinc concentration. During the first rainfall after the construction of the test field, samples were taken from the drainage system to determine the time that zinc leaching occurred.

2.2.4 Heavy metal partitioning

Heavy metal partitioning in the Budel gradient soils was described calculating the partition coefficient K_p according to Equation 1:

$$K_P = \frac{C_s}{C_w} \qquad (1)$$

where K_p is the partition coefficient (l/kg), C_s is the metal concentration in the solid soil phase (mg/kg) and C_w is the metal concentration in the pore water, $CaCl_2$ or water extracts (mg/l).
For the OECD artificial soil and the experimental field plot soil, which are homogeneous in their characteristics, heavy metal partitioning was analysed for a range of concentrations using the Freundlich isotherm given in Equation 2:

$$C_s = K_f * C_w^{1/n} \qquad (2)$$

where C_s is the metal concentration in the soil (mg/kg), K_f is the Freundlich sorption coefficient (l/kg), C_w is the metal concentration in the pore water, $CaCl_2$ exchangeable or water-soluble fraction (mg/l) and 1/n is the shape parameter of the Freundlich curve. When 1/n equals unity and variation in soil factors is random or negligible, the Freundlich K_f of a soil sample is equal to the partition coefficient K_p of that sample.

2.2.5 Derivation of site-specific soil quality objectives

Site-specific soil quality objectives for zinc were calculated for Budel and experimental field plot soil according to the method of the Dutch Soil Protection Act (VROM 1994ab). This method has been developed to differentiate soil quality objectives (Target and Intervention Values) with respect to organic matter and clay content of the soil and is based on the statistical correlations between heavy metal concentrations in topsoils of Dutch reference areas and the organic matter and clay contents of the respective soils. For zinc, this relationship is (Equation 3):

$$[Zn] = 50 + 1.5 * (2*\%clay + \%OM). \qquad (3)$$

For natural occurring substances like zinc, the Target Values (TVs) for good soil quality at which a multifunctional use of the soil is considered to be possible, are set at the 90th percentile of the natural background concentration (Vegter, 1995). Accordingly, the local TV can be calculated by substituting the measured clay and organic matter content in the above formula. The site-specific Intervention Value can be calculated as follows (Equation 4):

$$IV_{ss} = IV_{st}\left[\frac{50 + 1.5*(2*\%clay + \%OM}{50 + 1.5(2*25 + 10)}\right] \quad (4)$$

where
IV_{ss} = site-specific intervention value
IV_{st} = intervention value for a standard soil containing 25 % clay and 10 % organic matter
These site-specific soil quality objectives serve to evaluate whether toxic effects on community endpoints studied on enchytraeids, nematodes and micro-organisms (see Chapters 6, 7 and 8) can be related to a local exceedance of the Target and Intervention values.

2.3 Results

2.3.1 Variation of metal partitioning in OECD artificial soil

- *The effects of acidity and of different lots of basic constituents.* According to the standard test protocols, the acidity of the OECD artificial soil is prescribed to be set within a narrow range. The range may differ between species because different species have a different pH-optimum. Nonetheless, prescribed variation of acidity may vary within the range of one pH-unit. In addition, acidity may change during experiments, for example in *E. andrei* tests, acidity changes often by approx. 0.5 to 1 pH unit upwards. In view of the influence of acidity on metal partitioning in soils (e.g., Janssen *et al.*, 1997), we investigated the quantitative importance of acidity variation in OECD artificial soil for the abiotic partitioning of metals over solid and liquid phase. To this end, we quantified pore water metal concentrations in a single batch of freshly contaminated artificial soil set at different pH, and we compared batches prepared from different stocks of peat and clay of different origin.

It appeared that metal partitioning over solid and liquid phase was sensitive for acidity differences among batches between pH of 4.5 and 6.5, irrespective of the origin of the constituents (Figure 2.2). The dissolved of zinc concentration appeared to change from 7.9 to 15.8 mg/l at the same total soil concentration, while soil pH was within the range of one pH-unit near the pH-range prescribed for e.g. *E. andrei*. The dissolved zinc concentrations differed by a factor of five for pH values that were half a unit outside the prescribed range. Similar relationships were found for cadmium, copper and lead. Tree other soil batches, although made of different lots of constituents, fitted within the function, suggesting a dominant role of pH in metal partitioning. These data suggest that variation in acidity of the artificial soil may have a measurable influence on toxicity if a test species is truly exposed through pore water transfer of metals and toxicity is expressed on the basis of total concentrations. This may account for part of the intra- and interlaboratory differences in toxic effect levels for a species (e.g., in ring tests or in repeated tests with the same organism, for example in different experiments with *Eisenia*; see Chapter 5). Also, it is a factor that may influence the apparent sensitivity of species, since species for which the prescribed pH is high will on the average tend to be less sensitive, due to the lower exposure concentration.

- *Mixture effects*. Different metals may show mutual interactions at the level of sorption to the soil solid phase. Competitive displacement of the most soluble metal is expected to occur when a second, less soluble metal is present. To investigate the quantitative importance of metal-metal interactions in OECD artificial soil, we quantified pore water metal concentrations in a single batch of freshly contaminated artificial soil, in the presence and absence of other metals. Methods were similar to those described in the previous section, with metal concentrations being added in equimolar amounts of two metals, ranging from 0 - 15 µmol/kg dry soil.

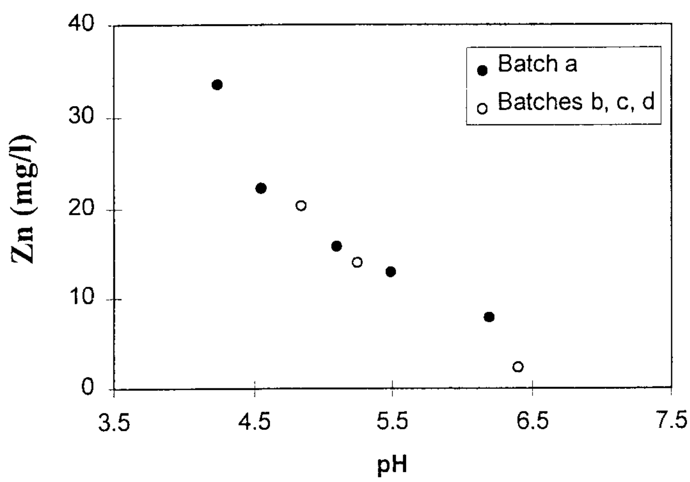


Figure 2.2. Partitioning of zinc (327 mg/kg dry soil added) over the solid and liquid phase as a function of pH established in OECD artificial soil (batch a), through addition of different amounts of calcium carbonate, and in three other batches (b, c, d) of OECD soils

Table 2.1. Parameters of the Freundlich sorption isotherms of metals (Zn, Cd, Cu) in OECD artificial soil at pH approx. 5.5. Bold values indicate the parameters for metals applied singly. The values in a column indicate the effect of the presence of secondly added metals (first column) on the sorption of the metal indicated in the upper row.

	Zn		Cd		Cu	
	Kf	*1/n*	*Kf*	*1/n*	*Kf*	*1/n*
Zn	**66**	**0.52**	68	0.47	190	0.70
Cd	47	0.54	**96**	**0.48**	202	0.76
Cu	43	0.53	64	0.49	**222**	**0.57**

Metal partitioning over solid and liquid phase appeared to be sensitive for the presence of other metals (Table 2.1). Sorption strength to OECD artificial soil increased in the series Zn < Cd << Cu, as shown by the increase of the Freundlich Kf-values. Addition of a second metal in the system caused a lower sorption compared to the single-metal situation in all binary mixtures. This effect was strongest for the weakly sorbing metals Cd and Zn, and less for the strongest sorbing metal Cu. When it is assumed that exposure of an organism is truly mediated by transport through the liquid phase, these results suggest that competitive displacement of metals in OECD artificial soil may be of quantitative importance for the outcome of mixture toxicity studies.
The experiment has demonstrated sorption interactions in OECD artificial soil by means of pore water concentrations to represent the liquid phase. The presence of abiotic metal-metal sorption interactions implies that mixture toxicity data based on total soil concentrations are not constant among soils, since the sorption characteristics of soils differ. Therefore, mixture toxicity data should be extrapolated among soils on the basis of joint-effect data that are insensitive of soil type influences, viz. mixture data based on truly bioavailable or body concentrations. It should be noted that interactions at the level of sorption cannot be established in contaminated field soils (e.g., Budel gradient soils), since mutual interactions among metals cannot be disentangled from the direct influences of soil characteristics on sorption.

2.3.2 Description of the Budel gradient soils

Table 2.2 summarises the main characteristics of the original Budel gradient soils together with total concentrations of a series of elements. Soil pH close to the factory (#1 to #4) was higher than at larger distances. Organic matter content showed considerable variation between soils and no consistent pattern with distance was observed. Heavy metal concentrations generally decreased with increasing distance. Figure 2.3 shows the total, $CaCl_2$ exchangeable and pore water concentrations of zinc, copper, cadmium and lead as a function of distance to the factory. Metal concentrations measured in pore water from pH adjusted soils #1a to #11a are shown in the same figure. The addition of $CaCO_3$ to optimise pH for the culturing of test organisms reduced the metal concentrations in the pore water.

The partition coefficients K_p for zinc, copper, cadmium and lead, calculated according to Equation 1, are given in Table 2.3, based on metal concentrations in the $CaCl_2$ exchangeable fraction. Partition coefficients based on concentrations in the pore water are given in Table 2.4 together with data on pH adjusted soil samples #1a to #11a. $CaCl_2$ based partition coefficients differed between metals, for copper and lead the sorption to the solid phase was proportionally stronger than for cadmium and zinc. Partition coefficients showed large variation between sites. No correlation between K_p and soil pH was observed, so differences in other soil characteristics may account for this.

Figure 2.3. Total, $CaCl_2$ exchangeable, water-soluble and pore water metal concentrations in the Budel gradient soils as a function of the distance to the factory. 'Pore pH' = pore water concentration of pH-adjusted soils.

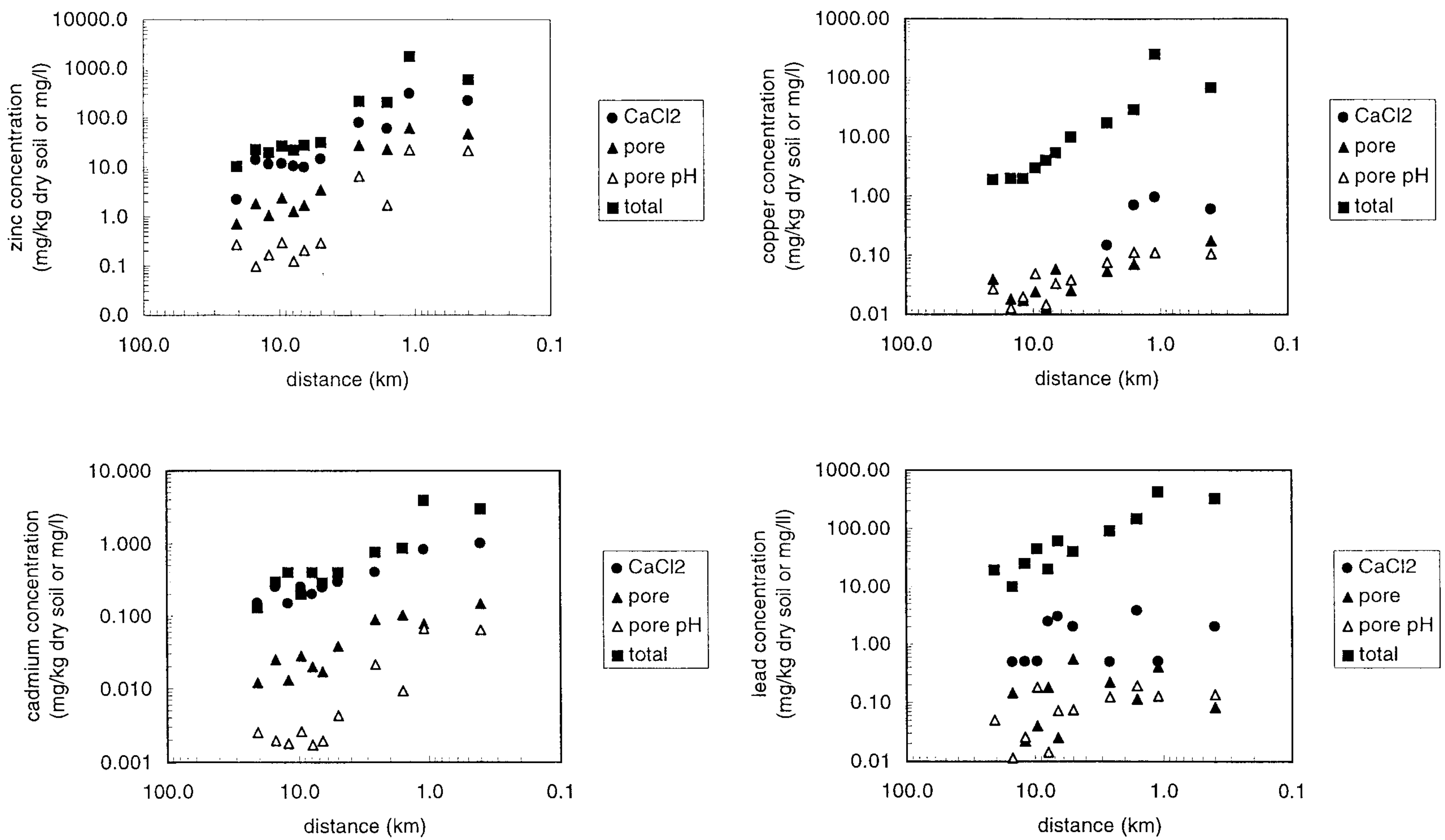


Table 2.2. Main characteristics of the Budel gradient soils #1 to #11 taken at different distances (D) from the factory. Metal concentrations indicate total amounts.

#	D (km)	pH (KCl)	OM (%)	clay (%)	Ca	Zn	Cu	Pb	Cd	Fe	Cr	TV	IV
					(mg/kg dry soil)								
1	0.4	4.7	1.9	2.0	300	598	68	325	3.0	6550	8.0	59	302
2	1.1	4.9	3.6	1.4	539	1787	250	424	3.9	15686	16	60	307
3	1.6	4.0	2.8	1.3	204	207	29	148	0.9	6130	41	58	298
4	2.6	4.3	2.4	1.2	289	219	17	91	0.8	4770	6.7	57	295
5	5.0	3.1	3.7	1.3	<50	32	10	40	0.4	1960	3.0	59	305
6	6.6	2.9	6.4	1.5	74	28	5.4	61	0.3	2935	12	64	329
7	7.9	3.2	4.5	1.3	<50	23	4.0	20	0.4	1920	4.0	61	311
8	9.6	2.9	4.3	1.3	100	27	3.0	45	0.2	2820	3.0	60	310
9	12.0	2.9	5.5	1.3	<50	20	2.0	25	0.4	1530	4.0	62	319
10	15.0	3.3	4.7	1.6	100	23	2.0	10	0.3	3360	n.d.	62	317
11	20.7	3.4	3.5	1.2	<50	11	1.9	19	0.14	2043	5.9	59	302

D=distance from the factory stack, TV=soil-specific Target Value for zinc, IV=soil-specific Intervention Value for zinc, see paragraph 2.3.5 for explanation of the latter two parameters.

Table 2.3. Partition coefficients K_p for zinc, copper, cadmium and lead based on metal concentrations in the $CaCl_2$ exchangeable fractions of Budel gradient soils.

#	K_p-$CaCl_2$ (ml/g)			
	Zn	Cu	Pb	Cd
1	17.0	1123	1615	19.8
2	48.1	2629	8495	36.6
3	24.3	400	384	11.3
4	17.6	1163	1839	15.4
5	11.8	>100	193	5.82
6	18.1	> 54	195	4.22
7	11.2	> 40	> 389	5.64
8	12.7	> 30	173	3.24
9	7.26	> 20	492	5.71
10	6.28	> 20	191	9.62
11	37.9	> 19	384	7.17

Table 2.4. Partition coefficients K_p for zinc, copper, cadmium and lead based on metal concentrations in the pore water fractions. pH* and K_p^* indicate pH-KCl and partition coefficients of pH adjusted soils #1a to #11a of Budel gradient soils.

#	pH	pH*	zinc		copper		lead		cadmium	
			K_p	K_p^*	K_p	K_p^*	K_p	K_p^*	K_p	K_p^*
1	4.7		12.7	27.6	384	645	1157	2381	20.3	46.6
2	4.9	5.30	28.8	79.2	2232	2250	5108	3287	50.2	58.9
3	4.0	5.32	8.94	119	410	257	363	768	8.42	93.1
4	4.3	5.13	7.86	33.1	326	229	794	731	8.64	35.9
5	3.1	5.26	9.09	109	400	262	180	530	10.5	92.2
6	2.9	5.33	16.4	135	93.1	163	110	839	17.0	149
7	3.2	5.45	17.4	181	308	274	800	1397	20.0	232
8	2.9	5.63	11.2	90.0	125	61.2	250	246	7.12	76.5
9	2.9	5.48	18.5	117	118	101	625	972	30.8	222
10	3.3	5.12	12.4	234	111	157	456	876	12.0	153
11	3.4	5.06	14.7	38.4	48.7	71.0	132	384	10.8	51.2

For zinc, partition coefficients based on pore water from pH adjusted samples were increased by a factor of 2.2 to 19 compared to the original values, whereas for cadmium a 1.2- to 13-fold increase was observed. For lead and for copper the partition coefficients tended to change slightly upward or downward after pH adjustment. This implies that the proportion of metals in the pore water in the homogenised, rewetted and pH adjusted soil is lower than in the original field soil for both zinc and cadmium. For copper and lead sorption along the gradient tended to increase when judged by Freundlich calculations, as an average over all samples (see Table 2.5). Freundlich adsorption coefficients based on metal concentrations in the $CaCl_2$ exchangeable and pore water fractions are shown in Table 2.5. For $CaCl_2$ exchangeable zinc and cadmium, Freundlich isotherms fitted reasonably well to the data, for copper and lead, values for K_f and 1/n could not be accurately estimated and showed a large variation. Partitioning of these metals is apparently strongly affected by local variation in soil properties and cannot be accurately predicted from information on total concentrations alone. The Freundlich shape parameter 1/n was near unity for zinc, indicating that adsorption is not dependent on the zinc concentration. For this metal, the average K_p value is thus grossly similar to K_f. This implies that the Freundlich adsorption isotherm may be used for an estimation of concentrations in the $CaCl_2$ exchangeable and pore water fractions in soil samples from Budel with a known total zinc concentration. The same holds for cadmium in the case of pore water K_f values of the original soil samples.

Table 2.5. Freundlich adsorption coefficients K_f and 1/n values for zinc, copper, lead and cadmium based on metal concentrations in the $CaCl_2$ exchangeable and pore water (PW) fractions of Budel gradient soils #1 to #11. PW* indicates the pore water of pH adjusted soil samples #1a to #11a. Values between brackets indicate 95 % confidence intervals.

	K_f	1/n	r^2
Zn			
$CaCl_2$	14.25	1.133	0.891
	(8.597-23.62)	*(0.833-1.432)*	
PW	13.90	0.977	0.948
	(9.415-20.52)	*(0.805-1.150)*	
PW*	81.91	0.768	0.905
	(56.50-118.8)	*(0.580-0.956)*	
Cu			
$CaCl_2$#	1009	0.954	0.564
	(-)	*(-)*	
PW	1573	1.594	0.676
	(99.46-24884)	*(0.762-2425)*	
PW*	2194	1.721	0.725
	(160.9-29913)	*(0.922-2.520)*	
Pb			
$CaCl_2$	159.6	0.470	0.115
	(14.93-1705)	*(-)*	
PW	197.7	0.590	0.286
	(37.82-1033)	*(-)*	
PW*	740.6	0.940	0.614
	(145.9-3791)	*(0.379-1.502)*	
Cd			
$CaCl_2$	111.9	1.709	0.822
	(17.78-704)	*(1.207-2.211)*	
PW	14.01	0.983	0.677
	(2.428-80.85)	*(0.471-1.496)*	
PW*	17.46	0.677	0.827
	(5.054-60.92)	*(0.444-0.910)*	

#: $CaCl_2$ exchangeable concentrations of sample #5 to #11 were below the detection limit and were excluded from the K_f calculation.

2.3.3 *Development of zinc contamination in the experimental field plot*

In July 1994, actual measured and nominal total zinc concentrations were very close to each other. The first rainfall after the construction of the experimental field plot occurred during the first week of August 1994. Zinc concentrations in the drainage water started to increase almost immediately, reached a maximum in September 1994 and decreased to normal levels in December 1994. This indicates that a substantial leaching of zinc from the soil occurred during that period. As a result, total zinc concentrations in the soil decreased between the first two sampling dates in July 1994 and February 1995, after which they stabilised over the following two years (Figure 2.4). Concentration decrease was most pronounced at nominal zinc concentrations higher than 560 mg/kg dry soil. At the highest concentration of 3200 mg Zn/kg dry soil, recovery of zinc at the last two sample occasions (October 1995 and May 1996) was 71 and 68 % of the amount measured in July 1994. Analysis of soil samples taken at various depths in October 1995, revealed that zinc was distributed homogeneously over the soil layer.

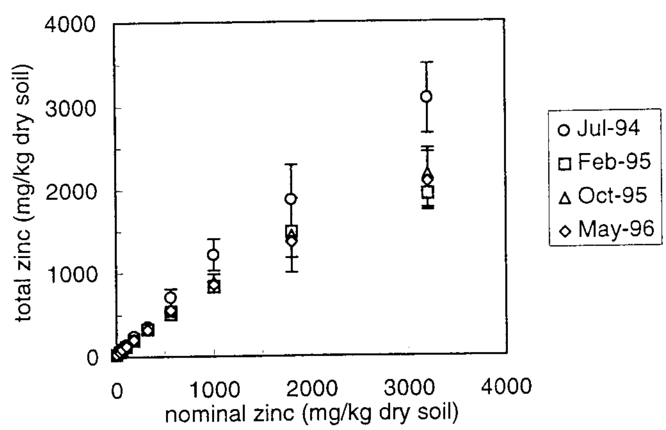


Figure 2.4. Average total zinc concentrations measured in the experimental field plots A-F in July 1994 (O) and the plots B-F in February 1995 (□), October 1995 (Δ) and May 1996 (◊) as a function of the nominal concentrations added.

Figure 2.5 shows the pH-KCl as a function of the measured total zinc concentration in the soil. The initial pH-KCl of the Panheel soil was 5.2. At the time of the first sampling in July 1994, pH in the control soil compartments of plots A to F was slightly raised to an average of 6.0 ± 0.2 (± SD). In February 1995, control soil pH was further raised to a value of 7.1 ± 0.1 (average of plot B to F; ± SD). This was due to the presence of $CaCO_3$ in shell particles in the sharp sand, which slowly dissolved. At the next sampling occasions, only minor pH changes were detected. pH was negatively related to zinc concentration at nominal concentrations up to 1000 mg/kg dry soil.

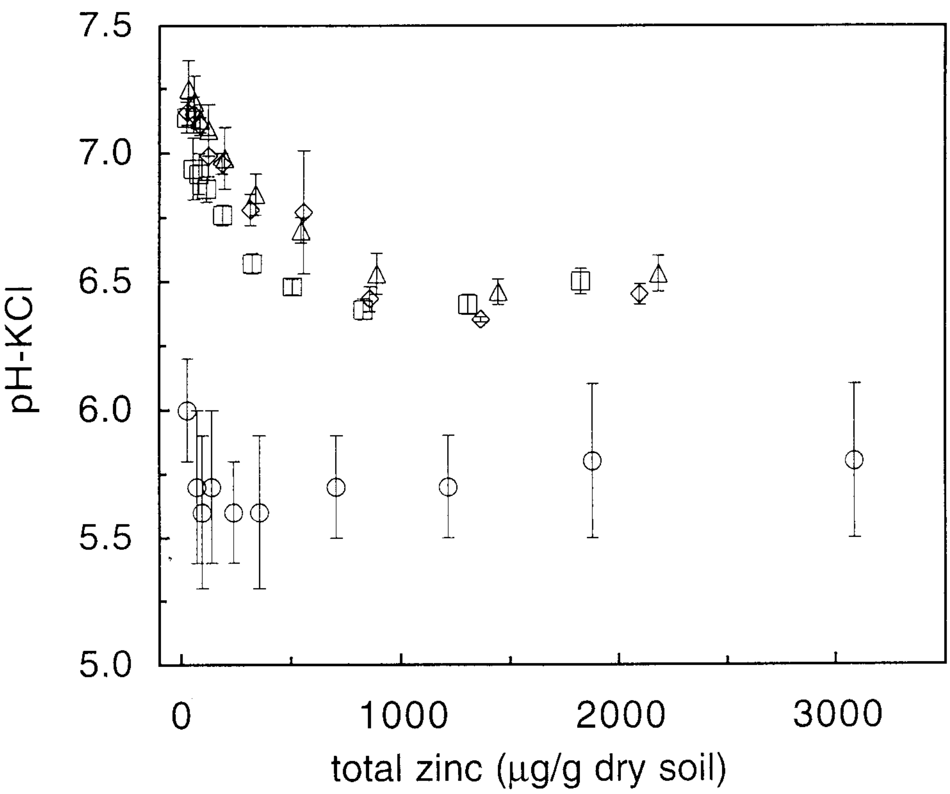


Figure 2.5. Average pH-KCl measured in plots A-F in July 1994 (O) and plots B-F in February 1995 (□), October 1995 (Δ) and May 1996 (◊) as a function of the total zinc concentration.

$CaCl_2$ exchangeable and water-soluble zinc concentrations measured in the test field soil are shown in Figure 2.6. as a function of the total zinc concentration. In July 1994, an average (± SD) of 40.4 ± 10 % of the total zinc was found in the $CaCl_2$ exchangeable fraction at the highest nominal zinc concentration. Variation between the compartments was relatively large. Average maximum $CaCl_2$ exchangeability decreased to 23.7 ± 1.5 and 19.2 ± 2.5 % in February and October 1995, respectively. In May 1996, a maximum of 19.1 % of the total zinc was found in the $CaCl_2$ extracts and variability between the compartments was reduced in comparison with the first sampling date. At the first sampling date, maximum water solubility was 1134 mg Zn/kg dry soil, corresponding with 36.8 % of the total zinc concentration at the highest nominal concentration of 3200 mg Zn/kg dry soil. In May 1996, only 1.5 % of the total amount of zinc was water-soluble at this concentration.

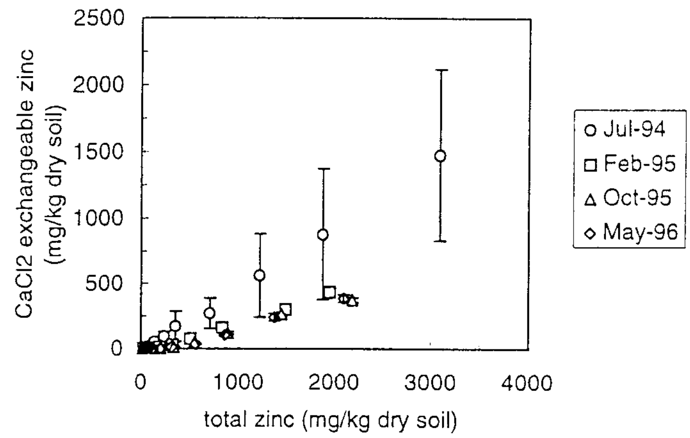


Figure 2.6. Average $CaCl_2$ exchangeable zinc concentrations measured in plots A-F in July 1994 (O) and plots B-F in February 1995 (□), October 1995 (Δ) and May 1996 (◊) as a function of the total zinc concentration.

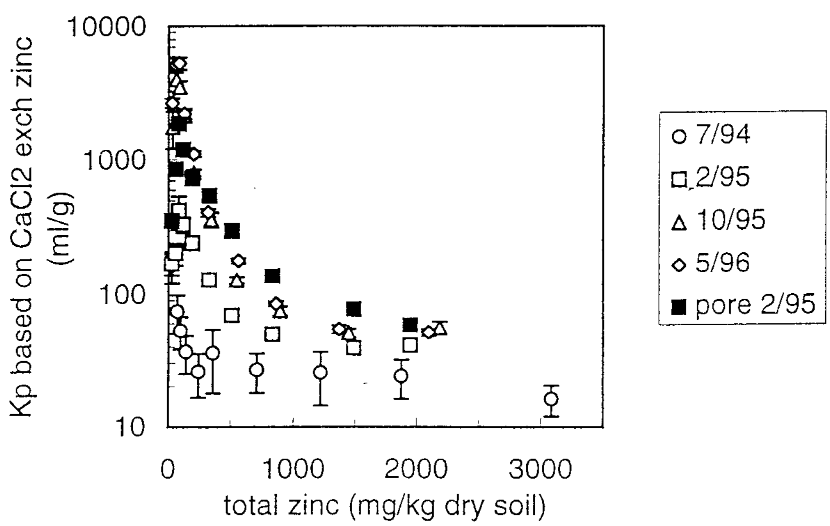


Figure 2.7. Average K_p values based on $CaCl_2$ exchangeable and water-soluble zinc concentrations measured in plots A-F in July 1994 (O) and plots B-F in February 1995 (□), October 1995 (Δ) and May 1996 (◊) as a function of the total zinc concentration. Filled squares indicate pore water K_p values determined in plot A in February 1995.

Partition coefficients and Freundlich adsorption coefficients with corresponding 1/n values were calculated according to Equations 1 and 2. Figure 2.7 shows the average K_p based on $CaCl_2$ exchangeable and water-soluble zinc concentrations as a function of the total zinc concentration. The K_p value based on pore water zinc concentrations determined in plot A in February 1995 are shown in the same figure. Partition coefficients decrease with increasing concentration, this effect was most pronounced when $CaCl_2$ exchangeable concentrations are considered. K_f values based on $CaCl_2$ exchangeable and water-soluble zinc concentrations are shown in Table 2.6 for the respective sampling dates. Except for the second sampling date, the 1/n values for the test field soil differed from unity, indicating that sorption was concentration dependent. The combination of decreased total zinc concentrations and increased sorption resulted in a rapid reduction of the $CaCl_2$ exchangeable and water-soluble zinc concentrations during the first month after construction of the test field. This implies that for the toxicity experiments which were carried out in the test field during this period, the prevailing exposure concentrations can be estimated only by approximation.

2.3.4 Variations in heavy metal behaviour in soils - comparison of experimental soils with literature data

The observations on OECD artificial soil, the Budel gradient soils and the experimental field plot soil as described in the preceding sections clearly demonstrate the complexity and variability of heavy metal sorption in different soils. Most literature data on heavy metal adsorption involve studies in which adsorption or partition coefficients are determined using batch equilibration methods. Soil samples are equilibrated with aqueous metal solutions of different strength. The sorption equilibrium is highly dependent on the type of soil and metal solution used, and literature data on the adsorption of heavy metals on soils show large variations. Baes and Sharp (1983) determined partition coefficients in a range of agricultural soils with different pH and found values of 0.1-8000, 1.26-26.8, 1.4-333, and 4.5-7640 ml/g for zinc, cadmium, copper and lead, respectively. Freundlich adsorption coefficients cover a similar wide range of values. For zinc, K_f values between 2 and 774 ml/g with 1/n values between 0.70 and 0.96 have been found by Buchter *et al.* (1989). Values between 5.5 and 8900 ml/g (1/n=0.60-2.08) have been reported for cadmium (Buchter *et al.*, 1989; Sanchez-Martin and Sanchez-Camazano, 1993). K_f ranges of 53.7 to 6353 ml/g (1/n=0.55-1.42) and 136 to $43.2*10^6$ ml/g (1/n=0.74 to 5.39) were determined for copper and lead by the same authors.

The reduced solubility of zinc in OECD artificial soil at increasing pH and the increase in K_p and K_f values after pH adjustment of the Budel gradient soils are in agreement with Barrow (1986a), who has shown that a one unit increase of pH by the addition of $CaCO_3$ increased the zinc retention capacity of natural soils with a factor of about 10. The influence of pH increase on adsorption is in the same order of magnitude for zinc and cadmium, which is consistent with results of Elliott *et al.* (1986) and Lo *et al.* (1992). The influence of pH on partitioning of copper and lead in the Budel gradient soils was relatively small. This was not expected, since for these metals, adsorption often shows a stronger pH dependency than for zinc and cadmium (Elliott *et al*, 1986; Lo *et al.*, 1992).

Table 2.6. Freundlich adsorption coefficients and corresponding 1/n values estimated for zinc contaminated soil from the experimental field plot, estimated from $CaCl_2$ exchangeable and water-soluble zinc concentrations.

sampling date	$CaCl_2$ exchangeable		water-soluble	
	K_f	1/n	K_f	1/n
July 1994	71.7	0.69	214	0.47
February 1995	182	0.59	586	0.96
October 1995	345	0.40	557	0.53
March 1996	363	0.39	1107	0.68

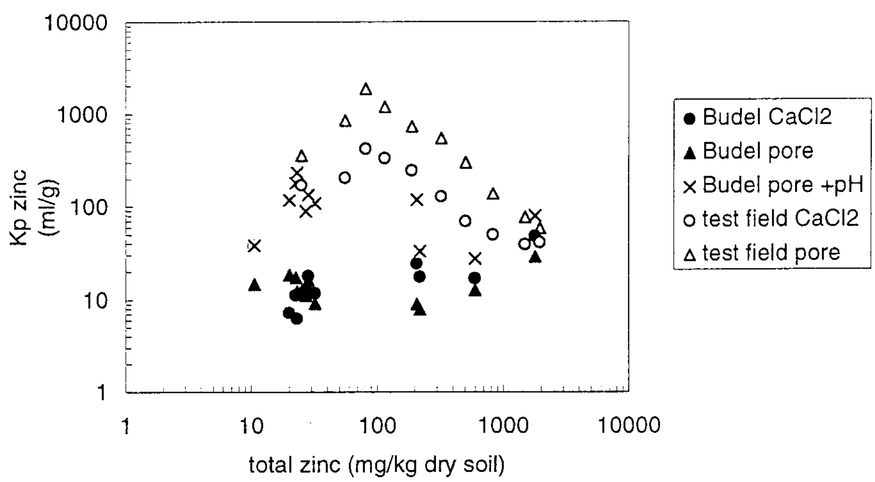


Figure 2.8. K_p values for the Budel gradient soils and test field soil based on $CaCl_2$ exchangeable and pore water zinc concentrations as a function of the total zinc concentration in the soil. Pore water K_p values for pH adjusted soil samples #1a to #11a are indicated by crosses.

In the experimental field plot, the initial leaching of excess, readily soluble $ZnCl_2$ and the simultaneous increase in pH resulted in a rapid increase in the sorption of zinc. Sorption K_f values continued to increase after February 1995, although pH and total zinc concentrations were stabilised from that moment on. This indicates that time is an important factor in heavy metal sorption as well. It is assumed that zinc sorption onto soil involves an initial, rapid adsorption reaction followed by diffusive penetration of the surface (Barrow, 1986b). This sorption process can be simulated artificially, as shown by Brümmer *et al.* (1983), who used an eight weeks period of alternate drying and rewetting to equilibrate zinc with soil after contamination with $ZnCl_2$. In several studies it was shown that an increase in contact time increased the retention of heavy metals by soils (Barrow, 1986b; Mann and Ritchie, 1994).
To compare the zinc partitioning in the test field soil with the Budel gradient soils, K_p values are plotted as a function of total zinc concentrations in Figure 2.8. From this figure and from the data in Table 2.5 and Table 2.6, it is clear that adsorption of zinc in the experimental field plot was higher than in the Budel gradient samples. Raising the pH in Budel samples #1a to #11a reduced the differences, especially at the highest zinc concentrations. The results obtained for the experimental soil used in this study indicate that especially pH and contact time are important factors determining the heavy metal partitioning in soils.

2.3.5 Site-specific soil quality objectives

The target and intervention values for zinc in the Budel gradient soils were calculated using the organic matter and clay contents given in Table 2.2. Due to small differences among the Budel gradient soils, local Target and Intervention Values differ slightly among soils, i.e. between 57 and 62 and between 295 and 329 mg Zn/kg for TV and IV, respectively. The TV is exceeded in soils #1 - 4, the IV in soils #1 - 2 (see for details: Table 2.2). For the pragmatic purposes of Chapters 7, 8 and 9, the small variation in TV and IV is regarded to be negligible, and summary values for Budel-specific Target and Intervention Values are given in Table 2.7. Similarly, the averaged soil-specific TV and IV have been calculated for experimental field plot soil, of which the organic matter and clay content are indicated in Table 2.7.

Table 2.7. Summary of soil-specific Target and Intervention Values (TV and IV) for zinc as calculated on the basis of the values for standard soil VROM (1994ab) and soil-type correction factors. The soil-specific TV and IV are given in round figures for pragmatic use in Chapters 6, 7 and 8.

Soil	Soil characteristics		Standard soil		Soil-specific	
	%clay	%OM	TV mg Zn/kg	IV mg Zn/kg	TV mg Zn/kg	IV mg Zn/kg
Budel	1.2 - 2.0	1.9 - 6.4	140	720	60	309
EFP	2.0 - 2.9	2.0 -2.4	140	720	61	312

EFP = experimental field plot, OM=organic matter.

3. EFFECTS OF ZINC ON THE GERMINATION AND GROWTH OF RED CLOVER, *TRIFOLIUM PRATENSE*, UNDER FIELD AND LABORATORY CONDITIONS

J.A. de Knecht, R.N. Hooftman, N.H.B.M. Kaag, N. van der Hoeven & L. Henzen

3.1 Introduction

In cell metabolism of plants, different heavy metals like cobalt, iron, manganese, molybdenum, nickel and zinc fulfil essential roles. The various functions of zinc in plants are among others: (1) protection of membrane lipids and proteins from oxidation (Bettger and O'Dell, 1981), (2) gene regulation through zinc-binding enzymes (Woolhouse, 1983; Marschner, 1986) and (3) active centres of metallo-enzymes, such as carbonic anhydrase, Cu-Zn superoxide dismutase and RNA polymerase (Clarkson and Hanson, 1980). On average, zinc is adequate for optimum plant growth at 0.3 μmol/g shoot dry weight (*i.e.* 20 μg/g; Marschner, 1986). Zinc deficiency is characterised by stunted growth and decreased leaf size, which may result from loss of the capacity to produce sufficient amounts of the hormone indoleacetic acid. Plants can also develop chlorosis in combination with necrotic spots, indicating a zinc requirement for chlorophyll biosynthesis (Taiz and Zeiger, 1991).

As with other essential heavy metals, zinc is phytotoxic when present in excessive amounts in the soil. Little is known about the nature of the primary lesions caused by heavy metals. It is generally believed that the toxicity is based on the ability to bind strongly to oxygen, nitrogen and sulphur atoms. Zinc preferably binds to oxygen- and nitrogen-containing ligands (Borovik, 1990). In the cell the occurrence of excessive amounts of zinc might result in a displacement of other essential metal ions from functional biomolecules, disruption of the integrity of biomolecules or a blockage of functional groups of biomolecules. Visible symptoms of severe zinc phytotoxicity are stunted growth and chlorosis (Woolhouse, 1983).

The concentration of heavy metals in plant parts is the result of uptake and translocation processes (Marschner, 1986). It is generally believed that uptake of zinc by plants is predominantly controlled by a metabolically mediated process, at least at the low concentrations which are normally found in soil solutions. At higher concentrations, the zinc uptake may also be partly passive (Mullins and Sommers, 1986). An important factor determining the uptake of heavy metals by plants is their bioavailability, c.q. the activity of the free divalent cations in the soil solution (Clarkson and Lüttge, 1989). The free ion activity in the soil solution will depend on the chemical speciation of the metal, which is determined by the physical and chemical properties of the soil, such as soil particle distribution, organic matter content, salinity, pH, redox potential and the mineral nutrients present in the soil (Salomons and Förstner, 1984; Xian, 1989). Consequently, plant uptake and toxicity can only be related to the total zinc concentration in the soil if these parameters are taken into consideration. Several extraction solutions have been proposed to assess the bioavailability of heavy metals to plants (Salomons and Förstner, 1984). The best relations are found with extractions using water or unbuffered solutions of salts, like $CaCl_2$, KNO_3 and NH_4NO_3 (Pierzynski and Schwab, 1993; Houba *et al.*, 1996; Lebourg *et al.*, 1996). Apart from the soil characteristics, uptake of zinc can also be influenced by climatic factors such as temperature, humidity and light intensity. Obviously, the uptake of zinc by plants can only be related to the zinc concentration in the soil when these factors are also taken into consideration.

The aims of the study reported here were:

- To get an insight in the validity of standard laboratory toxicity tests performed with soil to predict the toxic effects of zinc in plants under field conditions.
- To investigate whether the toxicological effects of zinc can be explained on the basis of the internal zinc concentration.

Special attention was given to chemical extraction techniques to determine the bioavailable zinc concentration independent of soil characteristics. With respect to the objectives of the Validation project as a whole, the study presented here focused on the representativity of laboratory toxicity data for the field situation (aspect 1, see Chapter 1).

Initially, *Trifolium pratense, Lolium perenne, Vicia sativa* and *Sinapis alba*, were tested on their sensitivity for zinc in Budel soil. These species were selected from the OECD guideline 208 (OECD, 1984b) on the basis of their representativity for wild plants growing on the tested soils. *T. pratense* appeared to be the most sensitive species and was therefore selected for the comparison between the effects of zinc under field and laboratory conditions. The test parameters in these toxicity experiments were germination and shoot growth. After this, the effects of zinc on *T. pratense* were investigated using three different soil types: Panheel and Budel natural soils and OECD artificial soil. Both experiments in experimental field plots on the campus of the Vrije Universiteit in Amsterdam and laboratory tests with freshly contaminated soil were conducted. In addition to this, laboratory tests were carried out in 1995 and 1996 with soil transferred from the experimental field plots to the laboratory, in order to improve comparison between the laboratory and field experiments.

The laboratory tests were carried out in a temperature-controlled room under a light-dark regime of 16 h light-8 h dark. A number of 10 seeds were sown in soil in plastic pots at a depth of ca. 1 cm in each replicate. The emergence of seedlings was recorded and in each pot five seedlings were allowed to grow further; the remaining seedlings were removed. These seedlings were selected from the first to germinate; healthy-looking individuals were chosen, not growing too close together.

The field experiments in the experimental field plots were started on July 14th, 1994, on July 13th, 1995 and on April 29th, 1996. PVC tubes were placed in each compartment up to a depth of ca. 25 cm. In each tube a number of 15 seeds was sown at a depth of ca. 1 cm. The tubes were covered with glass petri dishes until a reasonable number of seeds had germinated. Ultimately 5 or 6 plants were allowed to grow further, the others were removed. During both laboratory and field tests, the visual appearance of the plants was assessed in comparison with the controls. The number of surviving plants was recorded. All surviving plants from the laboratory and field tests were harvested after 24 and 41 or 42 days, respectively and the wet weight of the shoots was immediately determined individually. The roots were cleaned and pooled per class of nominal zinc concentration before weighing. In 1994 and 1996 a part of the shoots and roots was used for chemical analysis of the internal zinc content.

3.2 Results

3.2.1 The influence of soil type on zinc toxicity determined in laboratory tests

The laboratory tests with Budel gradient soils showed that *T. pratense* is more sensitive to zinc and cadmium than *Vicia faba* and *Lolium perenne.* For this reason all the experiments were performed with the former species. *Sinapis alba* was not suitable as test species, because its growth appeared to be highly dependent on the pH value of the soil. At pH 7.6, the optimum pH value for the growth of this plant species, there was no correlation with the zinc gradient in the Budel soils; this is most likely caused by a strongly decreased bioavailability of zinc at this high pH (for more detailed information the reader is referred to Notenboom and Posthuma (1994, 1995)).

Comparison of the tests carried out with freshly contaminated Panheel, Budel and OECD artificial soils shows considerable differences in effect values for both shoots (Table 3.1) and roots (Table 3.2). The EC50s for the effects of zinc on shoot growth expressed in external total soil concentrations in Panheel soil had ca. half the value of those in the freshly contaminated Budel soil; those in the Budel gradient soil were ca. three times higher than in the freshly contaminated Budel soil. These differences were less pronounced for both shoots and roots when the effects were expressed on the basis of internal zinc concentrations (Table 3.1 and Table 3.2).

Table 3.1. EC50s for the effect of zinc on shoot growth of *Trifolium pratense* estimated for different soil types under laboratory or field conditions expressed on the basis of external total soil concentrations and internal zinc concentrations in the shoots

Soil type	External EC50 mg/kg dw soil	Internal EC50 mg/kg fw plant shoot
Panheel soil (Lab 1994)*	76[a]	1060
Panheel soil (Field 1994)	117	200
Panheel soil (Field 1996)	526	569
Budel soil, spiked (Lab 1994)*	131	1600
Budel gradient soil (Lab 1994)	347	1460
OECD soil (Lab 1994)*	356[a]	1020

[a] Expressed as nominal zinc concentration; * Freshly contaminated

Table 3.2. EC50s for the effect of zinc on root growth of *Trifolium pratense* estimated for different soil types under laboratory or field conditions expressed on the basis of external total soil concentrations and internal zinc concentrations in the roots (nd = not determined).

Soil type	External EC50 mg/kg dw soil	Internal EC50 mg/kg fw plant root
Panheel soil (Lab 1994)*	53[a]	6600
Panheel soil (Field 1994)	nd	nd
Panheel soil (Field 1996)	nd	nd
Budel soil, spiked (Lab 1994)*	68	3200
Budel gradient soil (Lab 1994)	347	3700
OECD soil (Lab 1994)*	276[a]	5300

[a] Expressed as nominal zinc concentration; * Freshly contaminated

Root growth appeared to be more sensitive to zinc than shoot growth when external concentrations in the three tested soils were considered. In compliance with this phenomenon, the accumulation of zinc in the roots was higher than in the shoots, which difference was increasing with the external concentrations (Van der Hoeven and Henzen, 1994b). Consequently, based on internal concentrations, root growth appears to be less sensitive to zinc than shoot growth.

The growth of roots and shoots in the Budel gradient soils was significantly reduced within a distance of 5 km from the zinc factory. The growth response correlated well with the 0.01 M $CaCl_2$ exchangeable zinc fraction (Figure 3.1). The EC50 value for shoot growth in the Budel

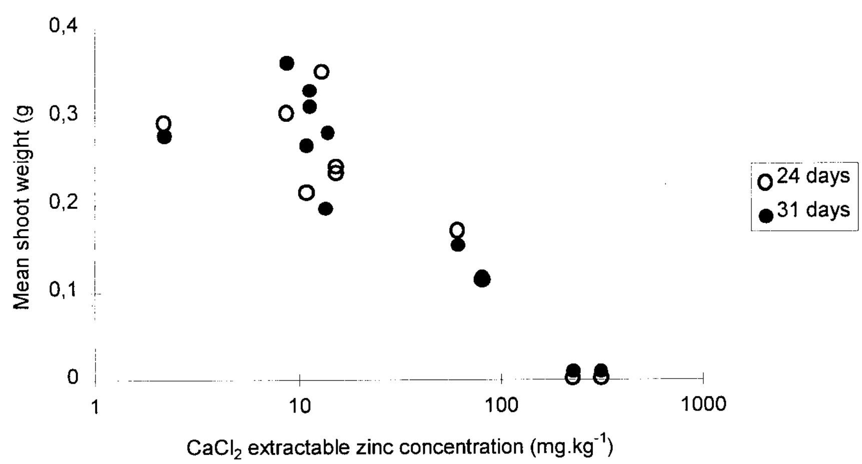


Figure 3.1. Mean shoot weight of *Trifolium pratense* after 24 and 31 days of growth on Budel gradient soils with different $CaCl_2$ extractable zinc concentrations (after Van der Hoeven and Henzen, 1994b).

gradient soil was better comparable with the value found in freshly contaminated clean Budel soil (121 mg/kg and 144 mg/kg respectively) when based on $CaCl_2$ exchangeable concentrations than when based on total concentrations (data not shown). Root growth appeared to be ca. twice as sensitive to zinc as shoot growth, but just as in the case of shoots, there were no differences between the Budel gradient and the freshly contaminated Budel soil when effects were based on $CaCl_2$ exchangeable concentrations. Comparison between the Budel gradient soil and freshly contaminated Budel soil on the basis of the total amount of zinc, showed that the EC50 values for both shoot growth and root growth were twice as high in the gradient soils than in the freshly contaminated soils. For more detailed information, the reader is referred to Van der Hoeven and Henzen (1994a and b).

3.2.2 Comparison of zinc toxicity under field and laboratory conditions

- Seed germination

The first experimental field plot experiment was carried out in July 1994, during an extremely warm and dry summer. The germination success of seedlings in the field was irregular through all zinc concentrations, being very low in the tubes positioned in the sun. From the control seeds placed in the sun, the mean germination percentage was only 9%; in the more shadowed areas this value was 83%. The NOEC for growth in this experiment was estimated from the shadow group to be 271 mg/kg (based on $CaCl_2$ exchangeable concentrations, see Table 3.3). In 1995 and 1996 discrepancies in germination were not observed; germination was good and there were no adverse effects on germination at any of the zinc concentrations tested (Table 3.3). All experiments were conducted on Panheel soil.

Table 3.3. Estimated NOECs for the effect of zinc on the germination of *Trifolium pratense* based on the nominal, total, $CaCl_2$ exchangeable and water-soluble zinc concentrations under field and laboratory conditions with Panheel soil. Exposure time in the field and laboratory experiments was 41 and 25 days, respectively (nd = not determined).

Experiment	Nominal (mg/kg)	Total (mg/kg)	$CaCl_2$-exch. (mg/kg)	Water-soluble (mg/kg)
Field 1994	560	706	271	66
Field 1995	> 1000	nd	nd	nd
Field 1996	> 1000	> 858	> 103	> 7
Lab 1994*	180	nd	nd	nd
Lab 1995*	320	nd	nd	nd
Field - Lab 1995[a]	> 1000	815	13.6	158
Field - Lab 1996[a]	> 1000	> 858	> 103	> 7

a Laboratory experiment performed with soil transferred from experimental field plot; * Freshly contaminated

- Shoot Growth

Estimated NOEC and EC50 values for the effect of zinc on shoot growth based on nominal, total, $CaCl_2$ exchangeable and water-soluble zinc concentration, obtained from laboratory and field experiments are given in Table 3.4 and Table 3.5, respectively. The tests performed under laboratory conditions gave well reproducible NOECs and EC50s for shoot growth. The position of the plants in the experimental field plots and the extreme weather conditions not only had a strong effect on the emergence of the seeds but also on plant development. Particularly in 1995 plant growth was poor and irregular. Furthermore the fresh weight of the shoots decreased every year. In 1996 the fresh weight was less than 10% of that in 1994 (Figure 3.2). Reasons for this may be:

- The season in which the field test was carried out: May-June in 1996 versus July-August in 1994 and 1995.
- Differences in meteorology: A rather cool spring in 1996 versus extremely warm summers in 1994 and 1995.
- Decrease in soil nutrient concentration.

Table 3.4. NOECs for the effect of zinc on the shoot growth of *Trifolium pratense* based on nominal, total, $CaCl_2$-exchangeable and water-soluble zinc concentrations under field and laboratory conditions with Panheel soil. Exposure time in the field and laboratory experiments was 41 and 25 days, respectively (nd = not determined).

Experiment	Nominal (mg/kg)	Total (mg/kg)	$CaCl_2$-exch. (mg/kg)	Water-soluble (mg/kg)
Field 1994	32	71	15	3
Field 1995	320	340	11	7
Field 1996	100	125	1	1
Lab 1994*	32	nd	nd	nd
Lab 1995*	32	nd	nd	nd
Field - Lab 1995[a]	320	325	11	7
Field - Lab 1996[a]	320	320	9	1

[a] Laboratory experiment performed with soil transferred from experimental field plot; * Freshly contaminated

Table 3.5. EC50 for the effect of zinc on the shoot growth of *Trifolium pratense* based on nominal, total, $CaCl_2$-exchangeable and water-soluble zinc concentration under field and laboratory conditions with Panheel soil. Exposure time in the field and laboratory experiments was 41 and 25 days, respectively (nd = not determined).

Experiment	Nominal (mg/kg)	Total (mg/kg)	$CaCl_2$-exch. (mg/kg)	Water-soluble (mg/kg)
Field 1994	86	117	34	5
Field 1995	320-560	340-546	11-46	7-8
Field 1996	558	526 (445-622)	19 (13-29)	2.5
Lab 1994*	76 (64-91)	nd	nd	nd
Lab 1995*	73 (61-87)	nd	nd	nd
Field – Lab 1995[a]	711 (631-802)	680	99	13
Field – Lab 1996[a]	1050 (853-1300)	890	111	8

[a] Laboratory experiment performed with soil transferred from experimental field plot; * Freshly contaminated

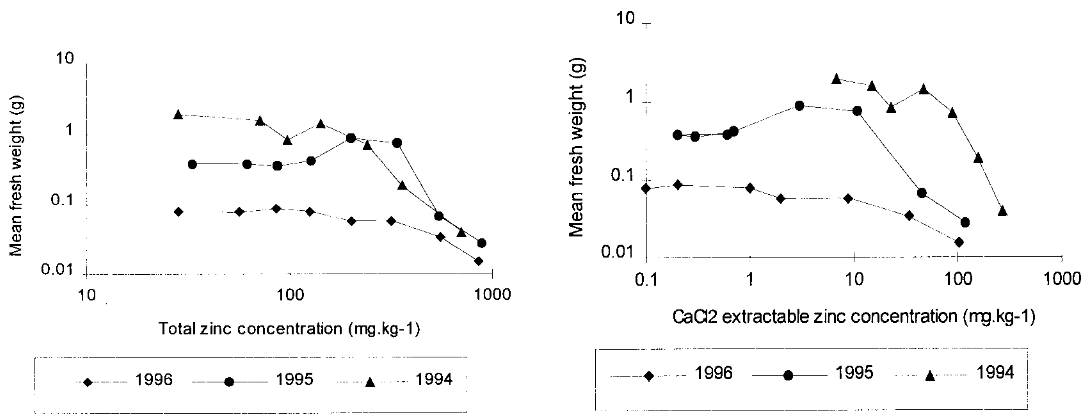


Figure 3.2. Mean fresh shoot weight of *Trifolium pratense* grown on the experimental field plots in relation to total and $CaCl_2$ extractable zinc concentrations.

The NOEC and EC50 values for shoot growth in laboratory and field tests were similar in 1994 (experiments carried out just after contaminating the Panheel soil). In 1995 the growth of the plants was irregular at all zinc concentrations, which can be attributed to the positioning of the field plots and the meteorological conditions. The variations in plant growth hamper a proper estimation of the effect values. Therefore the EC50 value for the second year can only be given as a range between a minimum and a maximum (Table 3.5). However the toxicity was clearly a factor four lower than in1994. In 1996 the toxicity was in the same range as in 1995 and it is therefore unclear whether the toxicity decreased any further or not. The reduction of toxicity of the field soil was clearly demonstrated in the laboratory tests with soil obtained from the experimental plots.

A reduction in toxicity of zinc to *T. pratense* was also demonstrated, although to a lesser extent, by Van Beelen and Notenboom (1996) in experiments with soil aged for 8, 19 and 110 weeks in the laboratory.

Expression of the effect concentrations on the basis of water-soluble and $CaCl_2$ exchangeable concentrations strongly reduces the absolute differences between the years. For the EC50 values the differences were less than a factor 5 both in the field and the laboratory experiments. In conformity, the EC50 value expressed in $CaCl_2$ exchangeable fraction, determined in the laboratory in the experimental field soil, was almost comparable with that found in freshly contaminated Budel soil, namely 99-111 versus 144 mg Zn/kg.

The EC50s expressed on the basis of the internal shoot tissue concentrations (data not shown) were quite comparable between the different soil types tested under laboratory conditions. By contrast the internal effect concentration in the field experiment of 1994 was almost 5 times lower. However, the absolute growth in the field experiment was also higher to more or less the same extent. In 1996 the internal effect concentrations in the field experiment was only two times lower.

3.3 Discussion

The results of the present plant studies indicate that the sensitivity for zinc based on the external zinc concentration was in the order germination < shoot growth < root growth. The relatively low metal sensitivity of germination is probably due to the low permeability of the seed coat for heavy metals. The relatively high sensitivity of root growth is misleading since based on the internal effect concentrations roots are actually more resistant against zinc than the upper parts. However, due to high zinc accumulation in the roots, inhibitory effects occur at lower external zinc concentrations. A higher zinc accumulation in the roots in comparison to the shoots is a common phenomenon (Lin *et al.*, 1996; Harmens *et al.*, 1993; Wheeler and Power, 1995), although a uniform distribution has also been observed (Wallace, 1989). In trees the distribution of zinc follows the pattern roots>foliage> branches>bowles (Lin *et al.*, 1996).
The effect concentrations for *T. pratense*, based on the total external zinc concentration, are within the range of values found for the wild species grey narrow-leaved plantain (*Plantago lanceolata*), white clover (*T. repens*) and perennial ryegrass (*Lolium perenne*). On acid sandy soil (pH 4.2; 9.5 mg Zn/kg) amended with zinc for 6, 8 and 4 weeks, the extrapolated EC50s for the effect on shoot inhibition were 800, 500-600 and >1000 mg Zn/kg dry soil for these three species, respectively (Dijkshoorn *et al.*, 1979).
The results of both the field and laboratory studies clearly demonstrated that ageing of the soil decreased the toxicity of zinc for plants. This can partly be explained by leaching of the readily soluble zinc after the first rain period (see Chapter 2). Since the total effect concentrations decreased as well, the retention of zinc by soil must also have had increased. The latter has also been observed in several other studies (Juste and Meant, 1992; Henry and Harrison, 1992). This common phenomenon is in agreement with the stronger sorption of zinc to soil particles reported in Chapter 2. As a consequence of the effect of leaching and ageing, the toxicity of zinc in plants can not be based on nominal and total zinc concentrations in the soil. Alternatively, the water-soluble and $CaCl_2$ exchangeable fraction seem to correlate well with the zinc toxicity, both being independent of soil type and age of contamination. In accordance with findings of Lebourg *et al.* (1996) and Novozamsky *et al.* (1993) this indicates that unbuffered salt solutions (e.g. $CaCl_2$, $NaNO_3$ and NH_4NO_3) are the most suitable extractants to estimate the mobility and the bioavailability of trace elements for plants and to assess the toxicological risks. Soil analyses for effect estimation, however, still remain dubious, especially in the case of plants, because no extraction technique can absolutely mimic the interaction between the plant and the rhizosphere.
An important factor which should also be taken into account when assessing metal phytotoxicity is the buffer capacity of the soil. If this is low the soil will be relatively sensitive to acidification by the plants. Since the uptake of zinc significantly increases at a low pH (Jones and Burgess, 1984; Chaney *et al.*, 1987), the phytotoxicity will be higher than in soils with a

high buffer capacity. This is one of the major reasons why plants living on metal enriched soils not automatically have to be metal tolerant (Verkleij and Schat, 1990).
According to Macnicol and Beckett (1989) some of these problems can be avoided by the use of internal tissue concentrations since these depend on the amount which is actually taken up by the plants. In our studies the internal effect concentrations (EC50s) were indeed almost the same on the different soil types. The EC50s derived from the field experiments, however, were in all cases significantly lower than those estimated for plants growing under laboratory conditions. A good comparison between the field en laboratory experiments, however, is not possible because the exposure times were not identical. This in fact also applies to the other effect concentrations. According to the law of Haber the effect concentration decreases in time, which indeed coincides with the lower effect concentrations estimated for the field experiments. Nevertheless, the EC10 based on dry weight estimated in the field experiment of 1996 is in agreement with critical leaf tissue concentrations affecting growth in most plant species, which are reported to range from 200 to 300 mg Zn/kg dw (Macnicol and Beckett, 1989; Balsberg Påhlsson, 1989).
The use of critical tissue concentrations is open to discussion. It can be argued that the total tissue concentration bears little toxicological significance, because most of the metal is stored in a physiologically inactive form (e.g. phytate or silicate complexes in the case of zinc) in the vacuoles. In addition, the developmental state should be taken into account. It is known that around the generative state nutrient uptake decreases, due to a decreased carbohydrate supply to the roots, resulting in a sharp decline of the mineral nutrient content in the vegetative parts (Marschner, 1986). This problem is also recognised by Macnicol and Beckett (1989), who additionally put forward that also the different organs of the plant can have a differential sensitivity to zinc. Therefore, the critical tissue concentration can only be used within a certain defined (short) exposure time, using very young plants, and preferably by sampling only a number of defined leaves.
In retrospect, the experimental design of the experimental field plots and thus the field experiments were not really suitable to perform plant toxicity tests. In particular the position towards the sun had a large influence on the emergence of the seeds and on plant development, which on its turn strongly hindered the estimation of effect concentrations. It would also have been necessary to have more knowledge about the growth curve of *T. pratense*. It can be excluded that the control plants already exceeded the exponential growth phase. In that case the differences with the zinc treated plants will decline with time, resulting in an underestimation of the effect concentrations. This, however, is probably only to be expected in the first year of the field experiments when high growth rates were recorded.
To overcome the problems mentioned above we recommend to assess the phytotoxicity of field soil by transferring the field soil to the laboratory and to carry out standardised laboratory plant growth tests with an exposure duration which is strictly limited to the (exponential) vegetative phase.

3.4 Summary and conclusions

As part of the Validation project, phytotoxicity tests with zinc were carried out using different types of soils to compare the effects on plants under laboratory and field conditions and to investigate whether these effects can be explained on basis of the internal zinc concentrations in the plant shoots or roots. In these experiments specific attention was given to the relation between observed toxic effects and zinc concentrations in the soil as obtained by different extraction methods. Because preliminary experiments with four wild plant species showed that *Trifolium pratense* was the species most sensitive to heavy metals, both field and laboratory experiments were carried out with this species.
The following conclusions can be drawn:

1. In 1994 and 1995 the germination of plant seeds and the plant growth were too heavily influenced by climatic factors to obtain reliable test results, which strongly hampered the correlation of field and laboratory experiments.
2. Standard laboratory tests with field soil seem to be the best and most reliable method to assess toxicity of contaminated field soils.
3. Both in experimental field plots and standard laboratory toxicity tests with field soil the bioavailability of zinc to plants decreases during long term residence in soil.
4. The zinc concentration as determined by 0.01 M $CaCl_2$ extraction seems to be most suitable to predict the phytotoxicity of zinc in soil, independent of soil type and age of contamination.
5. Plant tissue concentrations look promising to assess phytotoxicity.

4. FIELD RELEVANCE OF THE *FOLSOMIA CANDIDA* SOIL TOXICITY TEST

C.E. Smit and C.A.M. van Gestel

4.1 Introduction

This chapter gives an overview of the research which was conducted with the springtail *Folsomia candida* (Willem) within the framework of the Validation project. *F. candida* is a parthenogenetic springtail species with a relatively short development time. Because of its mode of reproduction, wide distribution and the ease with which the animals can be cultured, it has proved to be a suitable organism for experimental research (Usher and Stoneman, 1977). An international draft guideline for the assessment of effects of soil pollutants on *F. candida* is under development by the International Standardization Organization (ISO, 1994).

This part of the project was focused on the first aspect of validation mentioned in Chapter 1 of this report, concerning the representativity of laboratory toxicity data for the field situation. An experimental approach was followed in which laboratory and semi-field studies were conducted to gain insight into the effects of zinc on *F. candida* in relation to (a)biotic factors. Research was focused on the following questions:

- Which (a)biotic factors influence the toxicity of zinc for *F. candida* ?
- Do these factors cause a difference between laboratory toxicity data and effects observed in the field ?
- Is it necessary and possible to establish a laboratory to field correction factor for the extrapolation of laboratory data to the field situation ?

From the issues summarised in Chapter 1, bioavailability and exposure conditions were identified as the factors most likely to determine the field relevance of this toxicity test. The experiments were therefore focused on these themes. The ISO draft guideline mentioned before was used as a starting point for the research. In this test, juvenile *F. candida* are incubated in small pots containing contaminated soil. After 4 weeks of incubation, water is added to the soil to allow the remaining animals and their offspring to float to the surface. The water surface is photographed, after which the animals are counted on the projected slides. Adult animals can be collected from the water surface for the determination of weight and for chemical analysis.

The test was applied to different zinc contaminated soils to investigate the influence of soil characteristics on the bioavailability and toxicity of zinc. The influence of food supply and temperature on zinc toxicity was determined to investigate the importance of test conditions as modifying factors and outdoor toxicity experiments were conducted in the experimental field plot at the VU university campus (see Chapter 2). Soil toxicity tests generally do not allow for a close investigation of the mechanisms which underly the observed effects. Therefore an additional study was performed in which the effects of zinc were determined after exposure of individual animals to zinc contaminated food. Data from this experiment were used to estimate the effects of zinc exposure on population development of *F. candida*.

This chapter summarises the main results of the research on *F. candida*. For a detailed description of this part of the Validation project, the reader is referred to the original publications (see a.o. Smit, 1997); appropriate references are indicated in the text.

4.2 Results

4.2.1 Bioavailability of zinc for Folsomia candida

To evaluate the importance of soil chemical factors and chemical application form on the ecotoxicity of zinc, the toxicity of zinc in artificially contaminated soils was compared with effects in soil collected from the Budel pollution gradient (Smit and Van Gestel, 1996). Growth and reproduction of *F. candida* were not affected in the Budel gradient soils. Even in the most contaminated samples, where zinc concentrations exceeded the EC_{50} observed in artificially contaminated soils by a factor of 4 to 9, reproduction was not significantly different from the control.

To further investigate the influence of soil characteristics and contamination history on the bioavailability and toxicity of zinc for *F. candida*, the effects of zinc were determined in four test soils, which differed in soil characteristics and treatment after contamination (Smit and Van Gestel, 1998a). The soils used were 1) OECD artificial soil, freshly contaminated with $ZnCl_2$, 2) control soil from the experimental field plot, freshly contaminated with $ZnCl_2$, 3) control soil from the experimental field plot, percolated with water after contamination with $ZnCl_2$ and 4) field plot soil which after contamination was subjected to ageing under outdoor conditions for one and a half years. Uptake and effects of zinc were related to total, $CaCl_2$ exchangeable, water-soluble and pore water zinc concentrations, and to internal concentrations in the animals.

Figure 4.1 shows the concentration-effect relationships for the effect of zinc on reproduction of *F. candida* in the 4 test soils, based on total zinc concentrations in the soil. Effects were most pronounced in the freshly contaminated OECD and experimental field plot soils, while reproduction in the test field soil was affected only at the highest zinc concentration. Ageing of zinc contamination could be partly simulated by percolating freshly contaminated soil with water before use in the toxicity experiment. Differences in toxicity between soils were reduced when water-soluble zinc concentrations were used to estimate effect concentrations (Figure 4.2), especially in the experimental field plot soil.

EC_{50} values for the effect of zinc on reproduction of *F. candida* are summarised in Table 4.1 for all experiments which were performed within the framework of the Validation project. Average effect concentrations are given for all soils, soils which were contaminated immediately before use in the experiments and soils which were percolated with water or were aged in advance. As a measure of variation, the coefficient of variation was calculated by the expression of the standard deviation as a percentage of the average EC_{50}. Effect concentrations for freshly contaminated soil (1-7) compared very well to each other when differences in organic matter and clay content were accounted for using the soil type correction which has been developed within the Netherlands soil protection policy (see Chapter 2). However, the use of these soils

Table 4.1 – EC_{50} values for the effect of zinc on reproduction of *Folsomia candida* in different test soils. Effect concentrations are based on zinc concentrations in soil and animals. Table includes the calculated EC_{50} according to the soil type correction for 'standard' soil. Average EC_{50}s and coefficients of variation (CV) are given for all soils (1-10), soils which were contaminated immediately before use in the experiments (1-7) and soils which were aged or percolated with water in advance (8-10).

nr.	test soil	soil type	OM	clay	temp.	EC_{50} (mg Zn/kg dry soil or mg Zn/kg dry weight)						*reference*
			%	%	°C	total	total st.*	$CaCl_2$ exch.	water sol.	pore water	internal	
1	OECD	artificial	10	14	18	473	619	62.2	11.9	10.7	150	*[1]*
2	OECD	artificial	10	14	20	626	819	-	8.00	-	96.5	*[2]*
3	PANH	sand	2.4	1.9	19	261	616	125	20.5	29.7	146	*[1]*
4	PANH	sand	2.4	1.9	18	184	434	99.8	19.2	-	147	*[3]*
5	PANH	sand	2.4	1.9	18	266	628	83.1	12.6	24.4	-	*[4]*
6	Budel	sand	3.0	1.4	20	185	442	48.6	2.55	-	-	*[5]*
7	LUFA	sand	3.3	2.9	20	348	751	64.7	10.1	-	-	*[5]*
8	PANH-perc	sand	2.4	1.9	18	534	1261	297	27.6	19.5	347	*[1]*
9	PANH-aged	sand	2.0	2.9	18	2178	4942	332	22.1	6.15	328	*[1]*
10	PANH-aged	sand	2.0	2.9	18	1705	3869	268#	19.3	-	-	*[6]*
	average nr. 1-10					676	1438	153	15.4	18.1	202	
	CV (%)					102	111	113	49	53	53	
	average nr. 1-7					335	616	80.6	12.1	21.6	135	
	CV (%)					49	23	35	51	45	19	
	average nr. 8-10					1472	3366	299	23.1	12.8	340	
	CV (%)					57	56	11	19	74	3	

*: total st.: EC_{50} normalized to a 'standard' soil containing 25 % clay and 10 % organic matter. #: unpublished result . References: [1] Smit and Van Gestel (1998b) [2] Van Gestel and Hensbergen (1997); [3] Smit *et al.* (1998a); [4] Smit and Van Gestel (1997); [5] Smit and Van Gestel (1996); [6] Smit and Van Gestel (1995)

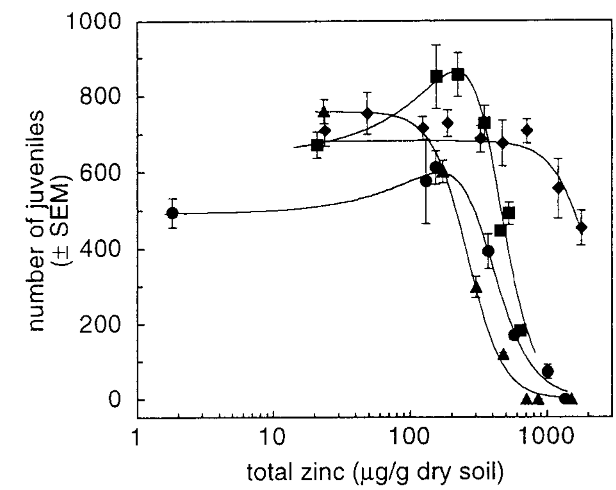


Figure 4.1

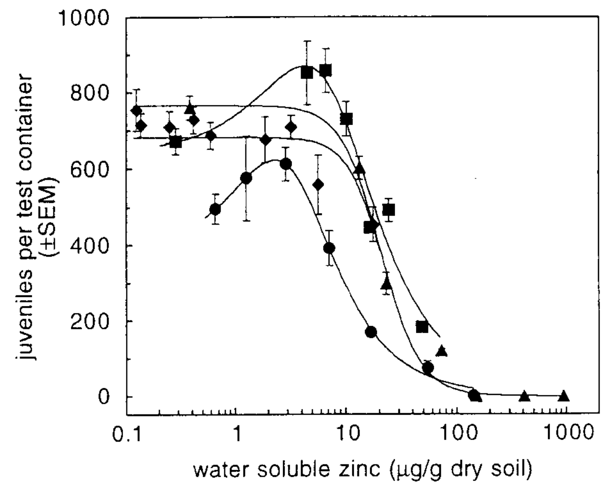


Figure 4.2

Figure 4.1 - Concentration effect relationships for the effect of zinc on the reproduction of *Folsomia candida* based on total zinc concentrations after 4 weeks of incubation in OECD (●), experimental field plot soil (fresh) (▲), experimental field plot soil (percolated) (■) and experimental field plot soil (1.5 years aged) (◆). Solid lines represent the curve-fit according to a logistic model (Haanstra *et al.*, 1985 or Van Ewijk and Hoekstra, 1993).

Figure 4.2 - Concentration effect relationships for the effect of zinc on the reproduction of *Folsomia candida* based on water-soluble zinc concentrations after 4 weeks of incubation in OECD (●), field plot soil (fresh) (▲), field plot soil (percolated) (■) and field plot soil (1.5 years aged) (◆). Solid lines represent the curve-fit according to a logistic model (Haanstra *et al.*, 1985 or Van Ewijk and Hoekstra, 1993).

overestimated the effects of zinc for *F. candida* by a factor of 5 to 8 compared to the test field soil. The enhanced toxicity of zinc in freshly contaminated soils remained present when effect concentrations were expressed on the basis of internal zinc concentrations in the Collembola. Expression of the EC_{50} on the basis of available concentrations reduced the differences between soils and, as was expected from the observation that accumulation was correlated with water-soluble concentrations, variation was least for effect concentrations based on water-soluble zinc concentrations, especially for the experimental field plot soil.

4.2.2 Interaction of zinc and cadmium toxicity

The effects of simultaneous exposure to zinc and cadmium on survival, growth and reproduction of *F. candida* were determined after 2, 4 and 6 weeks of exposure in OECD artificial soil. The distribution of zinc and cadmium over the solid and aqueous soil fraction was studied to gain insight into the way these metals influence each others sorption and bioavailability (Van Gestel and Hensbergen, 1997).

Table 4.2 - EC_{50} values for the effect of mixtures of cadmium and zinc (added as chloride salts) on the growth (fresh weight and dry weight) and reproduction of *Folsomia candida* after 2, 4 and 6 weeks of exposure in artificial soil. EC_{50} values are expressed as Toxic Units (TU) and based on total and water-soluble metal concentrations in the soil and internal metal concentrations in the animals.

parameter	time (wk)	EC_{50} (95% confidence interval) in Toxic Units based on total conc.	water-soluble conc.	internal conc.
fresh weight	2	1.00 (0.86-1.16)	0.11 (0.00017-72.0)	1.44 (0.98-2.13)
	4	1.25 (1.16-1.36)#	2.53 (1.55-4.15)@#	2.03 (1.72-2.39)*
	6	1.46 (1.33-1.60)@	8.03 (5.19-12.4)*	3.53 (2.89-4.31)*
dry weight	2	0.77 (0.64-0.94)	0.16 (0.0017-16)	1.42 (1.02-1.96)
	4	1.43 (1.31-1.56)*	2.99 (1.75-5.11)*	2.27 (1.86-2.78)*
	6	1.59 (1.40-1.81)@	7.38 (3.41-16.0)*	3.06 (1.83-5.10)@
reproduction	4	0.68 (0.46-1.02)@	-	1.11 (0.43-2.87)
	6	1.11 (0.94-1.32)	0.56 (0.11-2.75)	1.53 (1.12-2.08)#

* EC_{50} significantly different from unity ($p<0.05$)

@ EC_{50} significantly different from unity ($p<0.05$), but slope of dose-response relationships for cadmium and zinc not similar

\# EC_{50} significantly different from unity ($p<0.05$), but slope of dose-response relationships for single metals and mixture not similar

Water solubility of cadmium was increased significantly by the addition of zinc in concentrations >400 mg/kg, whereas 1600 mg/kg cadmium did not affect the water solubility of zinc (Figure 4.3). No interactions of zinc and cadmium were observed regarding uptake of metals in the animals. Table 4.2 shows EC_{50}s for the effect of mixtures of zinc and cadmium on growth and reproduction expressed on the basis of toxic units. Although a proper assessment of mixture effects was sometimes hampered by the non-similarity of the individual concentration effect relationships, it was concluded that in general the effects of zinc and cadmium on growth of *F. candida* are antagonistic. The effects of zinc and cadmium were concentration-additive when reproduction was considered. Expression of effect concentrations on the basis of water-soluble concentrations or internal concentrations in the animals led to similar conclusions.

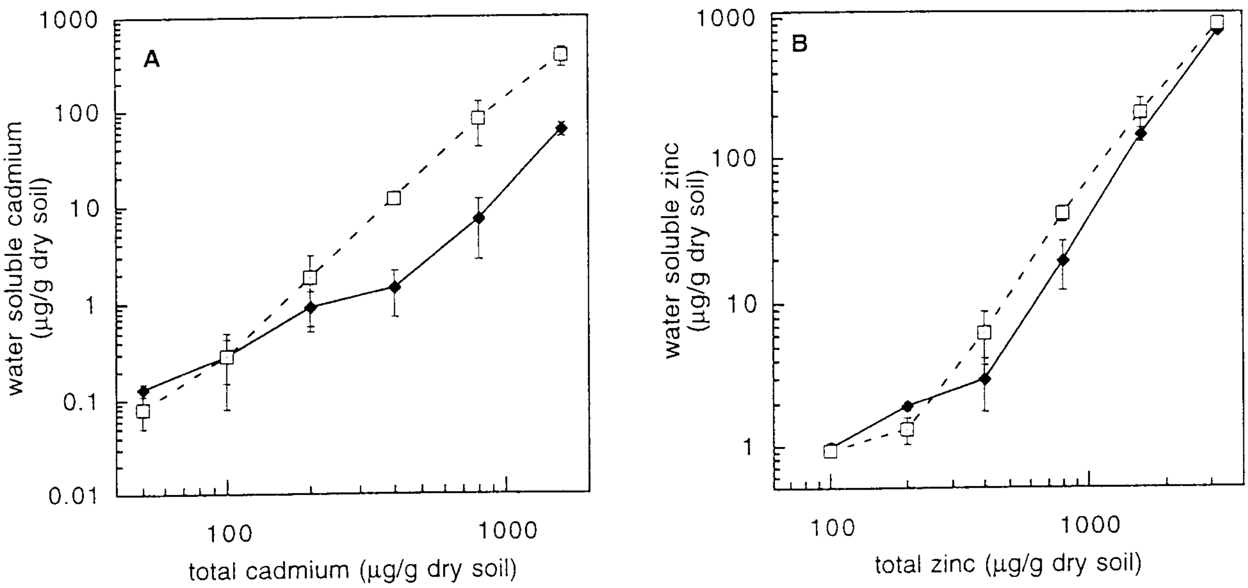


Figure 4.3 - Water-soluble concentrations of cadmium (A) and zinc (B) in the artificial soil used in toxicity tests with *Folsomia candida*, after application of the single metal (◆) or a combination of both metals (□) (error bars indicate standard deviation; n=2).

4.2.3 Influence of test conditions on zinc toxicity

Experiments were conducted to investigate whether toxicity of zinc for *F. candida* is modified when animals are exposed under suboptimal or fluctuating test conditions. The influence of food supply on zinc toxicity was investigated by incubation of animals in zinc contaminated experimental field plot soil with four different food regimes (Smit *et al.*, 1998b). The first group was fed dried baker's yeast which was supplied on top of the soil, for the second treatment, yeast was mixed homogeneously in with the soil, the third group of animals received pollen grains for food, and for the last treatment, no additional food was supplied during the toxicity experiment.

Animals that received yeast on top of the soil were less able to regulate the internal zinc concentration and were most sensitive to zinc when growth or reproduction were considered. Reproduction was affected when animals were still able to regulate the internal zinc concentration. Effect concentrations were not significantly different from the pollen treatment. Reducing the availability of food had a drastic effect on control growth and reproduction. Toxicity of zinc was, however, not enhanced by the absence of readily available food of good quality. The results of this experiment indicate that both food shortage and zinc stress have a major influence on population development of *F. candida*, the interaction between food supply and zinc toxicity is small.

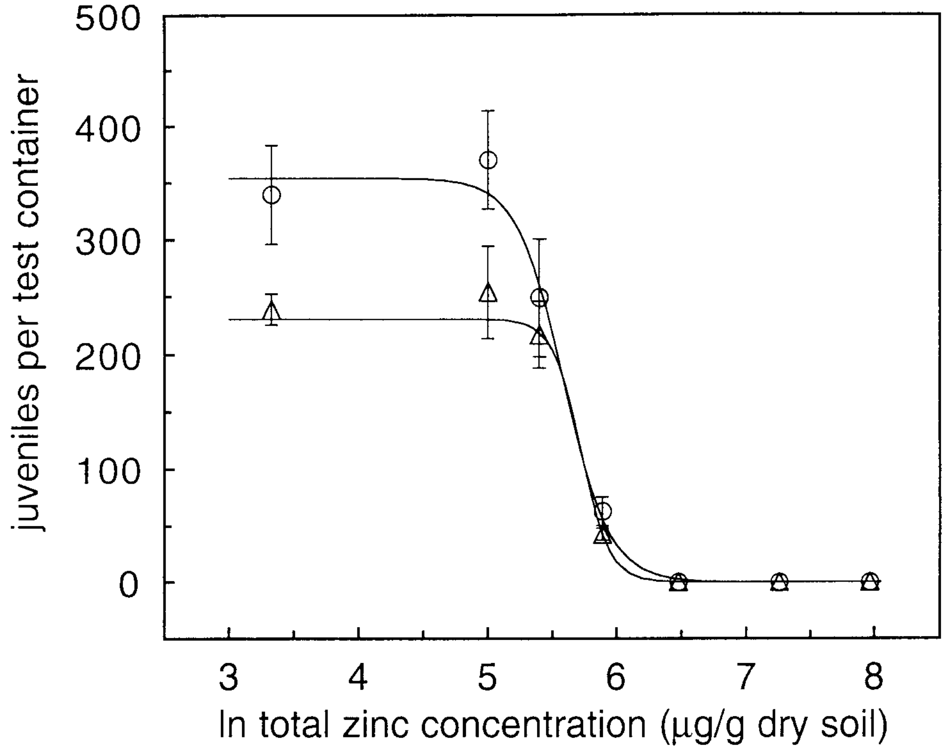


Figure 4.4 - Concentration-effect relationships for the effect of zinc on the reproduction of *Folsomia candida* after 4 weeks of exposure under constant (○) or alternating (Δ) temperature conditions. Error bars indicate standard error of the mean, the solid line is a curve-fit according to the logistic model (Haanstra *et al.*, 1985).

The influence of temperature on toxicity of zinc was investigated by exposing *F. candida* to zinc contaminated soil at different temperatures (Smit and Van Gestel, 1997). In one experiment, the toxicity of $ZnCl_2$ was determined at a constant temperature and under alternating temperature conditions. Although control performance of *F. candida* was influenced by the temperature treatment, effect concentrations for reproduction were not significantly different between treatments (Figure 4.4). Levels of internal zinc were higher in animals exposed to alternating conditions. A second experiment was performed to determine the sensitivity of *F. candida* to zinc at constant temperatures of 13, 16, 19 and 24 °C. LC_{50}s based on total zinc concentrations in soil decreased from 741 (690-795) at 13 °C to 580 (547-615) mg/kg dry soil at 24 °C. Sublethal toxicity of zinc increased with decreasing temperature, although significant differences in zinc toxicity were observed only between 13 and 24 °C (Figure 4.5). Internal zinc concentrations increased with decreasing temperatures and when the effect of zinc on reproduction was based on internal zinc levels, differences between temperatures were not significant.

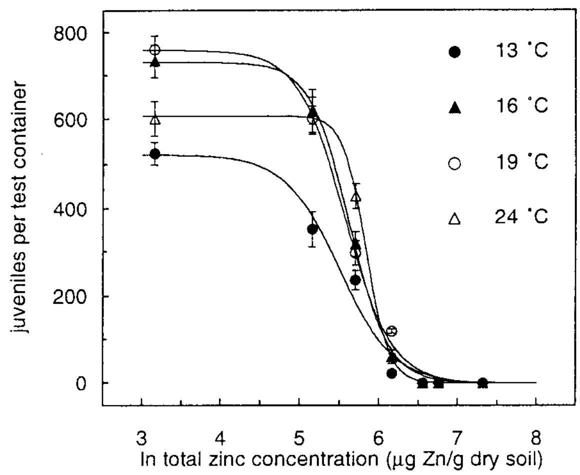


Figure 4.5. Concentration-effect relationship for the effect of zinc on the reproduction of *Folsomia candida* after exposure to zinc contaminated soil at four different temperatures. Error bars indicate standard error of the mean, the solid line is a curve-fit according to the logistic model (after Haanstra *et al.*, 1985).

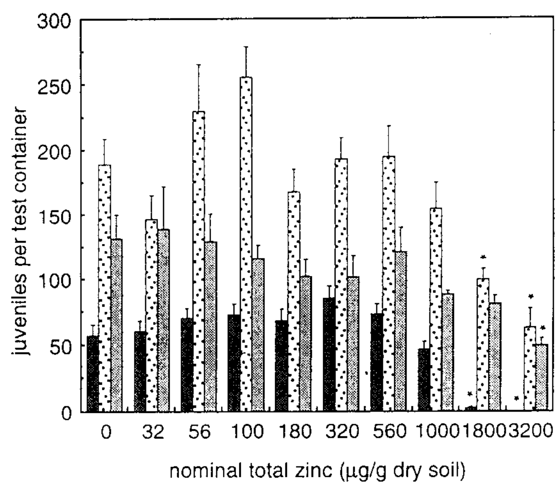


Figure 4.6 - Average number of juveniles produced per test container as a function of the nominal zinc concentration after incubation of *Folsomia candida* in artificially contaminated soil during the field experiments performed in 1994, 1995 and 1996 (left to right per concentration). Error bars indicate standard error of the mean. Asterisks indicate significant differences from control (P<0.05).

4.2.4 Toxicity of zinc under outdoor conditions

Toxicity tests were performed in the experimental field plot during the late summers of 1994, 1995 and 1996, respectively (Smit *et al.*, 1997). The first experiment in 1994 was performed using juvenile *F. candida*, whereas in 1995 one out of four plots was used to incubate adult animals. Since incubation of adults did not result in significantly different effect concentrations and control performance of the adults was higher, only adult animals were used in the last experiment performed in 1996.

Table 4.3 – EC_{50} values and 95 % confidence intervals for the effect of zinc on the reproduction of *Folsomia candida* after incubation in zinc contaminated test field soil in laboratory and field experiments during three consecutive years. Effect concentrations are based on total, $CaCl_2$ exchangeable and water-soluble zinc concentrations.

Date of experiment	lab or field experiment	EC_{50} (95 % confidence interval) total	CaCl$_2$ exch. zinc (mg/kg dry soil)	water sol.
Aug. 1994	field	979*	238*	70.2*
		(892-1074)	(177-322)	(48.3-102)
		901#	179#	13.1#
		(670-1213)	(144-222)	(-)
Dec. 1994	lab	1705	268	19.3
		(1246-2329)	(204-350)	(15.1-24.2)
Sep. 1995	field	1491	255	30.1
		(1143-1947)	(177-367)	(17.8-51.0)
Feb. 1996	lab	2178	332	22.1
		(1527-3106)	(269-368)	(14.5-33.8)
Sep. 1996	field	1749	359	20.2
		(886-3450)	(125-1033)	(1.81-225)

*: effect concentrations based on soil analysis data of August 1994 (before leaching of excess zinc)

#: effect concentrations based on soil analysis data of February 1995 (after leaching of excess zinc)

The number of juveniles is shown as a function of the nominal zinc concentration in Figure 4.6. In 1994 and 1995, a significant reduction of the number of juveniles was observed at nominal exposure concentrations of 1800 and 3200 μg Zn/g dry soil, whereas in 1996 reproduction was decreased significantly at 3200 μg Zn/g dry soil. Effect concentrations for the effect of zinc on reproduction estimated on the basis of measured total, $CaCl_2$ and water-soluble zinc concentrations are shown in Table 4.3, together with results obtained from laboratory tests performed with soil from the same field plot. The first toxicity experiment in 1994 was carried out at the time zinc concentrations in the test field plot were decreasing due to leaching of zinc

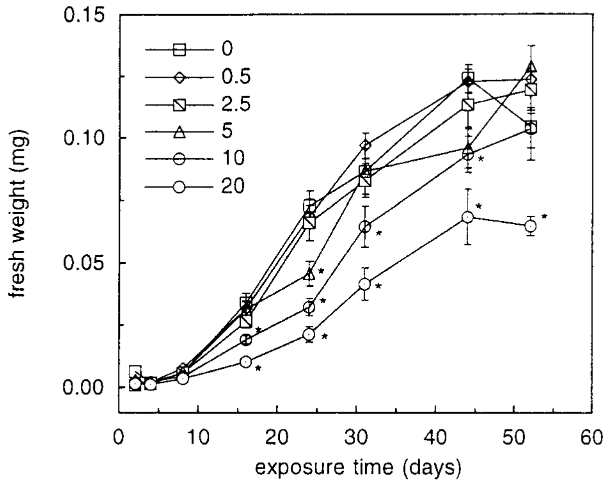


Figure 4.7 - Fresh weight of *Folsomia candida* at different exposure concentrations as a function of incubation time. Figures in legend indicate exposure concentrations in mg Zn/g dry yeast, error bars indicate standard error of the mean. Asterisks indicate significant differences from control treatment (Williams' test, $P<0.05$).

from the soil with rainwater. Therefore, soil analysis data of August 1994 and February 1995 were both used to estimate effect concentrations. It can be argued that the actual EC_{50}'s are situated in between the two estimates. Assuming that the actual EC_{50} based on water-soluble zinc for the 1994 experiment is indeed lower than the estimated value of 70 mg/kg dry soil, the EC_{50}s based on water-soluble zinc concentrations differed by less than a factor of two from effect concentrations obtained in the laboratory, although variation between replicates was substantial. EC_{50}'s based on water-soluble zinc concentrations were comparable with effect concentrations estimated for other soil types.

4.2.5 Effects of zinc on life history characteristics of Folsomia candida

Aside from studies in soil, additional experiments were conducted to investigate the processes that are responsible for the inhibition of the reproduction of *F. candida* by zinc (Smit, 1997). Animals were exposed individually to zinc contaminated food and effects on several life history characteristics were determined. Zinc caused a decrease in the body growth of the springtails, which was apparent from a decrease in the estimated final fresh weight at the highest exposure concentration (Figure 4.7). Growth retardation caused a significant extension of the juvenile period and a reduction in the number of animals reaching the reproductive state. A significant effect of zinc on the size of the first egg clutch and on the egg development time and egg viability was observed (Table 4.4). The intrinsic rate of population increase was estimated by combining laboratory toxicity data with life history information on *F. candida*. The direct effect

Table 4.4 – *Upper part* Effect of zinc on survival, the number of egg laying animals, length of the juvenile period, first clutch size, the egg development time and the average hatching success (number of juveniles as % of the number of eggs) of *Folsomia candida*. Standard deviations are given between parentheses, significant differences from control values are indicated by asterisks (Williams' test, $P<0.05$). *Lower part* EC_{10} and EC_{50} values (in mg Zn/g dry yeast) are presented together with 95 % confidence intervals for each parameter.

exposure concentration	survival	egg laying animals	juvenile period	clutch size	egg devel. time	hatching success
(mg Zn/g dry yeast)	(%)	(% of surv. animals)	(days)	(number of eggs)	(days)	(%)
0	95	89	18.5	20.8	20.1	64
			(3.66)	(11.6)	(3.30)	(38)
0.5	100	100	18.0	16.1	20.9	65
			(3.24)	(7.62)	(2.43)	(32)
2.5	90	100	19.2	18.8	21.7*	53
			(3.52)	(5.19)	(3.23)	(38)
5	80	83	18.5	18.9	22.1*	49
			(2.42)	(6.21)	(3.03)	(38)
10	95	89	20.0*	15.8*	25.1*	35*
			(3.41)	(5.36)	(4.28)	(34)
20	75	73	37.2*	11.7*	38.9*	24*
			(9.64)	(7.90)	(10.2)	(11)
EC_{10}	-	-	10.4	8.77	6.97	-
			(7.68-14.1)	(3.20-24.0)	(4.57-10.6)	
EC_{50}	-	-	20.6	25.3	23.9	13.4
			(18.3-23.1)	(13.0-49.3)	(18.9-28.9)	(4.66-38.5)

of zinc on reproduction combined with the indirect influence via growth retardation caused a 55 % reduction of the estimated intrinsic rate of population increase.

4.3 Discussion

4.3.1 Bioavailability of zinc for Folsomia candida

The importance of soil chemical factors and contamination history as demonstrated in the present study is in agreement with observations of Spurgeon and Hopkin (1995) who found that toxicity of zinc for the earthworm *Eisenia fetida* was much higher in laboratory contaminated soils than in polluted field soils. The uptake of zinc for *F. candida* could be described reasonably well using water-soluble concentrations. The same conclusion was drawn for the earthworm *E. fetida* by Spurgeon and Hopkin (1996b). This supports the hypothesis of Van Gestel *et al.* (1995) that uptake of metals by soil inhabiting soft bodied animals occurs mainly via the soil pore water fraction and indicates that bioavailability of zinc for *F. candida* is determined by the sorption equilibrium between the solid and aqueous soil phase.

The use of OECD artificial soil in toxicity experiments is sometimes criticised because the organic matter and clay type are not representative of natural soils in Europe (Marinussen, 1997; Spurgeon and Hopkin, 1995). The results of the present study show that zinc toxicity is overestimated in all laboratory contaminated soils, regardless of soil type or origin. Furthermore, effect concentrations for *F. candida* determined in OECD artificial soil compared very well to those obtained using natural soils when differences in organic matter and clay content were accounted for using a correction factor. The great discrepancy in toxicity between experimental soils and field soils, which was observed when effect concentrations were based on total zinc concentrations, is therefore assumed to result mainly from the influence of ageing on bioavailability of zinc rather than from the composition of the soil.

Ageing of zinc contamination could be partly simulated by percolating freshly contaminated soil with water before use in the toxicity experiment, thus creating a more realistic exposure situation. Another advantage of this method is that excess chloride, which is introduced by using $ZnCl_2$ as a contaminant, is removed from the soil pore water. The results of the experiments indicated that zinc toxicity for *F. candida* is enhanced by the presence of chloride. This is most likely due to a direct effect of chloride on *F. candida*, since effects of zinc appeared at lower internal zinc concentrations in the presence of chloride. The importance of anion type and concentration when using metal salts in toxicity experiments was also demonstrated for the earthworms *E. fetida* and *E. andrei* (Hartenstein *et al.*, 1981; Weltje *et al.*, 1995).

It is generally assumed that heavy metal toxicity for soil animals is determined by the amount of free metal ions present in the soil solution. A relationship between free metal ion concentrations, uptake and effects remains to be established for *F. candida*: differences in cadmium toxicity observed for *F. candida* in three different soils could not be explained by the amount of free cadmium ions present in the soil solution (Vonk *et al.*, 1996). The analytical methodology for a direct quantification of heavy metal speciation in soil has not been developed yet (Ure and Davidson, 1995). It has recently been proposed to apply speciation models in the risk assessment of heavy metals to account for differences in bioavailability between soils (De Rooij and Smits, 1997). Uptake patterns and mechanisms of toxicity in soil arthropods are species specific and metal dependent (Crommentuijn *et al.*, 1994, 1995a; Hames and Hopkin,

Table 4.5 - Effect of mixtures of cadmium and zinc (added as chloride salts) on the growth (fresh weight and dry weight) and reproduction of *Folsomia candida* after 2, 4 and 6 weeks of exposure in artificial soil at the level of EC_{10} values based on total and water-soluble metal concentrations in the soil and internal metal concentrations in the animals. The cases where the combined action at the EC_{10} level deviates from that at the EC_{50} level are indicated in bold.

	(wk)	total conc.	water-soluble conc.	internal conc.
fresh weight	2	additive	**synergistic**	additive
	4	antagonistic	**additive**	**additive**
	6	antagonistic	antagonistic	antagonistic
dry weight	2	additive	**synergistic**	additive
	4	antagonistic	**additive**	**additive**
	6	**additive**	**additive**	**additive**
reproduction	4	synergistic	-	additive
	6	additive	additive	**additive**

1991; Janssen *et al.*, 1991). It will be difficult to develop a model in which bioavailability can be related to toxicity for soil biota in general, without making gross simplifications and introducing large uncertainties. In the present study, differences in toxicity between soils were reduced when water-soluble zinc concentrations were used to estimate effect concentrations. It is concluded that at the moment, this relatively simple extraction technique can serve as a practical tool to correct for differences in bioavailability between soils.

4.3.2 Mixture toxicity

Cadmium sorption appeared to be affected by zinc, whereas sorption of zinc was not affected by cadmium (Figure 4.1). This can be due to a strong competition of zinc for cadmium binding sites (Christensen, 1987), but more likely the increased cadmium water solubility at increased zinc concentrations can be explained from the speciation of cadmium in the presence of chloride. It is well known that cadmium may complex with chloride (Wolt, 1994), whereas chloride complexation is of minor importance for zinc (Elrashidi and O'Connor, 1982). The excess chloride added in the mixtures may have led to an increased complexation of cadmium, thus leading to an increased water solubility. Apparently, the cadmium chloride complexes are not available for uptake by the Collembola. In the mixture toxicity study, zinc and cadmium did

not affect each others uptake and cadmium accumulation was not increased by the increased water-soluble concentrations in the presence of zinc.
The dissimilar shape of the concentration-response relationships (Table 4.2) suggests that the mixture may have a different mode of action compared to the single metals. Therefore also combined effects at the EC_{10} level were evaluated (Table 4.5). Comparison of Table 4.2 and Table 4.5 shows that the mixture toxicity may differ at the EC_{10} and the EC_{50} level. There seems to be a trend towards additive effects at lower concentrations, but this trend is not as consistent as suggested for organic chemicals (Warne and Hawker, 1995).
Effects on growth occur at relatively high concentrations, whereas effect concentrations for reproduction are low, and this explains why effects of cadmium and zinc are mainly antagonistic when growth is considered as an endpoint, and mainly additive when considering reproduction.
In the literature, mainly concentration-additive or antagonistic effects of cadmium and zinc mixtures have been reported, with most studies being restricted to aquatic organisms and the few terrestrial studies found only using one or two mixture concentrations of cadmium and zinc. In the latter, decreasing effects of cadmium with increasing concentrations of zinc have been described for the terrestrial species *Acheta domesticus* and *Tenebrio molitor* (Migula *et al.*, 1989ab; Vogel, 1988).
Concentration-additivity might be a reason to apply an extrapolation factor greater than unity to single metal toxicity data, although in most field situations effects will be determined by one, dominantly present toxicant. The fact that mixture effects differ for different parameters and effect levels implies that it is not possible to decide whether mixture effects of heavy metals are underestimated or overestimated by the use of single metals in laboratory tests.

4.3.3 Influence of test conditions on zinc toxicity

As was outlined in Chapter 1, section 1.3, food limitation may modify toxicity in field situations compared to the laboratory. The results of the present study indicate that food is a major factor determining the population development of *F. candida* in uncontaminated soil. In accordance with results of Stam *et al.* (1996), control performance of *F. candida* as measured by growth and reproduction was reduced when animals were fed pollen. Reducing the availability of food had a drastic effect on control growth and reproduction, which confirms the assumption that the animals have fairly narrow tolerance limits with respect to food conditions. A number of literature data indicate that food limited animals are more susceptible to toxic stress than well fed ones (Chandini, 1988, 1989; Enserink *et al.*, 1995; Ristola, 1995; Van Gestel *et al.*, 1991). The results of the present study do not support the validity of such a general rule for *F. candida* as the toxicity of zinc for *F. candida* was not enhanced by the absence of readily available food of good quality. A similar conclusion has been drawn for the effect of dimethoate on reproduction of *F. candida* by Krogh (1995).
The role of contaminated food as a possible route of exposure to zinc was not examined in this study. Belfroid *et al.* (1995) have demonstrated that the relative importance of dietary uptake for bioaccumulation of organic contaminants in earthworms increases when bioavailability of

chemicals in the soil pore water is low. In view of this, it can be reasoned that for *F. candida* uptake of zinc from the food might contribute substantially to bioaccumulation when adsorption of zinc onto soil particles is high. Literature data indicate that *F. candida* is able to use different food sources to meet its nutritional demands. Apart from yeast and pollen as used in this study, the animals have been shown to feed on various fungal species (Klironomos *et al.*, 1992) and nematodes (Lee and Widden, 1996). It thus appears that *F. candida* is not a very selective feeder, and if the animals are able to distinguish between heavily contaminated and less contaminated food, this might enable *F. candida* to avoid exposure by switching between food types.

The influence of temperature on toxicity of zinc was more pronounced than the influence of food supply. The increased sensitivity of *F. candida* at low temperatures may be explained by the fact that the juvenile period of *F. candida*, which represents the most sensitive life stages, is extended by a decrease in temperature (Johnson and Wellington, 1980; Snider and Butcher, 1973). Another explanation can be found in an increase of accumulation at low temperatures, which was likely due to a reduction of zinc elimination. The effect of temperature on zinc toxicity was parameter dependent: where a decrease in temperature led to an increase in sensitivity based on sublethal parameters like growth and reproduction, animals were less sensitive at low temperatures when mortality was regarded as a test parameter.

Crommentuijn *et al.* (1995 b) have proposed the use of a Sublethal Sensitivity Index (SSI), defined as the ratio between the lethal and sublethal effect concentration, as a tool to evaluate species specific population-level effects of toxicant stress. High SSIs indicate a priority of survival over reproduction after toxic stress, whereas a low SSI indicates that the animal continues to reproduce until death. Calculation of the ratio between LC_{50} and EC_{10} for reproduction resulted in values of 6.4, 4.4, 5.1 and 2.4 for exposure at 13, 16, 19 and 24 °C, respectively. The differences in SSIs indicate that the strategies to cope with toxic stress are not only species specific, but are also dependent on the exposure conditions.

An influence of temperature on zinc toxicity was observed only when animals were exposed under constant conditions. Fluctuations in temperature reduced control performance of *F. candida* compared to exposure under constant conditions, but did not cause a change in zinc toxicity. This is in agreement with Demon and Eijsackers (1985), who studied the effect of two pesticides on the survival of the isopod *Philoscia muscorum* and did not find differences between exposure at constant and fluctuating temperatures. This indicates that constant temperatures form a good basis for the extrapolation of toxicity levels. Since average temperatures can alter the sensitivity of *F. candida* for zinc, and changes in sensitivity are parameter dependent, temperature is regarded as an important factor when comparing results from tests which are conducted at different, constant temperatures.

From the results of this study, it appears that the modification of zinc toxicity by test conditions depends on the size of the range of climatic and habitat conditions normally encountered by *F. candida*. When tolerance limits are small, as is the case for feeding conditions, changes in test conditions within the ecologically relevant range will have a minor influence on toxicity. This was also shown for the influence of moisture content on cadmium toxicity for *F. candida*

(Van Gestel and Van Diepen, 1997). The importance of temperature for the extrapolation of laboratory toxicity data to the field situation is assumed to be small, since an overall effect of temperature will be diminished by the considerable fluctuations in temperature which are encountered in nature.

4.3.4 Internal concentrations as a measure of risk

The adverse effects of toxicants on biota depend on the amount of toxicant present at the site of action in the organism. It is therefore assumed that the toxicokinetic behaviour of a chemical provides a better estimate of the potential hazard of a toxicant than the concentration in the environment. This concept has been used in many aquatic and terrestrial toxicity studies on organic chemicals (Belfroid *et al.*, 1995; Mackay *et al.*, 1992; McCarty *et al.*, 1992; Moriarty et al, 1984) and has been applied by Crommentuijn *et al.* (1994) to explain differences in cadmium toxicity between soil arthropod species.

Van Wensem *et al.* (1994) have proposed to compare accumulation levels measured in field animals with internal effect concentrations determined in laboratory experiments to evaluate the ecotoxicological risks of heavy metals for field populations. This approach is based on the assumption that an animal will experience negative effects at a fixed internal concentration, irrespective of the way and rate this concentration is reached (Van Wensem *et al.*, 1994). Van Straalen (1996) has argued that the use of internal concentrations in risk assessment would be restricted to non-regulated metals, since residues of essential elements, which are subjected to strong regulation, will not necessarily reflect the uptake rate or exposure concentration.

Expression of zinc toxicity on the basis of internal zinc concentrations in *F. candida* showed that effects of zinc cannot be fully explained by accumulation. This implies that the existence of a fixed internal threshold concentration of zinc above which physiological functions are impaired is not likely for *F. candida*. Effects of zinc on growth and reproduction occurred at internal concentrations which were very close to or even lower than the regulated level. The assumption of Van Tilborg and Van Assche (1996), who state that the range of external concentrations over which an organism is able to maintain a stable internal concentration represents a no-risk area, is obviously not valid for *F. candida*. A similar conclusion has been drawn by Van Gestel *et al.* (1993) when studying the effects of zinc on the earthworm *E. andrei*. To gain insight into the relationships between zinc accumulation and effects in soil organisms, a detailed study on the mechanisms of toxicity should be conducted, including investigations on uptake routes and internal distributions.

4.3.5 Toxicity of zinc under outdoor conditions

In all outdoor experiments, the number of juveniles was relatively low. This can be partly explained by the low average temperatures during the tests (13.7, 15.4 and 12.4 °C in 1994, 1995 and 1996, respectively). The development time of *F. candida* is temperature dependent (Johnson and Wellington, 1980; Snider and Butcher, 1973) and the optimum temperature for reproduction is between 15.5 and 21 °C (Snider and Butcher, 1973). At lower temperatures, animals will reproduce later and eggs will need a longer time to hatch. Another explanation may be that animals might have experienced problems reaching the food which was placed on top of the soil. In general, performance was better when experiments were started with adult animals. Apparently, juvenile animals, which have to complete several moulting cycles before reaching the reproductive state, are more susceptible to drought and other adverse conditions than adults. EC_{50}s based on total zinc concentrations were 3 to 10 times higher than effect concentrations reported from studies with freshly contaminated soil (see Table 4.1). When based on water-soluble concentrations, EC_{50}s obtained from the field experiment were in the same order of magnitude as reported for freshly contaminated soils. This supports the conclusion of Van Gestel and Van Straalen (1994) that acute field effects may be predicted from laboratory studies provided that the test species is the same and the exact exposure concentration is known.

4.3.6 Consequences of zinc exposure for population development under field conditions

The consequences of the observed life history changes for the development of a population under field conditions are yet to be established. Results from laboratory experiments with cadmium and triphenyltin hydroxide showed that effects on reproduction of *F. candida* could be compensated for by an increased life span (Crommentuijn *et al.*, 1997). Among Collembola, it may be assumed that predation and unfavourable nutritional or climate conditions result in a much shorter life span in nature compared to the laboratory, and the likelihood of a field population to recover on the long term will be reduced when maturation is delayed. The results of the present study are consistent with the observation from the soil toxicity experiments that juvenile production is the most sensitive parameter for evaluating the effects of heavy metals on *F. candida* and support the conclusion of Van Straalen *et al.* (1989) that reproduction is an ecologically relevant parameter to judge effects of toxicants on soil arthropods.

4.4 Conclusions

1. Zinc toxicity for *F. candida* was overestimated in all laboratory contaminated soils, regardless of soil type or origin. The great discrepancy in toxicity between experimental soils and field soils is assumed to result mainly from the influence of ageing on bioavailability of zinc rather than from the composition of the soil.
2. Differences in toxicity between soils were reduced when water-soluble zinc concentrations were used to estimate effect concentrations. This relatively simple extraction technique can serve as a practical tool to correct for differences in bioavailability between soils. When based on water-soluble concentrations, EC_{50}s obtained from the field experiment were in the same order of magnitude as reported for freshly contaminated soils. This supports the conclusion of Van Gestel and Van Straalen (1994) that acute field effects may be predicted from laboratory studies provided that the test species is the same and the exact exposure concentration is known.

3. In a mixture toxicity study, zinc and cadmium did not affect each others uptake and cadmium accumulation in *F. candida* was not increased by the increased water-soluble concentrations in the presence of zinc. Mixture effects differed for different parameters and effect levels, indicating that it is not possible to decide whether mixture effects of heavy metals are underestimated or overestimated by the use of single metals in laboratory tests.
4. The results of the present study indicate that food is a major factor determining the population development of *F. candida* in uncontaminated soil. The toxicity of zinc for *F. candida* was, however, not enhanced by the absence of readily available food of good quality.
5. The influence of temperature on the toxicity of zinc was more pronounced than the influence of food supply. Since average temperatures can alter the sensitivity of *F. candida* for zinc, and changes in sensitivity are parameter dependent, temperature is regarded as an important factor when comparing results from tests which are conducted at different, constant temperatures. From the results of this study, it appears that the modification of zinc toxicity by test conditions depends on the size of the range of climatic and habitat conditions normally encountered by *F. candida*. The importance of temperature for the extrapolation of laboratory toxicity data to the field situation is assumed to be small, since an overall effect of temperature will be diminished by the considerable fluctuations in temperature which are encountered in nature.
6. Expression of zinc toxicity on the basis of internal zinc concentrations in *F. candida* showed that effects of zinc cannot be fully explained by accumulation. This implies that the existence of a fixed internal threshold concentration of zinc above which physiological functions are impaired is not likely for *F. candida*.

5. FIELD RELEVANCE OF THE *EISENIA ANDREI* REPRODUCTION SOIL TOXICITY TEST

L. Posthuma and R. Baerselman

5.1 Introduction

This chapter presents a summary of the research which was conducted with the oligochaete *Eisenia andrei* within the framework of the Validation project. This species, and its close relative *E. fetida*, are widely used for determination of the (relative) toxicity of chemicals in standardized laboratory tests on survival (OECD, 1984a; EEC, 1985) and reproduction. For the latter test various experimental designs are presently used (e.g., Van Gestel *et al.,* 1989, ISO, 1993; BBA 1994). The species was chosen for standardized test purposes because of its ease of culturing and its short generation time compared to other earthworm species (Van Rhee, 1970).
The species occurs predominantly in compost heaps, or other sites rich in organic material. Within the framework of the Validation project, it is therefore unlikely that true comparisons can be made between toxic effect levels on individuals exposed under laboratory conditions and those on populations under field conditions. The purport of this chapter is to compare sensitivity of this species between different exposure conditions, while ecological relevance is addressed through comparisons with literature data on the sensitivities of other earthworm species.
The standard test protocols for *Eisenia* spec. prescribe the use of an artificial soil substrate (OECD, 1984a, see further Chapter 2). Demineralized water must be added until field capacity is reached (55% w/w). Some calcium carbonate must be added to raise the pH from originally 3 - 4 to the desired value of approximately 6.0 ± 0.5. In reproduction tests, ground cow manure is added as food, either as a core in the soil or on the soil surface.
The research conducted with *E. andrei* focused on the first aspect of the project mentioned in Chapter 1 of this report, viz. on the representativity of laboratory toxicity data for the field situation. Species characteristics of *E. andrei* render the species unsuitable for studying population effects in field conditions. Various experiments were conducted to elucidate the representativity question. The experiments were designed on the basis of the reproduction test protocol developed at RIVM (Van Gestel *et al.,* 1989). Reproduction, as influenced by metals (alone or in mixtures), was studied in different soil types, and under different conditions. Reproduction of *E. andrei* is, however, severely hampered under acidic conditions (pH < 5, Van Gestel *et al.,* 1992a). Therefore, acidity of the soils used in the experiments was set to the pH-value prescribed for the artificial soil substrate. In addressing the representativity question, emphasis was put on measuring exposure concentrations (both out- and inside the animals), and on the quantification of toxic effects on various individual endpoints, viz. reproduction, growth and survival. Toxicity data are summarized by EC50 values to allow for quantification of effect-level divergence among experimental conditions. Other effect concentrations (such as EC10) are not given, due to statistical uncertainties associated to estimate low-effect parameters. The

toxicity data are compared to literature data to analyse the possible causes of divergence, to shed light on future developments and to formulate recommendations.
The experiments with *E. andrei* were mainly addressing the following themes:

- bioavailability
- effects of variable environmental conditions
- mixture effects.

The data have partly been published elsewhere (see: Posthuma *et al.,* 1994, Weltje *et al.,* 1995, Posthuma and Notenboom, 1996); the original publications contain detailed information. Concurrent with the present work, Spurgeon and colleagues performed a series of studies that addressed similar themes, with the species *E. fetida*. Results of our work are therefore primarily verified by comparisons to Spurgeon's data.

5.2 Results

5.2.1 Overview of experiments and results

In line with the three aims of the work with *E. andrei*, a pilot experiment was initially performed to determine whether differences in sensitivity existed between the standardized reproduction test and the same test in a contaminated field soil (pH adjusted with $CaCO_3$) collected near the Budel metal factory. Both soils have highly different characteristics (absence/presence of a metal mixture, short/long contamination histories, rich/poor soil characteristics). These observations demonstrated that cocoon production in the aged and mixed-metal contaminated soil from Budel was hardly affected, although the EC50 for reproduction, determined in freshly contaminated OECD artificial soil, was four times exceeded for zinc alone (Posthuma *et al,* 1994). This magnitude is exemplary for the magnitude of differences found in the experiments performed thereafter, from which it appeared plausible that the concentration of zinc having an effect on cocoon production of *E. andrei* differed up to one order of magnitude between laboratory and pH adjusted field soil (see below, see Table 5.1).
Various experiments were performed to address the issues of bioavailability, variable environmental conditions and mixture toxicity. In those experiments, *E. andrei* was exposed to zinc alone or in mixtures, in different soils and under various exposure conditions. Except for the specified changes, test conditions and set-up were as close to the standard OECD artificial soil test as feasible. This report focuses only on cocoon production as toxicity endpoint. EC50 values were estimated with a sigmoid response model (Haanstra *et al,* 1985). The soils used were:

- OECD artificial soil, freshly contaminated with $ZnCl_2$ or $ZnNO_3$ (laboratory conditions), experimental field plot soil, contaminated with $ZnCl_2$ approx. 2 and 3 years before the test (field conditions);
- Soils collected from the gradient around the zinc factory in Budel (aged, contaminated with mixtures of pollutants, laboratory conditions).

Table 5.1. EC50 values for the effect of zinc on cocoon production of *Eisenia andrei* in different soils and under different exposure conditions. Effect concentrations-50% are based on measured concentrations in soil (solid phase, liquid phase estimates) and body. A recalculation of EC50s (total soil concentrations) into values for 'standard soil' is included, based on soil type correction factors applied in the Netherlands. Averaged EC50s and Coefficients of Variation are given for each measure where applicable.

nr.	test soil	soil type	OM	clay	temp.	pH	duration	EC50 (mg Zn/kg dry soil or mg Zn/kg dry body weight)					
			%	%	°C	(=initial/e=end)*	(weeks)	total	total st. **	$CaCl_2$ exch.	water sol.	internal***	reference
1	OECD	artificial	10	14	20	4.8(i)/5.1(e)	3	429	561	133	n.d.	n.d.	[1]
2	OECD	artificial	10	14	20	5.0 (i)	3	921	1206	172	n.d.	200 - 300	[2]
3	OECD	artificial	10	14	20	n.d.	3	659	862	n.d.	n.d.	150 - 231	[3]
4	PANH-aged (95)	sand	2	2.9	var	7.2 (i)/7.0(e)	7	1282	2909	214	33.0	307	[4]
5	PANH-aged (95)	sand	2	2.9	var	7.2 (i)/7.0(e)	8	1277	2898	219	20.6	356	[4]
6	PANH-aged (96)	sand	2	2.9	var	7.2 (i)	3	1597	3624	287	21.5	305	[4]
7	PANH-aged (96)	sand	2	2.9	var	7.2 (i)	7	1676	3803	307	23.3	312	[4]
8	Budel gradient	sand	2.4	1.4	20	4-9-6.0(i)/5.9-6.9(e)	3	1790	4336	appr. 300	n.d.	n.d.	[5]
9	Budel gradient	sand	2.4	1.4	20	5.1-5.6(i)/5.6-6.4(e)	3	2553	6184	336	n.d.	>130	[1]
							average	1354	2931	238	25		
							C.V.	48	62	31	23		

*: initial: soil pH (KCl) in control soil prior to exposure or at the end of exposure; end: *ibid.* at the end of exposure; for the pH-adjusted field soils from Budel the range of the experiment is given; unmodified field-pHs are lower..

**: total st.: EC50 normalized to a 'standard soil' containing 25% clay and 10% organic matter.

***: for internal zinc concentrations the sigmoid concentration-effect model often did not converge; in those cases an EC50 range is optically estimated from the observations.

References are: [1] Posthuma and Notenboom, 1996, [2] Weltje *et al.,* 1995, [3] Van Gestel *et al.,* 1993, [4] Bakker *et al.,* 1997., [5] Posthuma *et al.,* 1994.

Table 5.2. Toxicity of metals for *E. andrei* in OECD artificial soil (alone and in mixture) and in soils from the gradient around the Budel zinc factory (mixture), based on total metal concentrations and on pore water concentrations. Calculation of pore water EC50s in OECD artificial soil was based on the isotherm values from Chapter 2. *: based on $CaCl_2$ exchangeable fractions.

Soil	Metal(mixture)	EC50	
		mg/kg	mg/l
OECD	Zn	429	35.1
OECD	Cu	191	0.77
OECD	Cd	95.1	0.98
		TU	TU
OECD	Cd+Zn	1.7	1.4*
OECD	Cd+Cu	1.8	n.d.
OECD	Zn+Cu	2.1	n.d.
Budel	Zn/Cu/Cd	10	1.4

The pH(KCl) in the soils from the Budel gradient ranged from 2.9 to 4.9 after sampling, which caused a large effect of acidity on cocoon production. This effect was superimposed on the metal effects, rendering the latter uninterpretable (Posthuma *et al.* 1994). For experiments with *E. andrei*, pH of these soils was therefore adjusted to a value of approx. 5.5 at the beginning of exposure. This might cause a very large underestimation of the toxic effects in not-adjusted field soils, because the addition of $CaCO_3$ increased both pH and Ca^{2+} ion concentration in the pore water.

Toxicity observations were summarized through EC50 values for each experiment, using various expressions to quantify the bioavailable concentration, viz. total, 0.01 M $CaCl_2$ exchangeable and water-soluble zinc concentrations in the soil (external measures of exposure), and body concentrations of zinc (internal measure of exposure). This approach is based on the assumption that sensitivity for zinc (expressed through the EC50) should be constant, irrespective of soil or exposure conditions, when the measure of exposure is highly correlated with the true exposure concentration of *E. andrei* (given a certain constancy of the relationship between body concentration and effect).

The experiments are summarized in Table 5.1, showing both the differences in experimental conditions (causal: soil type, pH, duration of exposure) and the sensitivity differences of *E. andrei* under those conditions (response). General trends regarding sorption characteristics of metals in the soils used are given in Chapter 2, experiment-specific data on sorption are given in this chapter. Results of the mixture experiments are summarized in Table 5.2. Gross inspection of Table 5.1 shows that the conclusions of the pilot experiment are confirmed: there are sensitivity differences among experiments. To address the relative importance of bioavailability, exposure conditions and mixture toxicity in causing the observed sensitivity differences among experiments, cross-sections through these tables are made in the following paragraphs. Since various aspects necessarily differed simultaneously among experiments, the explanations cannot unequivocally be attributed to a single cause. Hence, conclusions are based on inference and plausibility.

5.2.2 *Cross-section: Bioavailability of zinc for Eisenia andrei*

Exposure of earthworms to soil contaminants is often assumed to be (at least in part) mediated by pore water transfer, for organic chemicals (Belfroid *et al.,* 1996) and metals (Van Gestel *et al.,* 1995, Spurgeon and Hopkin, 1996b). Soil characteristics that influence the partitioning of a chemical over the solid and liquid soil phases may therefore be important modulators of uptake and toxicity. In the case of metals, soil acidity is often hypothesised to be a crucial factor, next to clay and organic matter contents (Kiewiet and Ma, 1991, Janssen *et al,* 1997a,b), Van Gestel *et al.,* 1992b, Van Gestel *et al.,* 1995).

- *Zinc partitioning in different experiments.* The bioavailability of a chemical is determined by both its (environmental) supply and the (biological) demand of the exposed organism. In this paragraph, the 'supply' of zinc in the experiments with *Eisenia andrei* is summarized (see Table 5.1).

Looking at 'supply', it has already been noted that acidity variation in OECD artificial soil has a large influence on metal concentrations in the pore water (Chapter 2). Sorption characteristics of the other soils used in the experiments were summarized in the Freundlich sorption parameter *Kf*, which is the concentration-dependent ratio of solid and liquid phase concentrations. A low value of this parameter implies weak sorption to the solid phase. In the Validation project, three methods have been applied to assess metal concentrations in the pore water, viz. shaking in 0.01M $CaCl_2$ and in water, and collection of pore water through centrifugation. Although the methods have different extraction efficiencies, they aimed to mimic the metal fraction that is (potentially) available in the pore water.

Kf values based on $CaCl_2$ exchangeable concentrations were lowest in OECD artificial soil (excluding experiment 3) freshly contaminated with zinc, viz. 47 and 59 l/kg for experiment 1 and 2. For the soils from the experimental field plot, with 2-3 years contamination history, the $CaCl_2$ based values were 182 and 345 (before and after experiment 4 and 5) and 363 l/kg (experiment 6 and 7) for May 1995, October 1995 and May 1996, respectively. For Budel gradient soils, after decades of contamination but shortly after pH-adjustment to 5.5, the $CaCl_2$-based value in this experiment was intermediate, viz. 153 l/kg (see Posthuma and Notenboom 1996). Sorption of zinc to experimental field plot soil and Budel gradient soils is stronger than to OECD artificial soil, despite the larger clay and organic matter content of the latter. Apparently, age of contamination is an important factor determining zinc solubility. Based on sorption characteristics alone ('supply') it was expected that the EC50 of *E. andrei* based on total concentrations would be lowest in OECD artificial soil, intermediate in soils from the Budel gradient, and highest in Panheel soil. An increase of total-zinc EC50 over time was expected from the loss of zinc and the increased sorption of remaining zinc demonstrated in the latter soil (Chapter 2).

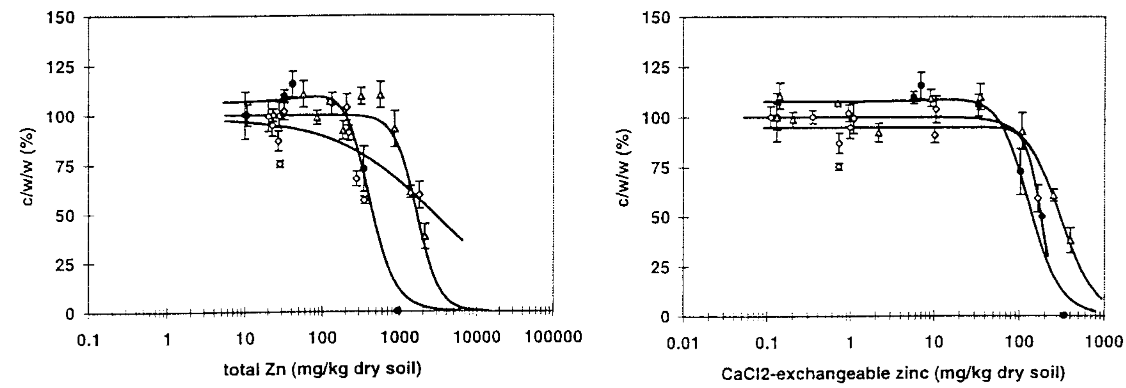


Fig. 5.1. Concentration-effect relationships for the effect of zinc on the reproduction of *Eisenia andrei* based on total zinc concentrations (left) or $CaCl_2$ exchangeable concentrations (right) in OECD soil (black circles), experimental field plot soil (data 1996, grey triangles) and Budel gradient soils (white markers). Solid lines represent curve-fits according to the model of Haanstra *et al.* (1985).

- *Bioavailability of zinc for Eisenia andrei.* The effects of bioavailability differences among soils and exposure conditions on zinc toxicity for *E. andrei* can be derived from the following cross-section of Table 5.1. Judged by total (external) concentrations, the EC50 values of the different experiments show that toxic effects increased in the range Budel - Panheel - OECD, irrespective of the exposure duration. In OECD artificial soil, the EC50 appeared to vary, but it was less than 1000 mg Zn/kg dry soil. Estimates for Panheel soil, derived from experimental field plot observations, varied between approx. 1250 and 1675 mg Zn/kg dry soil, and for Budel gradient soil the values vary between approx. 1800 and 2550 mg Zn/kg dry soil. An example is given in Figure 5.1, showing a comparison of concentration-effect curves for both total and exchangeable zinc concentrations in soil. Thereby, it should be noted that the true value for Budel may be higher than the value calculated in this way, since other metals also contribute to toxicity.

The variation in estimated sensitivities among experiments has been summarized by the coefficient of variation (C.V.), which is the standard deviation expressed as percentage of the mean. Based on total soil concentrations (for experiment 1-9) the C.V. value was 48%. The C.V. value increased after application of the soil type correction that is used in the Dutch soil protection policy. Similar to the observations with the springtail *Folsomia candida* (Chapter 4), soil type correction does not correct for differences between freshly contaminated and aged soils.

Judged by $CaCl_2$ exchangeable concentrations the differences in sensitivity among experiments reduced from 48% to 31% (no value only for experiment 3). This suggests that exchangeable zinc more accurately neutralizes differences among soils and exposure conditions than total concentrations, *i.e.* it reduces uncertainty in laboratory-to-field extrapolation of toxicity data in the case of *E. andrei*. A similar reasoning may hold for water-soluble zinc, similar to the observations on the springtail *F. candida* (Chapter 4), but the data for *E. andrei* are insufficient to judge this. The same holds true for body concentrations, but for this measure of exposure it appeared difficult to estimate EC50 values for experiments 2, 3 and 9. This is caused by the particular pattern with which the zinc body concentration changes at increasing exposure concentrations. This pattern suggests that internal concentrations are regulated in the range 100-130 mg/kg dry body weight, irrespective of soil type and exposure conditions. When the regulatory capacity is exceeded, the body concentration of zinc abruptly exceeds the above mentioned range, with large variation among individuals. The data, however, suggest that the variation in sensitivity based on body concentrations is also smaller than that based on total soil concentrations; if sensitivity differences existed among experiments they maximally covered a factor of two, with the animals from the experimental field plot being the least sensitive.

The importance of availability differences among soils was further investigated in a comparison of metal kinetics among soils. Kinetic observations showed clear accumulation patterns of zinc and other metals in one of the soils from the Budel gradient. In OECD soil freshly contaminated with the same metal mixture (Zn, Cu, Pb and Cd at similar total concentrations) mortality was observed almost immediately, and no kinetic observations could be made. This divergence of effects suggests strong differences in availability among both soils.

Comparison of sensitivity data with the sorption data suggests that zinc extractability is not the only factor that has influenced toxicity. Although the use of exchangeable zinc appeared to reduce uncertainty in laboratory-to-field extrapolation, it does not fully compensate for differences among soils and exposure conditions. Further compensation might be reached when addressing variable exposure conditions and mixture toxicity. Nonetheless, the cross-section on bioavailability suggests that uptake or toxicity studies with organisms may, in specific cases, be needed to reduce uncertainties in laboratory-to-field extrapolation, next to an abiotic characterization of bioavailability by expressing exposure through liquid phase concentrations.

In conclusion, accuracy of laboratory-to-field extrapolation for zinc toxicity data is improved when exchangeable rather than total soil zinc concentrations are used. Using exchangeable zinc reduces dissimilarity of effect levels between soils with different contamination histories (days *vs.* 2 or 3 years *vs.* decades, and $ZnCl_2$ *vs.* zinc from metal ores) and soil characteristics (low % organic matter (OM) and %Clay *vs.* high %OM and %Clay). It is likely that the same holds true for water-soluble and body concentrations, although the data are insufficient to calculate CVs. A refined assessment might consist of uptake and toxicity studies with organisms.

5.2.3 Cross section: influence of exposure conditions on zinc toxicity

A cross-section of the experiments addressing the effect of environmental conditions on zinc sensitivity consists of a comparison of results obtained under constant environmental conditions (experiments 1-3 and 8-9: climate room, constant 20 °C, soil humidity set to field capacity) with those obtained under variable conditions (experiments 4-7: outdoor conditions; variable temperature and humidity). Details of these experiments are summarized in Bakker *et al.* (1997). It should be noted that experiment 4 consisted of observations on a single replicate for all concentrations, whereas five replicates were used to derive EC50 values in experiment 5. This choice related to the 'black box' characteristic of the field experiment, in which experiment

4 was used for preliminary observations on the behaviour of worms in the prevailing conditions. In 1996, the experiments consisted of 3 weeks exposure and 7 weeks exposure (3 and 2 replicates/concentration, respectively).

The sensitivity of *E. andrei* for zinc was remarkably constant for the successive experiments of 1995 and 1996 in the outdoor field plot enclosures when EC50s were based on body concentrations. The EC50 values ranged from approx. 300 to 350 mg/kg dry body weight. This is remarkable, since the exposure conditions of both years were different. On average, 1995 was relatively dry and cold. The difference in abiotic conditions between both years has resulted in a large difference in cocoon production in control conditions: cocoon production (per worm per week) in 1995 was approx. one-fourth of that in 1996. Nonetheless, the sensitivity was similar in both years. Thus, for *E. andrei* the body concentration seems to be a good indicator of toxic effects in this species, irrespective of stressful exposure conditions. This suggestion is further confirmed by their similarity with the observations obtained with OECD artificial soil and Budel soil under constant conditions. It should be noted that the estimated effect concentration in pH adjusted Budel gradient soil is relatively low compared to the other values. This may indicate the contribution of other metals to toxicity (at a body concentration of 130 mg Zn/kg there were also other metals present in the body which might contribute to toxicity). It may also be the result of additional stress experienced by the animals when incubated in Budel soils. This possibility is not hypothetical, since the number of cocoons produced in clean Budel soil was approx. 25% lower than in clean OECD artificial soil.

Zinc sensitivity differed between 1995 and 1996 (experiments 4 and 5 *vs.* 6 and 7) when judged by total and exchangeable external concentrations. This is in agreement with the increased sorption of zinc that occurred during that period (see Chapter 2). Based on the exchangeable fraction, zinc sorption increased between February 1995, October 1995 and March 1996, as shown by the increase of the Freundlich K_f from 182, via 345, to 363 l/kg. Based on water-soluble zinc, these values were 586, 557 and 1107 l/kg. Increased K_f-values found with both methods indicate that sorption increased with time, at an almost constant total concentration. This explains the increase of the EC50 based on total zinc concentrations in soil from 1282 - 1277 to 1597 - 1676 mg/kg dry soil from 1995 to 1996. In addition, it may partly explain the similar increase observed when judged by exchangeable concentrations. Based on a C.V.-analysis for the experiments 5-7, EC50s derived from body concentrations and from water-soluble concentrations seem to be the most constant predictors of toxic effects (C.V. resp. 9 and 6%); total and exchangeable zinc appeared to be less constant (with C.V.-values of 14 and 17%).

Although controlled experiments have not been performed to study the influence of variable exposure conditions on zinc sensitivity of *E. andrei*, it can be concluded that the EC50 body concentration of zinc is relatively constant irrespective of the exposure conditions encountered in the experiment (this section) or soil and contamination characteristics (previous section). The EC50 based on water-soluble zinc concentrations might be similarly constant, although data for comparisons among soils have not been obtained (for further information on water-soluble zinc: see section 5.3, and the papers of Spurgeon *et al.* indicated therein).

5.2.4 Cross-section: effects of metal mixtures in different soils

The previous analyses neglected the influence of other metals than zinc alone: the toxic effects on cocoon production in the soils from the Budel gradient (experiment 8 and 9) were interpreted as if only caused by zinc. It may, however, be questioned whether other metals have also contributed to toxicity (either stimulatory, additively or counteractive). This introduces the problem whether all substances should be taken into account in laboratory-to-field extrapolation, and, if so, in which way. In this paragraph we address the issue of mixture toxicity, starting from the principle that mixture toxicity studies in soil cannot be made without proper assessment of the issue of availability. In line with this, Calamari and Vighi (1992) have identified three major interaction levels for (soil) ecotoxicity studies, viz. interactions in the exposure soil, interactions during uptake, and interactions in the organisms' body. This paragraph addresses mutual interactions among metals at the level of mixture effects on cocoon production in *E. andrei*; sorption interactions have already been demonstrated in Chapter 2. The paragraph is concluded by an analysis of the toxicity of the metal mixture in the factory soils (experiments 8 and 9) in view of availability and mixture aspects simultaneously.

Various mixture toxicity studies were performed with *E. andrei* under laboratory conditions in OECD artificial soil. The results of these studies were used to address the aspect of joint toxicity of metals in Budel gradient soils (experiment 8 and 9). The studies in OECD artificial soil focused on mixtures of Cd, Cu and Zn. No data were obtained for Pb, since high anionic concentrations caused severe effects on cocoon production, rendering experimental results on Pb uninterpretable. The high anionic concentrations were present in the experiment due to the low toxicity of Pb. This implies that the contribution of Pb to the joint toxicity of metals in Budel gradient soils could not be established. Due to its low toxicity, probably partly attributable to strong sorption, the contribution of Pb to toxic effects in Budel soils is considered negligible.

Joint effects of metals in OECD artificial soil were determined first on the basis of total metal concentrations, expressed as Toxic Units (TU, see Chapter 1, section 1.3.2) on the basis of single-metal exposure experiments in the same medium. Observed mixture effects of the three binary mixtures were less than concentration additive, since all EC50s (in TUs) were larger than unity (Table 5.2). These observations suggest that the net toxic effects of the mixtures in soil were less than was expected from concentration additivity. The results do not give a clue towards the cause of this mixture effect, i.e. the response cannot be attributed to specific interactions in soil, during uptake, or in the body. Such a disentangling of causes is needed to extrapolate the data from OECD artificial soil to the mixture in Budel soil (or other soils), for which metal bioavailability was shown to be different from that in OECD artificial soil.

Only for the mixture of Cd+Zn data were obtained to disentangle the different interaction levels in part, viz. the mixture effect was calculated not only for total soil concentrations (all interaction levels included) but also for exchangeable concentrations (interactions in soil largely excluded, due to the probable linkage to the bioavailable concentrations). The reduction of the EC50 from 1.7 to 1.4 TU for this measure of exposure substantiates the previous observation

that interactions in soil influence the joint effects of metals. Apparently, the joint effects of metal mixtures in soils are influenced by soil (sorption) characteristics, in this case the effect of soil sorption interactions has softened the response that occurs at the level of body concentrations. Similar observations have been made for *E. crypticus* (Chapter 6), although in that species the net effect of a Cu+Zn mixture was determined by less than concentration additive responses at the level of body concentrations (TU_{body}=1.8) and a reinforcing effect of sorption interactions ($TU_{total\ soil}$=1.2) (Posthuma *et al.,* 1997).
The experiments in Budel gradient soils (experiments 8 and 9) also bear on a mixture situation, but in a different soil type. Extrapolation of the findings of the binary mixture to Budel gradient soil can best be performed through body- or through bioavailable concentrations. Since insufficient data were collected on body concentrations, we used the Freundlich isotherm parameters of the metals in OECD artificial soil to transfer EC50s based on total concentrations into pore water EC50s, which thus defined TUs based on the liquid phase concentrations. The procedure and results are summarized in Table 5.2. For each Budel gradient sample the metal concentrations in the pore water (Posthuma and Notenboom, 1996) were summed as fractions of their EC50s in OECD artificial soil, and the sum-TU of each Budel soil was calculated. Numerically, the expected EC50 of the mixture in Budel soil would equal 1 TU, if concentration addition truly applies and if the true bioavailable concentration is used; in view of the data obtained on binary-mixture effects in OECD artificial soil, however, slightly less than additive effects were expected. The calculations show that the divergence of sensitivity for the mixture of Zn, Cu and Cd between Budel gradient soil and expectations from experiments in OECD artificial soil equalled a factor of 10 when based on total concentrations. This factor reduces to a value of 1.4 when $CaCl_2$ exchangeable metal concentrations are used.
It is concluded that the divergence of sensitivity among soils reduces to a value below a factor of two when mixture effects and an approximation of the bioavailable concentration are taken into account. The factor of 1.4 would suggest that, at the EC50 level, the joint effect in the organisms' body in Budel soils is slightly less than concentration additive. Confirmation that this may occur in oligochaetes has been obtained in a detailed study on *E. crypticus*, for which the joint effect of Cu+Zn judged at the level of body concentrations was 1.9 TU (Posthuma *et al.,* 1997). It is likely that the toxic effects on *E. andrei* in Budel soils have been influenced mostly by the interaction of these two metals, as judged from a comparison of the TU-composition of the three metals present in the liquid phase of Budel gradient soil (at adjusted pH, in TUs; Cd:Cu:Zn = 1: 3: 10).

5.3 Discussion

Experiments done with *E. andrei* within the framework of the Validation project focused on quantifying the toxic effects of metals in a relatively small number of soils and exposure conditions, to be able to explain the observations in terms of some of the factors mentioned in Chapter 1, and to assess the relative importance of those factors in laboratory-to-field extrapolation. This approach has resulted in a detailed explanation of the effects in a

contaminated field soil, based on observations in the standardized test system and the experimental field plot. It has led to the conclusions that (1) water-soluble and exchangeable soil concentrations and body concentrations of metals are more closely related to toxic effects than total soil concentrations, (2) that variable exposure conditions do not strongly affect toxicity, and (3) that laboratory-to-field extrapolation for *E. andrei* should take availability differences and joint effects into account simultaneously. The importance of these conclusions is emphasized for this species group by some field observations on the occurrence of earthworm populations at severely contaminated sites. While the total zinc concentration in Budel soils only reached up to approx. 1750 mg/kg, and given an LC50 in the range 68 - 188 mg Zn/kg in OECD artificial soil (Spurgeon and Hopkin, 1996b), the occurrence of earthworms in soils containing 3,000 mg Zn/kg (Avonmouth smelter), or 40,000 and 180,000 mg Zn/kg (mine spoil, Morgan and Morgan, 1991, Corp and Morgan, 1991) is surprising. Based on our experimental observations, it is unlikely that mixture toxicity or variable environmental conditions have caused this enormous divergence. Moreover, sensitivity differences among species are unlikely to bridge this gap (see below). Hence, bioavailability differences, among the factors studied, seem of paramount importance for laboratory-to-field extrapolation.
Below, our results are further compared with literature data, to investigate the robustness of our conclusions for more extreme exposure situations, other soils, field-relevant worm species and the occurrence of earthworm populations at contaminated field sites.

5.3.1 Bioavailability of metals

Applicable data on the aspect of bioavailability differences among soils have been reported in various papers by Spurgeon and colleagues. These authors have compared toxic effects of metals on *E. fetida* in standard laboratory tests with OECD artificial soil and in contaminated field soil, the latter collected in a gradient around the smelter at Avonmouth. Major differences compared to the observations with Budel gradient soils are (1) pH adjustment was not necessary due to the relatively high indigenous pH (approx. 6), (2) metal concentrations were much higher near the smelter and (3) organic matter contents were higher (cf. Table 5.1 and Table 5.3).
The comparison of zinc sensitivity in OECD artificial soil tests and in Avonmouth gradient soils supported our finding that assessment of exposure through the liquid phase concentration reduces laboratory-field divergence (Table 5.3). The EC50s based on the water-soluble zinc concentrations were 14.2 mg/kg in Avonmouth soils, whereas they were 17.4 and 10.5 mg/kg in OECD artificial soil at similar pH (approx. 6) and organic matter contents (10 and 15%, comparison of experiment 14 with 5 and 6). This divergence is negligible compared to the values obtained on the basis of total concentrations (3605 vs. 462 and 592 mg/kg respectively).

Table 5.3. EC50 values for the effect of zinc on cocoon production of *Eisenia* spec. in different soils and under different exposure conditions. Effect concentrations-50% are based on measured concentrations in soil (solid phase, liquid phase estimates) and body. Averaged EC50s and Coefficients of Variation are given for each measure where applicable.

nr.	test soil	soil type	OM	clay	temp.	pH	duration	(mg Zn/kg soil or body weight)			remarks	reference
			%	%	°C	(=initial/e=end)*	(weeks)	total	water sol.	internal		
1	OECD	artificial	10	20	20	6.3(i)-6.7(e)	8	276			no food added	[1]
2	OECD	artificial	10	20	20	6.1	3	357			Zn-singly	[2]
3	OECD	artificial	10	20	20	6.1	3	1001			mixture series with Cd, Cu, Pb, non-add. model	[2]
4	OECD	artificial	5	20	20	6	3	136	7.7			[3]
5	OECD	artificial	10	20	20	6	3	462	17.4			[3]
6	OECD	artificial	15	20	20	6	3	592	10.5			[3]
7	OECD	artificial	5	20	20	5	3	199	12.9			[3]
8	OECD	artificial	10	20	20	5	3	343	31.6			[3]
9	OECD	artificial	15	20	20	5	3	548	43.9			[3]
10	OECD	artificial	5	20	20	4	3	142	9.6			[3]
11	OECD	artificial	10	20	20	4	3	189	10.2			[3]
12	OECD	artificial	15	20	20	4	3	320	12.4			[3]
13	OECD	artificial	10	20	20	5.5-6.5	3	623				[4]
14	factory A.		12.9 - 27.1	n.d.	20	6.3-7.3	3	3605	14.2			[2]
15	factory A.		12.9 - 27.1	n.d.	20	5.54 - 7.37	20	637			started: juveniles: at 12 weeks only 70% adults	[5]
16	factory A.		12.9 - 27.1	n.d.	20	5.54 - 7.37	12	4950			started: juveniles: at 20 weeks all animals adults	[5]
17	manure				25		8			90**	deviating approach	[6]
18	manure/soil						8	3000			deviating approach	[7]
19	manure/soil						20	400			deviating approach	[7]
						average (2-14)	***	655	17			
						CV (%)		140	68			

For explanation of notes: see next page

Notes of Table 5.3

*: initial: soil pH (KCl) in control soil prior to exposure or at the end of exposure; end: *ibid.* at the end of exposure; for the pH-adjusted field soils from Budel the range of the experiment is given; unmodified field-pHs are lower.

**:estimated from internal concentration expressed on wet weight basis, based on approx. 15% dry wt.

***: averages and CVs based on 3-weeks experiments only.

References are: [1] Spurgeon *et al,*. 1994, [2] Spurgeon and Hopkin, 1995, [3] Spurgeon and Hopkin, 1996b, [4] Spurgeon and Hopkin, 1996c [5] Spurgeon and Hopkin, 1996a, [6] Reinecke and Reinecke, 1996, [7] Malecki *et al.,* 1982.

Freshly contaminated soil can overestimate toxic effects in some field soils due to differences in metal availability. It should be noted that other metals may contribute to toxicity in the Avonmouth soils. This means that the divergence of EC50s for this soil based on zinc alone is underestimated.

Spurgeon and Hopkin (1996b) also made comparisons of water solubility and toxic effect levels of zinc in OECD artificial soil tests in which the proportion organic matter and the soil acidity were manipulated. Water-soluble zinc appeared to change as a function of pH and organic matter content, in the same magnitude as demonstrated in the pore water fractions that we have collected by centrifugation (see Chapter 2). In their study, zinc solubility changed for example by a factor of 6 between pH 4 and 6 at 10%OM. Furthermore, based on total Zn concentrations, LC50s, NOECs and EC50s for zinc systematically increased with increasing pH or organic matter content for the 10 and 15% OM treatments; this effect was reduced when using water-soluble zinc concentrations. Based on the CV of all tests, however, there was no reduction of CV when comparing all experiments (4-12) for total and water-soluble based EC50s. These CVs were 54 and 71% respectively. The use of water-soluble zinc may thus importantly reduce inter-experimental variation of sensitivity between freshly contaminated soils and field soils, but it does not fully compensate for the effects of soil characteristics when differences among soils are more subtile.

The importance of bioavailability differences among soils has also been addressed in other recent studies on *E. andrei* (Van Gestel *et al.,* 1995, Janssen *et al.,* 1997ab). In the former study, the authors reiterated original data from various authors into multivariate regressions, that relate the Biota-to-Soil Accumulation Factors (BSAFs) to soil characteristics on the basis of uptake studies in various soils. Zinc uptake appeared to be mainly determined by soil pH, while the quantitative influence of clay and organic matter seemed small or negligible. For other metals the relative importance of soil factors for the BSAF was different, although soil acidity was always the factor with the largest influence. In the latter study a similar approach was followed, but both BSAFs and abiotic partitioning of metals were analysed as functions of soil characteristics (Janssen *et al.,* 1997b). This study demonstrated that abiotic partitioning and uptake were mainly modulated by the same soil factor, mainly soil acidity. This suggested that uptake via the liquid phase is likely. It should be noted, however, that the variation of soil characteristics of the 'training sets' that were used to derive the regression equations not only reflects mechanistic phenomena (acidity influences speciation) but also the relative variability of the soil factors in the training set (for example: acidity variation in the training set is larger than variation in clay content, so that a larger fraction of statistical variation is explained by acidity).

5.3.2 *Influence of variable exposure conditions on toxicity*

Our results suggest that the influence of variable environmental conditions on sensitivity was small, and that the differences between EC50s for the experimental field plot in 1995 and 1996 were likely attributable to differences in sorption behaviour of zinc in the soil. We found the internal concentration to be closely related to toxic effects in different conditions, although for statistical reasons internally based EC50s or NOECs are difficult to establish.

There are only few literature data from which the importance of variable environmental conditions on zinc sensitivity of *Eisenia* spec. can be derived. Spurgeon *et al.* (1994), for example, determined the zinc sensitivity in OECD artificial soil without adding food (Table 5.3, experiment 1). The EC50 for cocoon production, 276 mg/kg after 8 weeks, is lower than the values found under similar conditions with added food, but after 3 weeks. Due to the difference in exposure duration, it is not clear whether the slightly increased sensivity has been caused by lack of food, or is caused by experimental error. Again, however, the sensitivity expressed by the EC50 varied only by a factor of approx. 2.

Conditions at the start of the test are also important for the outcome of the test. Most studies of Table 5.3 bear on experiments starting with clitellate (adult) worms, but one outlier is present (experiment 15). Spurgeon and Hopkin (1996a) started experiments with juvenile worms (age: 5 weeks), and determined the EC50 of cocoon production after 12 and 20 weeks incubation in Avonmouth gradient soil. The EC50s were highly different, and the low EC50 for the 12-weeks exposed group suggests a high sensitivity of juvenile worms. It should be noted, however, that this EC50 is determined by reproductive activity per se as well as by delayed maturation in the most exposed worms. To what extent the high sensitivity of the younger animals reflects high sensitivity of the juvenile phase cannot be established. The data however suggest that the EC50 for cocoon production is not the only factor that is important for population performance. Next to number of cocoons, the generation time and growth are important sublethal endpoints to address when considering laboratory-to-field extrapolation at the level of population performance rather than cocoon numbers only (see also Klok and De Roos, 1996).

5.3.3 *Joint effects of metals in earthworms*

Research on the joint effects of metals in oligochaetes has generally revealed less than concentration additive effects when judged by total soil concentrations. Weltje *et al.* (1995), Posthuma *et al.,* (1995), and Posthuma *et al.* (1997) demonstrated this for *E. andrei* and *E. crypticus* (see also Chapter 6) by exposing these species to equitoxic mixtures of two metals in OECD artificial soil. Spurgeon and Hopkin (1995) studied the joint effects of four metals in OECD artificial soil, in a series of mixtures that mimicked the Avonmouth gradient through total concentrations of 7 sampling sites, and found an increased EC50 for cocoon production compared to zinc only treatments (experiments 2 and 3 of Table 5.3), which suggests significant

less than concentration additive effects. Khalil *et al.* (1996a,b) studied joint effects of tertiary metal mixtures (Cd, Cu and Zn) on the earthworm *Aporrectodea caliginosa* exposed in artificially contaminated field soil (%OM=21.6%, pH(H_2O)=7.05), and demonstrated slightly less than concentration additive effects on growth and cocoon production. These data suggest that mixture effects of metals, even in different soil types, are generally less than concentration additive at the EC50 level (mostly $1<TU_{observed}<2$).
For low metal concentrations, however, joint effects may differ from the above observations. For mixtures of micronutrient metals (Cu+Zn), or mixtures of a micronutrient and a xenobiotic metal (Zn+Cd) for example, it can be hypothesised that, at relatively low exposure concentrations, regulation of body concentrations within narrow limits will prohibit toxic effects, even if both metals are present at concentrations near their NOEC. This means that a response-additive effect might be expected at low concentrations. This is substantiated by the differences between observed joint effects judged at EC50 and at EC10 level found by Posthuma *et al.* (1997) and Van Gestel and Hensbergen (1997). From a mechanistic point of view, this might imply that laboratory-to-field extrapolation of mixture data at low exposure levels (concentrations much smaller than EC50) is different from that at higher exposure levels. In the former case, one might take into account only the most toxic metal and judge a contaminated situation on a per-substance basis (response addition), whereas in the latter case all metals should be taken into account simultaneously, although application of concentration addition might be (slightly) overprotective.

5.3.4 Sensitivity of Eisenia andrei in comparison to other oligochaetes

Spurgeon and Hopkin (1996c) investigated the toxic effects of metals for *Lumbricus rubellus* and *A. rosea*, next to *E. fetida*, following the standard protocol also used in the studies of the Validation project. For the newly introduced species, the exposure temperature was 15 °C, due to their lower optimum temperatures, and the food consisted of a mixture of manure with clean sandy loam soil. Judged by LC50s in OECD artificial soil, the ratio of *A. rosea*: *L. rubellus*: *E. fetida* was 1.0:1.3:2.0, for EC50s cocoon production 1.0:1.8:n.d., and for EC50 values for the effect on growth *E. fetida* was most sensitive, since no EC50 was reached in the other species. *A. rosea* did not produce cocoons in control soil, and the aberrant behaviour of the latter species may indicate that OECD artificial soil has a direct adverse effect on this species. These data show that the species order of sensitivities may differ per toxicity endpoint. Only for mortality the order is clear, with a sensitivity increase from *E. fetida*, via *L. rubellus* to *A. rosea*, with a maximum difference of a factor of 2.
Next to the study of Spurgeon and co-workers, the sensitivity of *E. andrei* can also be compared to *E. crypticus* (Chapter 6). From this comparison it appears that a sensitivity difference of a factor of 2 among oligochaetes is confirmed.

5.3.5 From sublethal fitness characteristics to population maintenance

Spurgeon and Hopkin (1996c) studied the population densities of worm species in the surroundings of the Avonmouth smelter. This offers the opportunity to study the relationship between toxic effects of metals or metal mixtures on fitness characteristics and the net toxic effects at the level of population size, as influenced not only by toxicants, but also by ecological interactions and variable environmental conditions. This study has shown that *L. rubellus*, *L. castaneus* and *L. terrestris* were found close to the factory, in soils in which *A. rosea*, *A. caliginosa* and *Allolobophora chlorotica* were absent. This shows that different species indeed have different net sensitivities for metals. The authors calculated the inter-species variability for population maintenance in the field to be a factor of 4. The data suggest, moreover, that the order of sensitivities as determined under laboratory conditions (previous section) was also present in the field (*L. rubellus* less sensitive than *A. rosea* when judged by LC50s). The authors suggest that the physiological make-up of the species was basic to this difference, i.e. the three 'sensitive' species had less active calcium-secretion glands than the three 'tolerant' ones.

5.4 Conclusions

1. The use of total soil concentrations of metals in standardized laboratory toxicity experiments overestimated the toxicity of the same total soil concentration in the experimental field plot and the pH adjusted Budel gradient soil. Using total soil concentrations, hence, introduces uncertainty in laboratory-to-field extrapolation.
2. Differences between predictions, based on laboratory toxicity experiments, and observations in different exposure conditions varied within one order of magnitude in the present experiments. The difference may be larger when soil and contamination characteristics differ more than in the present experiments (for example: earthworm species occur near the Avonmouth smelter).
3. The use of body concentrations, water-soluble concentrations or $CaCl_2$ exchangeable concentrations reduced uncertainty in laboratory-to-field extrapolation for *Eisenia* spec., both in the present experiments and the Avonmouth results.
4. The zinc sensitivity of *E. andrei* was largely unaffected by variation in exposure conditions (variation of temperature and humidity).
5. Similarity of toxic effects of metals in different soil types was largest for *E. andrei* when both bioavailability and joint effects were taken into account simultaneously. Laboratory-to-field extrapolation should take both factors into account.

6. RELEVANCE OF LABORATORY TOXICITY DATA OF *ENCHYTRAEUS CRYPTICUS* FOR SINGLE SPECIES BIOASSAYS AND COMMUNITY CHANGES IN METAL IMPACTED FIELD SOILS

J. Notenboom

6.1 Introduction

6.1.1 Objectives

An outlook of the work performed with enchytraeids in the context of the Validation project is given in this chapter. Enchytraeids (potworms) are a family of aquatic and terrestrial annelid worms related to the earthworms (Lumbricidae), both are Oligochaeta. Enchytraeids occur in a variety of soil systems: grassland, moorland, forest soils and arable land (Didden, 1993). In Europe more than 140 species belonging to over 20 genera are known (o'Conner, 1971). For arable Dutch soils densities of 11000 to 43000 ind/m^2 are reported (Didden, 1993).
In recent years Enchytraeids received increasing interest in soil toxicity testing (Römbke, 1989, 1995). International harmonised test guidelines are under development and laboratory cultures of several species (all belonging to the genus *Enchytraeus*) are available (Römbke, 1997a).
The work performed with enchytraeids focused on both aspects of the Validation project. Several issues related to these aspects (see Chapter 1) were tackled: bioavailability, multiple substances versus single contaminants, uncontrolled versus controlled conditions, and test species versus field species. In relation to the first aspect, *i.e.* the representativity of laboratory toxicity data for the field, the following two research questions were addressed:

- What are differences in toxicity of zinc for *Enchytraeus crypticus* in different soils under indoor and outdoor conditions, and to what extent are differences related to bioavailability ?
- Can the performance of *E. crypticus* exposed to metal contaminated field soils be explained from standard toxicity data if bioavailability and joint toxicity of metals are taken into account ?

In relation to the second aspect, *i.e.* the ecological significance of risk limits, the question was addressed:

- Is the enchytraeid community in metal contaminated soils impacted by metal toxicity and, if so, how is the level of impact related to the toxicity of the soils as judged by laboratory toxicity data and environmental standards ?

Emphasis was on the research questions related to the first aspect. The experimental approaches followed were very similar to those for the earthworms (Chapter 5). In addition, observations on composition and density of the enchytraeid community in soils at different distances from the Budelco zinc factory near Budel (see Chapter 2) were performed.

6.1.2 Approaches

The work of Dirven-van Breemen *et al.* (1994) on a soil toxicity reproduction test with *E. crypticus* served as a starting point for the experimental work. In our laboratory, this species is cultured (± 20 °C) on agar plates based on peat soil extract with oat meal porridge as added food. For the experiments adult worms were used, recognisable by clearly visible, white coloured eggs in the transparent body. Exposure of worms to (contaminated) soil was done in small containers (15 ml) filled with 7-10 g (wet weight) of soil (or OECD artificial soil). At the beginning of the experiments 10-15 adult worms were placed on top of the soil in the containers; animals were fed weekly with a pinch of grounded oat flakes. The OECD artificial soil used was similar to that applied in experiments with *Eisenia andrei* and *E. fetida*, except for the peat particle size which was ≤ 0.5 mm and as a consequence only about 35% water was necessary to reach a pF value of about 2 (recommended value for OECD artificial soil). In addition the pH(KCl) was set at 6.5 ± 0.5. Humidity of field soils was set at ±20 %, corresponding to field capacity. Mostly 4 weeks of exposure were appropriate for sufficient production of juveniles. The number of juveniles produced was calculated per single worm in one week (juveniles/worm/week).

For the outdoor exposure of *E. crypticus* in the experimental field plot (Chapter 2), containers were used of 5 cm diameter and 12 cm length, top and bottom provided with gauze of 50 μm mesh. The containers were filled with 10 cm long soil cores, and carefully placed in the cored holes. In order to have enough nutritional support for reproduction, grounded oat flakes were put in the enclosures before they were filled with soil. Enclosures were grafted by 50 individuals of *E. crypticus*. Weekly, containers of the control compartments were opened to determine the time after which sufficient reproduction occurred; additional containers were placed in the control plots for this purpose.

Observations on enchytraeid communities in metal contaminated soils were done at the zinc factory impacted area near Budel. A refined sampling program of 30 sites was carried out to study enchytraeid abundance and species composition. The sites were situated along a transect between 1.1 km and 5.6 km in north-east direction of the zinc factory (a subtransect of the transect described in Chapter 2). The samples were taken in coniferous (pine) forest areas, which beside distance to the emission source also appeared to differ in age of the plantation. Moreover, it was unavoidable that the sampled sites experienced different degrees of anthropogenic impact, like truck traffic, military exercise, bivouac activities etc. These influences, mainly associated with the military activities in the area, made that soil conditions could change over short distances. Sampling took place twice, in early summer and in fall of 1996. At each site three subsamples (litter layer, 0-4 cm and 4-8 cm mineral soil layer) were taken. Summer and fall samples were situated in the same plot of approximately one square meter.

Parallel to all biological experiments and observations, soils were characterised physico-chemically (see Posthuma & Notenboom, 1996) for pH, organic matter, moisture content, and various metal fractions (total, pore water and $CaCl_2$ exchangeable concentrations).

Doses-response relationships were described with the aid of a log-logistic model (according to Haanstra *et al.* 1985) which enabled calculation of EC_x values with confidence intervals. A multivariate statistical data analysis has been applied on the data of the community study in Budel. The program SIMCA-S version 6.0 (Umetri AB, Umeå, Sweden) has been used, for backgrounds the reader is referred to De Zwart (1997). This program is solely operated to analyse assumed linear relationships between environmental predictor variables (X) and biological response variables (Y). The procedure consists of two main steps, first, a Principal Component Analysis (PCA) as the first step in an indirect gradient analysis, and Projection to Latent Structures (PLS) which is also called Partial Least Squares modelling as a method of direct gradient analysis.

6.2 Field representativity of laboratory toxicity data

6.2.1 Zinc toxicity for Enchytraeus crypticus in laboratory tests

Results of reproduction toxicity tests with zinc (EC_{50} values on the basis of total concentrations) under controlled laboratory conditions in OECD artificial soil and experimental field soil differed not more than a factor 2 (experiments 1-6 of Table 6.1). The homogeneity of the data hardly changed when EC_{50}'s were recalculated for a standard soil according to current soil type correction factors (VROM, 1994ab).

$CaCl_2$ exchangeable concentrations were used in this project as external concentrations that supposedly better approached bioavailability. Therefore, it was expected that differences between soils were reduced when EC_{50}'s were based on exchangeable concentrations. The opposite was found, however, as is illustrated by an increase of the coefficient of variation from 23 to 119 within the group of experiments performed in OECD artificial soil (experiments 1-5 of Table 6.1). No clear factors, explaining this large variation could be identified, after a conscientious check of the analytical procedures. On the basis of the data collected with the validation project for this species, it cannot be decided whether the experimental conditions have induced true variation, or that the data should not be interpreted due to analytical uncertainties.

The initial pH of the experiments performed in OECD artificial soil differed less than about half a pH unit between experiments. The field soil had a pH at least one unit higher than OECD artificial soil. Zinc was applied to the soils as chloride salt. In experiments 3-5 the anion concentration of the treatments were balanced (800 - 1400 mg Cl^- / kg).

After the protocol for the reproduction test with *E. crypticus* was drawn up (Dirven-van Breemen *et al.*, 1994), some small amendments were incorporated, particularly with regard to the improvement of extraction efficiency of enchytraeids from the test soil and the accuracy of the counting method. Nevertheless, no systematic trend was seen between the tests performed with the original counting technique (experiments 1-2) and those with the adjusted technique (experiments 3-5).

Table 6.1. EC50-values for the effect of zinc on juvenile production of *Enchytraeus crypticus* in different soils and under different exposure conditions. Effect concentrations-50% are based on measured concentrations in soil (solid phase, liquid phase estimates) and body. A recalculation of EC50s (total soil concentrations) into values for 'standard soil' is included, based on soil type correction factors applied in the Netherlands. Averaged EC_{50}s and Coefficients of Variation are given for each measure where applicable.

experiment	test soil	soil type	OM	clay	temp.	pH	duration	EC50 (mg Zn/kg dry soil or mg Zn/kg dry body weight)					
			%	%	°C	(i=initial/e=end)[1]	(weeks)	total	total st. [2]	$CaCl_2$ exch.	water sol.	internal	reference[5]
1	OECD[3]	artificial	10	20	17	6.2(i)	4	251	281	n.d.	n.d.	n.d.	[1]
2	OECD	artificial	10	20	17	6.2(i)	4	186	208	n.d.	n.d.	n.d.	[1]
3	OECD	artificial	10	20	17	5.9-6.4(i)	4	254	284	18	n.d.	n.d.	[2]
4	OECD	artificial	10	20	17	5.9(i)	4	212	237	144	n.d.	n.d.	[2]
5	OECD	artificial	10	20	17	6.5-6.6(i), 6.4(e)	4	336	376	20	3.9	372	[3]
6	EFP[4], fresh	sand	2	2.9	17	7.2(i)	4	262	595	149	n.d.	n.d.	[4]
7	EFP[4], aged (96)	sand	2	2.9	var	7.2(i)	5	844	1918	102	n.d.	n.d.	[4]
8	Budel gradient	sand	2.4	1.4	17	4.9-5.5(i)	4	205	513	57[6]	n.d.	n.d.	[2]
						average (1-5)		248	277	61			
						C.V. (1-5)		23	23	119			
						average (1-8)		319	551	93			
						C.V. (1-8)		68	103	64			

Notes:

[1] initial: soil pH (KCl) in control soil prior to exposure; end: *ibid.* at the end of exposure; for the pH-adjusted field soils from Budel the range of the experiment is given; unmodified field-pHs are lower.

[2] total st.: EC50 normalized to a 'standard soil' containing 25% clay and 10% organic matter.

[3] OECD: artificial soil according to OECD.

[4] EFP: experimental field plot soil.

[5] [1] Dirven *et al.*, 1994; [2] Posthuma & Notenboom, 1996; [3] Posthuma *et al.*, 1997.; [4] Folkerts & Zweers, 1996 (recalc. Bakker, unpublished).

[6] Value based on final analysis deviates from the preliminary value given by Posthuma & Notenboom (1996).

6.2.2 Zinc toxicity for Enchytraeus crypticus under variable outdoor conditions

Worms were exposed for 5 weeks in enclosures placed into the compartments of the experimental field plot (Chapter 2). The average reproduction in control compartments (15 juv./worm/week) was in the same range as found in laboratory tests after 4 weeks of incubation. The EC_{50}'s for total and exchangeable concentrations are given in Table 6.1 (experiment 7). Compared with the laboratory experiment performed in the same soil (experiment 6) but freshly contaminated with zinc the EC_{50} based on total soil concentrations was 3-4 times higher in the outdoor experiment. When based on exchangeable concentrations, the EC_{50}'s were in the same order of magnitude.
Considering the experiments performed in experimental field soil (experiments 6 and 7) only and assuming that exchangeable concentrations come closer to true bioavailability, the results suggest that differences between both experiments are largely attributable to differences in bioavailability. Variable environmental outdoor conditions (temperature, moisture) seem to result only in a more variable response pattern and lower worm performance in the controls. Nevertheless, when corrected for bioavailability, in this case by applying exchangeable zinc fractions, the relative effect levels indoor and outdoor appear to be similar.

6.2.3 Bioassays with contaminated Budel soils

Field soils taken along a metal pollution gradient near Budel (Chapter 2) were taken to the laboratory for metal uptake and reproduction studies with *E. crypticus*. Premise for this approach is that a gradual change in metal contamination related to zinc factory emission (point source) occurred over the sampled transect and that other environmental variables show low randomly distributed variation (Posthuma, 1997). Pre-treatment of the soils consisted of sieving and homogenisation, and in adjusting the pH to about 5.5. The latter appeared to be inevitable to fulfil experimental prerequisites, because the pH differed considerably between soils and was negatively correlated with distance. Moreover, due to low pH in some soils a direct impact on enchytraeid survival could not be excluded.

Figure 6.1 illustrates that the reproduction of *E. crypticus* in the pH adjusted Budel soils decreases with decreasing distance to the factory. This field distance-response relation enables to establish the distance to the factory with 50 % reduction in worm performance (ED_{50}) at 3.5 km. Due to the steepness of the distance-response curve the uncertainty in the estimate of the ED_{50} is large (Posthuma & Notenboom, 1996).

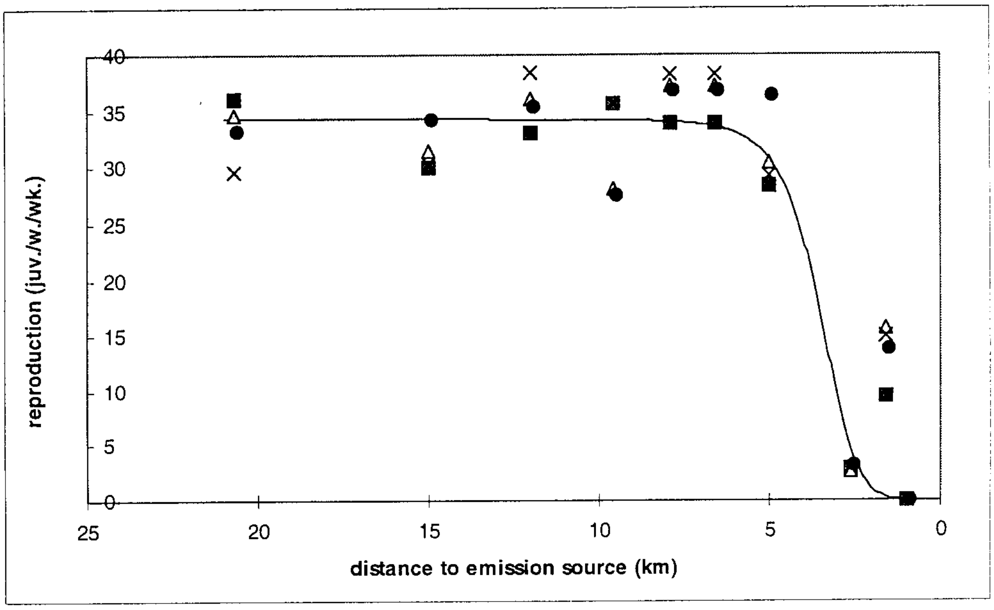


Figure 6.1. Reproduction of *Enchytraeus crypticus* in pH adjusted Budel gradient soils in relation to the distance to an emission source. Replicates indicated by different symbols.

6.2.4 Zinc as dominant toxic metal in Budel soils

In Budel soil #4, at 2.6 km from the factory, the total concentrations of zinc, cadmium, and copper were 219, 0.8, and 17 mg/kg dry wt, respectively (Chapter 2). In the soils collected closer to the factory (# 1-3), metal concentrations were higher. When compared to available laboratory data, the EC_{50} of *E. crypticus* for zinc in OECD artificial soil, on the basis of total concentrations, is reached or even exceeded in the soils sampled within 2.6 km of the factory. For cadmium and copper the EC_{50} is not reached at all in the studied Budel soils (EC_{50} values in OECD artificial soil are given in Table 6.3). For lead and chromium laboratory toxicity data for *E. crypticus* are lacking. Their contribution is considered negligible because it is known from other studies with Oligochaeta that these metals have a relatively low toxicity. Based on a judgement of the total external concentrations, and the relatively high mobility of zinc compared to lead and copper, this indicates that zinc is the dominant toxic metal for *E. crypticus* in the study area near Budel.

When the assumption is made that zinc contributes only to metal toxicity along the Budel gradient, a field dose-response relationship for *E. crypticus* can be established and a field EC_{50} for zinc can be calculated. Results reveal a rather large similarity between this field EC_{50} value (exp. 8 Table 6.1) and the laboratory values (exp. 1-5 Table 6.1), whether they were based on total or exchangeable concentrations. This is remarkable because we supposed that the $CaCl_2$ exchangeable concentrations were a better estimate of true bioavailability and that part of the laboratory to field differences were attributed to differences in bioavailability. Considering only zinc and accounting for bioavailability one would therefore expect the EC_{50} of zinc on the basis of total concentrations to be higher than the reported field value of 205 mg/kg (exp. 8 Table 6.1).

6.2.5 Metal bioavailability in Budel soils

The uptake of cadmium and zinc was determined in *E. crypticus* exposed to the above mentioned Budel soil #4 and in OECD artificial soil freshly contaminated with similar amounts of zinc and cadmium. The data enable estimation of apparent equilibrium concentrations for both metals applying a one-compartment model (Table 6.2). The net bioavailability of zinc and cadmium for *E. crypticus* in the pH-adjusted Budel soil #4 is obviously lower than in OECD artificial soil freshly contaminated with metals at the same concentrations.

The initial pH in the uptake experiments was for the Budel soil about one unit higher than for the OECD artificial soil. In Chapter 2 the dominance of pH over other soil characteristics is illustrated regarding the influence on metal partitioning. In our case this means that if Budel soil #4 would have had a similar pH than the OECD artificial soil the liquid phase concentrations of zinc and cadmium would be higher. The bioconcentration factors of both metals would increase too with decreasing pH (Janssen *et al.* 1997b). The difference in apparent equilibrium concentrations for zinc and cadmium in both soils at similar pH would probably be smaller than the data in Table 6.2 suggest. It seems, however, improbable that after pH correction the bioavailability of zinc and cadmium for *E. crypticus* would be higher in the Budel soils in comparison to OECD artificial soil.

Table 6.2. Equilibrium concentration $(Q_0 + {}^{a}/_{k})$, (mg/kg) estimates of a one-compartment model fitted to uptake and depuration data for Cd and Zn in *Enchytraeus crypticus* in Budel gradient soil #4 and in freshly contaminated OECD artificial soil with similar total metal concentrations. pH of the soils is determined at the start of the accumulation phase.

Soil type	pH (KCl) initial	Zinc	Cadmium
OECD	5.7	358	22.2
Budel #4	6.7	210	7.72

6.2.6 Metal mixture toxicity in Budel soils

Available data on single and mixture metal toxicity for *E. crypticus* in OECD artificial soil under laboratory conditions were used to consider the aspect of simultaneous toxic action of metals in the Budel gradient soils (experiment 8 in Table 6.1). For *E. crypticus* data on two binary mixtures are available (Cd+Zn and Cu+Zn). In Budel #4, the ratio between toxic units, (TU's*) for these metals on the basis of pore water concentrations is Cd:Cu:Zn = 1 : 4 : 300. This indicates that in addition to the large contribution of zinc, copper may contribute more to total metal toxicity than cadmium and that of the binary mixture toxicity data the Cu+Zn

* For the concept of toxic units (TU) see section 1.2.2

interaction appears the most significant one. The pore water concentration ratio's corroborate also our previous conclusion on the dominance of zinc in the metal toxicity of the Budel soils. Considering the toxicity of the mixtures in OECD artificial soil on the basis of total concentrations, the observed effects of the binary mixtures of Cd+Zn and Zn+Cu were less than concentration additive, since all EC_{50}'s were larger than unity (Table 6.3). This means that higher metal concentrations in the mixture are necessary to obtain a similar effect as in the singles.

Table 6.3. Toxicity of metals in OECD artificial soil (alone and in mixture) and in soils from the gradient around the Budel zinc factory (mixture), assessed through total metal concentrations, pore water concentrations and internal concentration.

Soil type	Metal (mixture)	EC_{50}
OECD	Zn	251 mg/kg (total)
OECD	Cu	477 mg/kg (total)
OECD	Cd	88 mg/kg (total)
OECD	Cd+Zn	1.4 TU
OECD	Zn+Cu	1.2 TU
Budel	Zn/Cu/Cd	0.57 TU

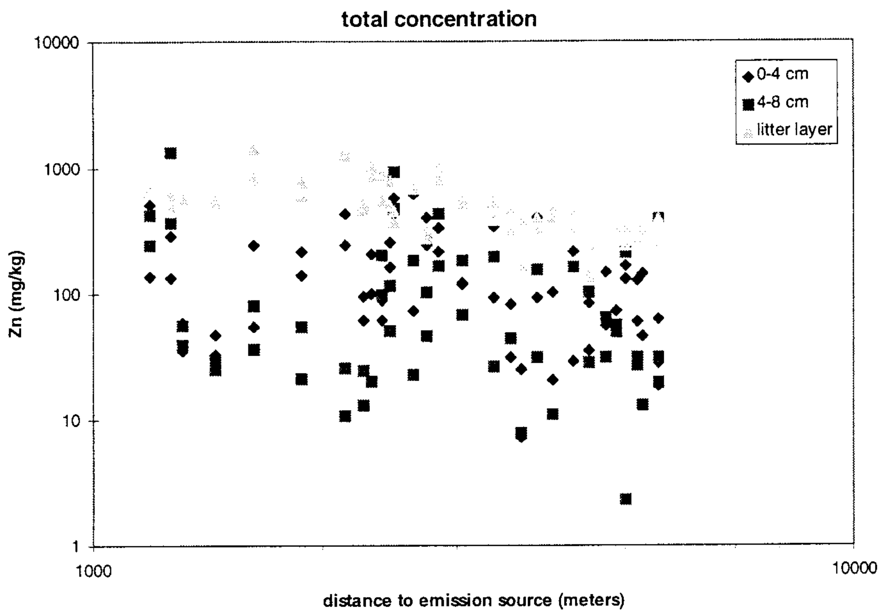
The reproduction of *E. crypticus* in de Budel gradient soils can be considered as the net result of a mixture toxicity study in another soil type. Next, this result can be compared with the findings in OECD artificial soil on the basis of total concentrations. Table 6.3 gives the EC_{50} in Budel soils expressed in TU. The EC_{50} is 0.54 assuming that only zinc and copper contribute to toxicity, and 0.57 when in addition the toxicity of cadmium is taken into account. Again this illustrates the insignificant contribution of cadmium to the toxicity of the Budel gradient soils. Remarkable, however, is that this EC_{50} value is smaller than unity. Also when the comparison is made on the basis of $CaCl_2$-exchangeable concentrations the results are similar (data not given). Apparently the metal in Budel gradient soils is more toxic than in a similar mixture in OECD artificial soil. There are, however, no indications that reduced performance in Budel soils has a solely toxicological cause; neither the presence of toxic levels of other metals nor bioavailability support this. Most plausible explanation appears that additional (physico-chemical) stress factors act on *E. crypticus* in the Budel gradient soils.

6.3 Ecological significance of risk limits

6.3.1 Field observations in Budel

Total metal concentrations of the soils sampled along the subtransect of the Budel gradient showed a much more variable pattern (Figure 6.2a) than expected from previous experiences, see Chapter 2. Also the pH of these soils in relation to distance to the factory showed a variable

a



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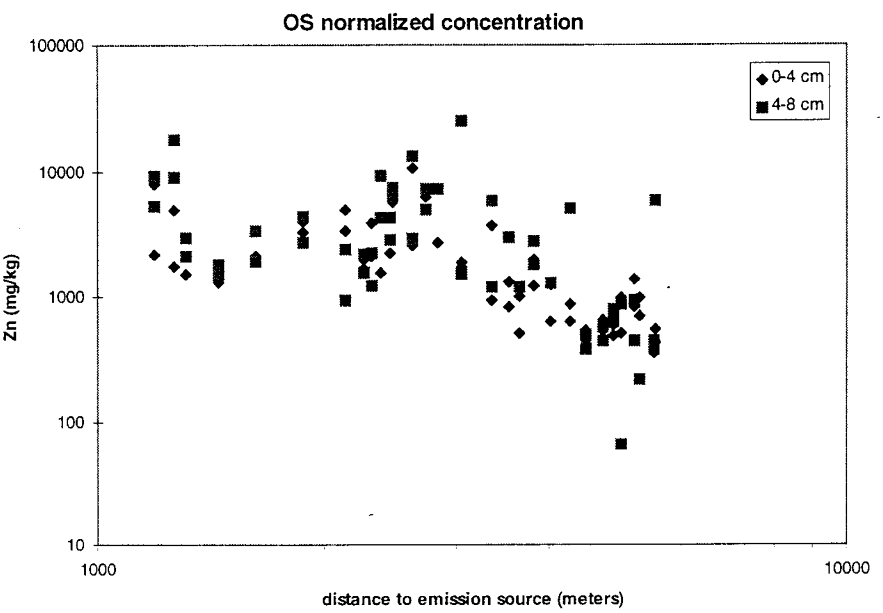


Figure 6.2. Budel enchytraeid gradient study. (a): Zinc concentrations in total concentration (mg/kg dry soil) of litter layer, 0-4 and 4-8 cm mineral soil layers in relation to distance to emission source. (b): Zinc concentrations in 0-4 and 4-8 cm mineral soil layer normalized for organic matter (mg/kg organic matter).

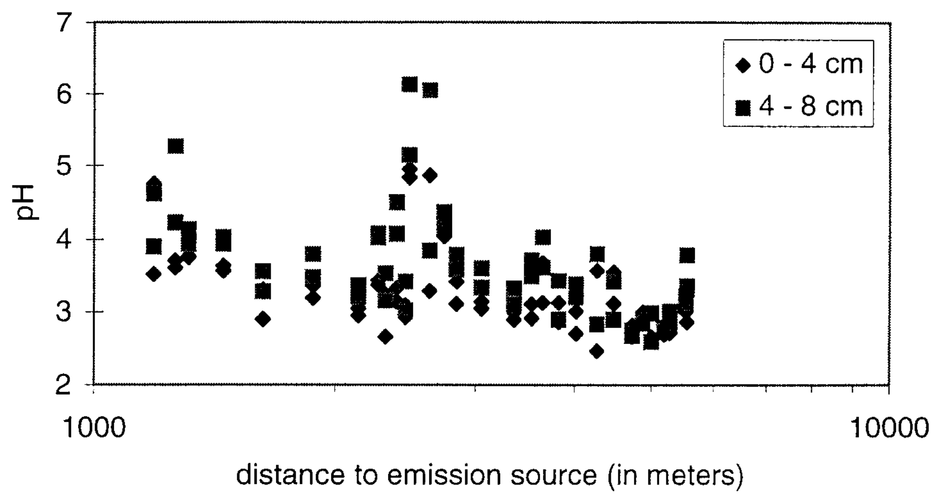


Figure 6.3. Budel enchytraeid gradient study. pH of 0-4 and 4-8 cm mineral soil layers in relation to distance to emission source.

pattern (Figure 6.3). Variation in metal concentrations was slightly reduced after normalisation for organic matter content (as % loss on ignition, LOI) (Figure 6.2b). Clay content differed hardly between these soils and is therefore not taken into account. Correlation between concentrations of the various metals (Zn, Cd, Cu, Pb) in the subtransect soils was generally high, correlation with distance to the factory was low. Pore water concentrations of zinc calculated with a generic formula for log K_p for Dutch soils (Janssen *et al.,* 1997a) showed a very similar pattern of variation as that of total concentrations.The enchytraeid species number and abundance pattern is rather variable along the transect (Figure 6.4), the general tendency appeared to be an increasing abundance and species number with increasing distance to the factory. However, the abundance and species numbers are low and therefore the robustness of the data poor. The total number of species encountered was only 6, maximum number in one sample 4. The number of specimens per sample ranged from 0 to 171 (recalculated 0 - 74.000 ind/m^2) and in 44 % of the sampled soil cores no enchytraeids were encountered at all. The species composition and abundance of enchytraeid populations in the Budel study area as a whole is comparable to what may be expected in other European coniferous sites, abundances appeared to be lower (Römbke, 1997b).

6.3.2 Field observations compared to target values

Assuming zinc to be the dominant toxic metal, the relationship of enchytraeid diversity pattern with zinc was investigated. Although variable, the general tendency of the pattern appeared to be that of decreasing abundance with increasing zinc content (Figure 6.5). Field EC_x values could, however, not be calculated. Based on organic matter content (as % LOI) of each sampled

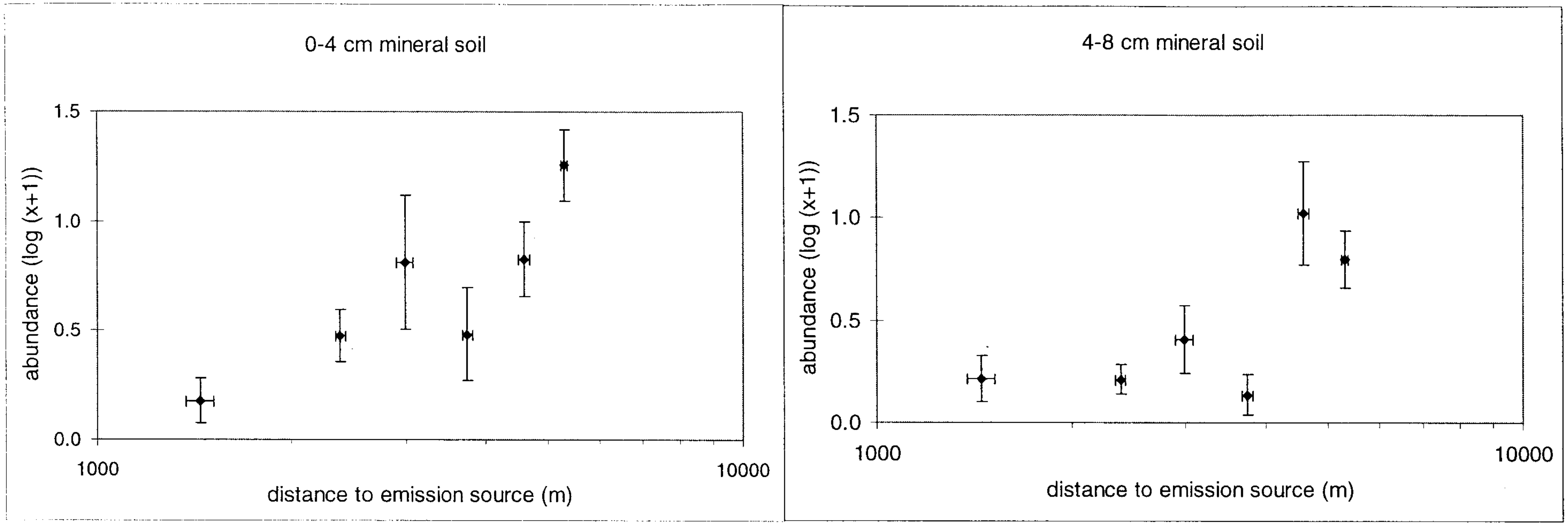


Figure 6.4. Budel enchytraeid gradient study. Abundance of enchytraeids in (a): 0-4 and (b): 4-8 mineral soil samples in relation to distance to emission source. Observations are grouped into similar size classes.

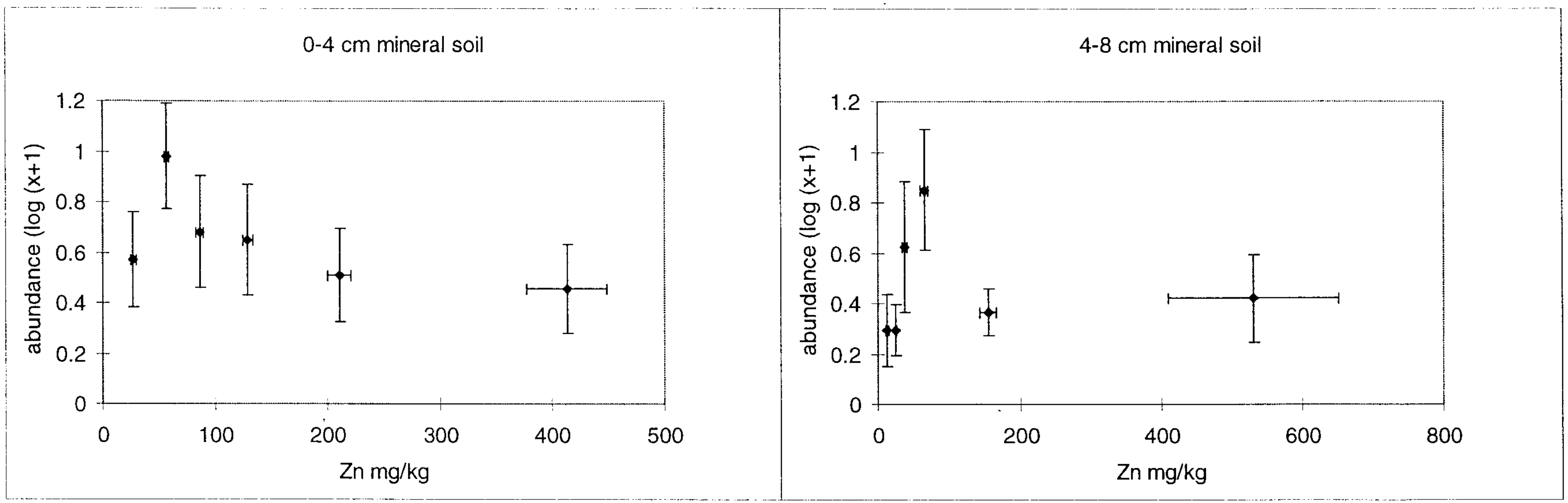


Figure 6.5. Budel enchytraeid gradient study. Abundance of enchytraeids in (a): 0-4 and (b): 4-8 mineral soil samples in relation to the total zinc concentration of the soils. Observations are grouped into similar size classes.

soil and assuming a clay content of 1.42 % (the mean of the samples described in Chapter 2), Target Values were calculated for each soil (according to VROM, 1994ab). The median Target Values for zinc was 62.5 mg/kg and 40 % of the 0-4 cm mineral soil samples had zinc concentrations lower than the 95-percentile (81.5 mg/kg) of the Target Values distribution. Due to the very limited number of observations (30 % in 0-4 and 23 % in 4-8 cm) in soils which exceeded > 2 times the Target Value for zinc and the large variation in the data, no conclusion could be drawn about the relation between the exceedance of the Target Values and the reduction in enchytraeid abundance.

6.3.3 Multiple factors act in the field

The variation in the data suggests that other methodological or ecologically relevant factors but zinc (metals) act in the field. Therefore, a multivariate analysis was performed to investigate if the enchytraeid diversity pattern along the transect is multivariably correlated to the metals, pH, humidity, and organic matter (as % LOI). When the environmental and biological data are analysed in concert by a subsequent PCA and PLS analysis, it shows that the first principal component is marked significant and the second marginally significant. The first component explains 57% of the variance in the environmental data, the second one just 14%. For the biological data only 19% and 15% are explained, respectively, by the first and second component. The variation of the enchytraeid diversity is explained for about 15% by the environmental data. Figure 6.6 shows the weight of environmental and biological data on the

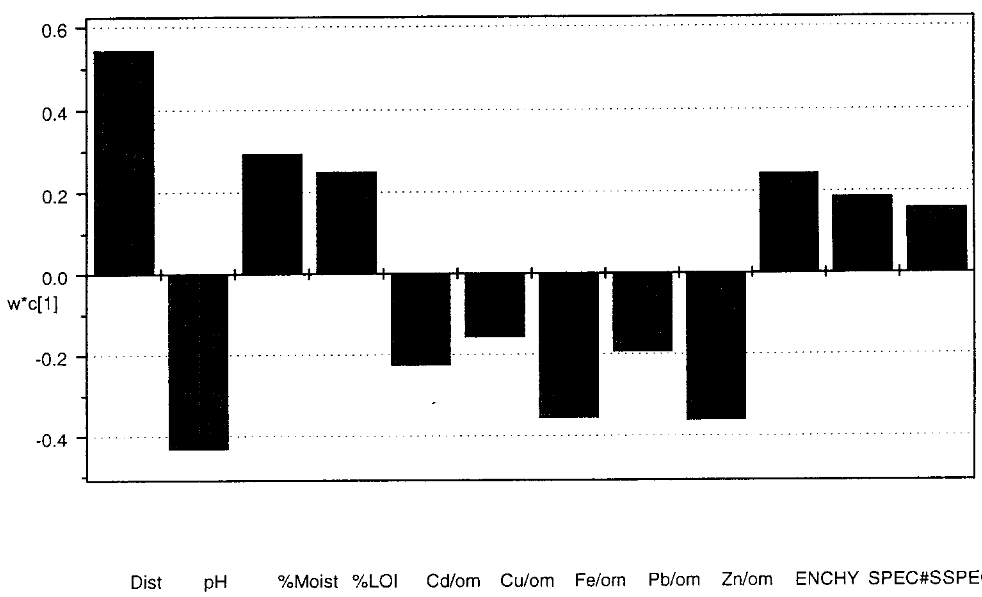


Figure 6.6. Budel enchytraeid gradient study. Weight or loading of environmental and enchytraeid variables detailed for the first axis of the PLS model. Environmental variables: Dist = distance to emission source; pH; %Moist = soil moisture in % water; %LOI = soil organic matter in % weight loss on ignition; Cd/om, Cu/om, Fe/om, Pb/om, Zn/om = metals in organic matter normalized concentrations. Enchytraeid variables: ENCHY = abundance of all enchytraeids; SPEC#S = species number per season; SPEC#Y = species number over the year.

first axis of the model. It shows that the pH and the metals (mainly zinc) correlate negatively with enchytraeid abundance and species number, while distance, moisture and organic matter content (%LOI) are positively correlated.
In a subsequent analysis the variation in biological and environmental data is investigated in relation to distance to the factory. Though much unexplained variation exists, the values of environmental and biological components of the first axis seem to be related to distance, for the second axis this appeared not the case (Figure 6.7). Thus, that part of the variation in environmental and biological factors explained by the first axis of the PLS model is clearly related to the distance to the emission source. Zinc content, pH, moisture and organic matter are the variables that give considerable weight to this axis. Important other sources of variance exist and as a consequence environmental variables associated with emission of contaminants by the factory are not simple to relate to changes in the enchytraeid community. Other influences may come from the dynamic and heterogenic nature of the area (blowing sands) and anthropogenic influences (military activities).

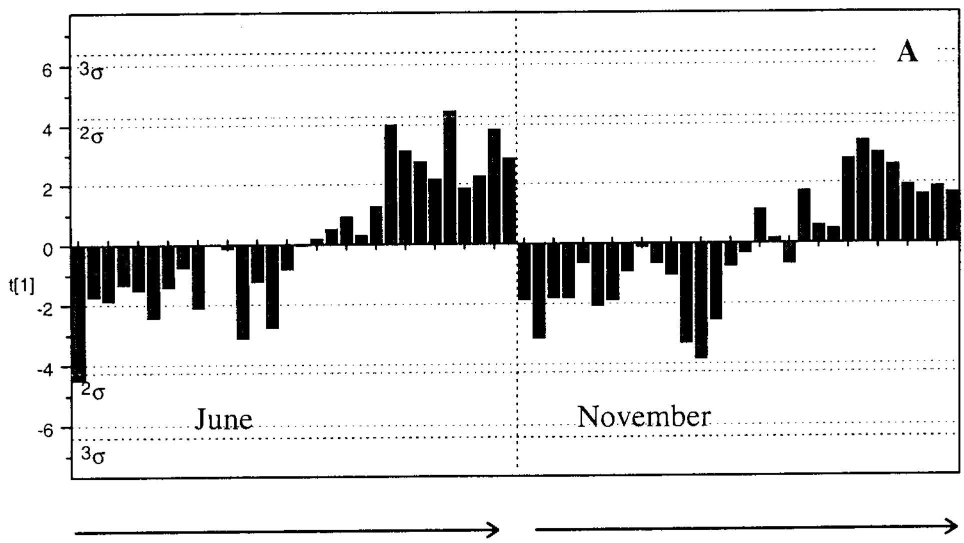
6.4 Discussion

6.4.1 Reproducibility of laboratory tests

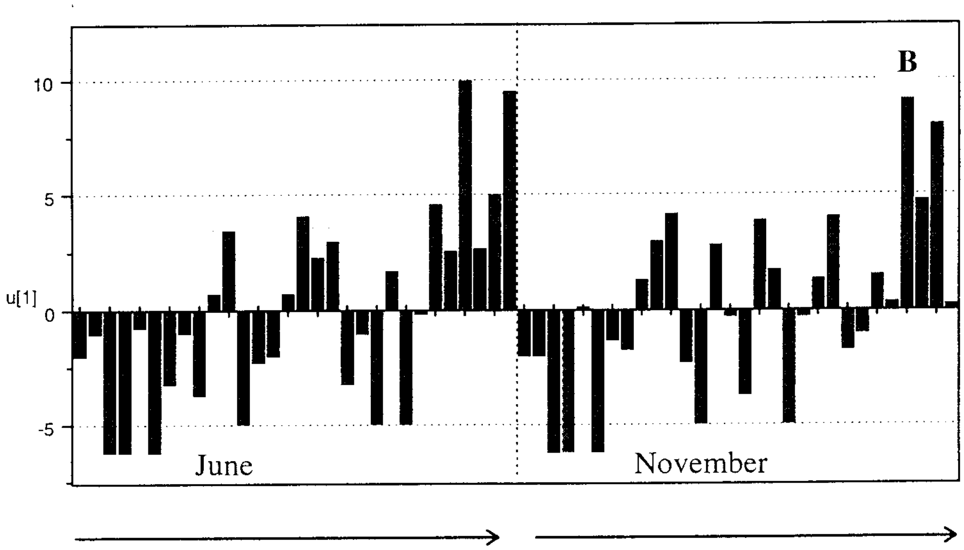
Available data on the toxicity of zinc for enchytraeids have all been obtained in our laboratory. Toxicity tests in which zinc is added to a clean soil (or substrate) with reproduction as endpoint were repeatedly performed in OECD artificial soil. These experiments reveal that when zinc toxicity is based on total concentrations, the test results differed less than a factor 2. This is a rather small variance given the fact that in the period these tests were performed, improvements in test methodology were implemented. A zinc reproduction test with *E. crypticus* has only once been performed in a natural soil, originating from the experimental field plot. Despite large differences in soil characteristics (organic matter, clay) the result based on total soil concentrations was in the same range as the results of tests performed in OECD artificial soil.
The available data are insufficient to conclude upon the variation in zinc toxicity for *E. crypticus* in different soils and the relationship with soil characteristics. The experience with our reproduction toxicity test with *E. crypticus* in OECD artificial soil reveals that this test gives well reproducible results. Its applicability to other soil types should be further investigated.

6.4.2 Bioavailability

Results of reproduction tests performed in various soils were also based on $CaCl_2$ exchangeable concentrations. The expectation was that these results would be more homogeneous because soil differences influencing the partitioning of the metals over solid and aqueous phase would be ruled out. In contrast to this expectation, Table 6.1 shows that the percentage of variation of EC_{50}'s for zinc bases on exchangeable concentrations did not decrease in comparison to total concentrations. Since the zinc EC_{50} of 205 mg/kg in Budel gradient soils is probably an underestimation, the coefficient of variation for EC_{50}'s on the basis of total concentration should probably be larger.



increasing distance to emission source



increasing distance to emission source

Figure 6.7. Budel enchytraeid gradient study. The scores of environmental (a) and enchytraeid (b) variables for the first axis of the PLS model with observation points arranged with increasing distance to the emission source.

The relatively large variance in the EC_{50}'s on the basis of exchangeable concentrations is for a large part due to two low values found in OECD artificial soil. Recent data on metal uptake in *E. crypticus* from contaminated field soils by this species is likely to depend on pore water transfer (Posthuma *et al.*, in press). Moreover, most data collected for $CaCl_2$ exchangeable zinc fractions (e.g. in the experiments with *E. andrei* (Chapter 5) suggest that the zinc exchangeabilty of experiment 4 is within the range , whereas the data for experiment 3 and 5 are outside the range.When these values are excluded as outliers, the coefficient of variation is 38 %. No good explanation is found for these two low values in OECD artificial soil, except that analytical procedures followed may have differed and that small pH differences between soils can have a relatively large influence on metal exchangeability.

In summary, and although some uncertainty remains in the data for E. crypticus, it is likely that application of operational techniques that focus on transfer via pore water have the power to come closer to true bioavailability and reduce the influence of differences in soil characteristics in exposure assessment.

Liquid-phase metal transfer is assumed to be the predominant exposure route for *E. crypticus*. When, however, food uptake appears to be a more important uptake route than presumed, food quality differences might be a factor of greater importance in the extrapolation of laboratory toxicity data to field situations. In our studies the enchytreaids received non-contaminated oatmeal porridge as food in the laboratory while in the field populations feed on completely different, probably contaminated, food resources.

Another approach for the assessment of bioavalability is the determination of internal concentrations. The uptake and depuration study with *E. crypticus* in OECD artificial soil and Budel gradient soil with similar metal content reveal different soil dependent equilibrium concentrations, suggesting in this case that ageing reduces bioavailability. Posthuma *et al.* (in press) studied metal uptake kinetics in earthworms exposed to natural soils with different metal contamination and the relationship with soil properties. They concluded that the association of metal uptake kinetics to soil properties is metal and organism specific. For each metal and species they were able to derive multivariate regression formulae describing the quantitative relationship between BCF and soil properties. Part of the variation, however, could not be described including an important soil-species interaction term. For zinc and *E. crypticus,* Posthuma *et al.* (in press) showed that pH($CaCl_2$) plays a dominant role. Within the range of investigated pH values, an increase of pH caused a decrease in BCF. New information (not yet reported) on metal uptake in OECD artificial soil reveals a rather different pattern from that found in natural soils. For natural soils, however, it appeared that rough estimations of BCF values can be made on the basis of soil properties, with pH being the most dominant one.

Internal concentrations were also determined in two enchytraeid species (*Cognettia sphagnetorum* and *Marionina clavata*) extracted from Budel gradient soil samples. These animals were generally much smaller than the laboratory animals. Together with the fact that they were stored in alcohol this led to important analytical problems with regard to detection limits and weight inaccuracies. The method was therefore not precise enough to make estimations of available concentrations and judgements of field exposure. Application of the above mentioned multivariate BCF equation to the Budel soils in order to estimate exposure of enchytraeids is not meaningful. The Budel soils show relatively small differences in physical properties in comparison to the range of soils for which these formulae are derived. Consequently, estimations of BCF for Budel soils fall within the noise of the method and have no predictive power.

Comparison of the results of the reproduction tests in the same soil under outdoor conditions with aged zinc contamination and indoor conditions with freshly amended zinc supports the hypothesis that bioavailability plays a dominant role in differences between laboratory and field toxicity. Variations in humidity and temperature, and differences in nutritional status led to more variation in the response pattern and differences in reproduction but not to a shift in effect levels. In our case, and assuming that experiment 3 and 5 have shown biased results for $CaCl_2$ exchangeable zinc fraction, differences between lab and field appear for a factor of 3 to 4 at the level of total soil concentrations attributable to bioavailability differences. When soil properties and contamination history in field situations are more distinct from the laboratory the factor of differentiation will certainly increase.

6.4.3 Multiple substances versus single contaminants

The response of enchytraeids exposed to Budel gradient soils was considered as the result of the joint action of metals. This approach did not reveal a clear picture about the importance of exposure to multiple substances (field) in comparison to single substances (laboratory). In our case there are good indications that zinc dominates as toxic metal. The contribution of copper and cadmium to total toxicity and the influence of metal interactions appeared to be overruled by zinc. In addition, enchytraeids exposed to Budel gradient soils appeared to suffer from additional stress. The response pattern in Budel gradient soils, with correction for bioavailability, could therefore not be fully related to metal toxicity.

From laboratory studies with metal mixtures (Weltje *et al.*, 1996; Posthuma *et al.*, 1997) the picture arise that, if corrected for bioavailability and disregarding concentration effects, binary metal mixtures are less than concentration additive. For the field this means that if concentration addition is used as nulhypothesis the number of active toxic compounds present determine the level of deviation between lab and field.

6.4.4 Field observations

The enchytraeid community seems adversely affected at the Budel sites close to the factory. This is seen in a reduced density and lower species number. Similar findings were also reported by Bengtsson and Rundgren (1982) in their study on the enchytraeid community in the vicinity of a brass mill (Gusum, Sweden). It appears that these anthropogenically induced changes are not unequivocally related to metals or metal mixtures. Other relevant environmental factors may also change with distance to the emission source and impose a direct or indirect stress on the community. In our study there is an obvious influence of the pH. Bengtsson and Rundgren (1982) assume a relation between decreased enchytraeid densities and deficiency of high-quality food resources in the vicinity of the brass mill. Due to the metal contamination, changes in species composition of microfungae and reduction in mycelial length were observed. Quantity and quality of humus in soils are important physical steering factors for enchytraeids and it is known that soil forming processes are affected by metals. Changes in the enchytraeid community are thus most likely a result of different directly and indirectly operating environmental factors. In the Budel study area these factors are not only related to the factory emission but also to other natural processes and anthropogenic activities. The results of the gradient study in Budel are therefore not to be considered as a field dose-response relationship which can simply be compared with laboratory data.

6.5 Conclusions

Issues in relation to the representativity of laboratory toxicity data for situations in the field:

1. The reproduction toxicity test with *E. crypticus* applied in OECD artificial soil gives well reproducible results. Test results (EC_{50}) based on total concentrations differed less than a factor two.
2. Similarity in results based on total soil concentrations of the *E. crypticus* reproduction test performed in various soils freshly contaminated with zinc seems high. The small number of different soils applied in this test restricts the general validity of these findings.
3. Although some unexplained aberrant results are found, extraction techniques seem applicable operational methods with the power to come closer to true bioavailability. Even though these methods have analytical limitations they have the power to reduce the influence of differences in soil characteristics in exposure assessment.
4. Bioavailability differences between freshly contaminated laboratory soils and (aged) field situations may cause differences in effect levels as judged through total concentrations of at least a factor of 3 but probably more.
5. The current soil type corrections applied in the Netherlands do not correct for bioavailability differences between soils.
6. Through fluctuating outdoor conditions the variability in the response pattern of metal exposed enchytraeids increases. When corrected for bioavailability, effect levels obtained under indoor and outdoor conditions are comparable.
7. When laboratory reared enchytraeids are exposed to field soils they will often experience additional, non-toxic, stress factors. When such factors operate they will cause dose-response patterns which are not unequivocally related to the presence of toxicants.
8. In the contaminated Budel gradient soils metal toxicity for enchytraeids is largely caused by zinc. Interactions of zinc with other metals, in particular copper, hardly contribute to the overall effect of the metals.

Issues in relation to the ecological significance of risk limits:

1. In the Budel study area additional stress factors overshadow the impact of metals on enchytraeid communities also when we are able to correct for bioavailability differences and mixture toxicity.
2. In general unknown environmental factors may have a direct or indirect influence on the structure of enchytraeid communities in the field. Therefore they complicate the extrapolation from laboratory to field.
3. Changes in the diversity pattern of the enchytraeid community in the vicinity of the zinc factory at Budel are statistically associated to the distance to the emission source. An influence on the community by deposited emission products appears therefore plausible.
4. Only a very small amount of the biological variation could technically be attributed to metal concentrations. This is because of the low abundance and species number and large variation in observations.

Acknowledgements

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7. EFFECTS OF ZINC ON COMMUNITIES AND BIODIVERSITY OF SOIL NEMATODES

A.J. Schouten, M.L.P. van Esbroek & J.R.M. Alkemade

7.1 Introduction

For the group of nematodes, attention was focused mainly on the aspect of 'validation of risk limits, due to their ecological characteristics and in view of practical possibilities for experimental approaches.
Nematodes are small (0.2 - 2 mm) worm-shaped animals with an eel-like way of moving. They occur in every soil sample, normally in high abundances (several million per m^2) and a considerable species diversity (20-60 per sample). Moreover, within the soil nematode fauna functional groups can be distinguished based on feeding type or life history characteristics. Bongers (1990) developed an ecological index which can be used to detect disturbances or ecological recovery of local nematode communities. In many aspects, nematodes can be regarded as intermediate between the microflora and meso/macro soil fauna. This intermediary position is not only based on size. Nematodes form an important link (transfer channel) in the soil food web. They are one of the most important consumers of bacteria and fungi, and they serve as a food source for larger animals like, springtails and mites.
Nematode sampling requires only small quantities of soil (50-100 g). Soil samples can be mixed to compose a bulk sample from a large area. Culturing is easy for a number of bacterial feeding species in growth media or on agar plates. Despite the fact that the taxonomy of certain groups is still difficult, many nematodes can be identified to species or genera. For experimental purposes individuals or communities can be manipulated very well. These ecological and practical characteristics render the nematode fauna suitable for investigations on community structure in contaminated soil. This was the core attention of the nematological research in the Validation project.

7.2 Research on freeliving nematodes

This chapter describes the research on the effects of zinc on free living soil nematodes within the Validation project. As a starting point, it was aimed to study the effects of zinc on nematodes at three levels of complexity: in laboratory tests; in the experimental field plot in Amsterdam; and by true field sampling near Budel. Detailed results are given in Rademaker *et al.* (1994), Alkemade *et al.* (1996) and Schouten *et al.* (in prep.). These three approaches offer, in principle, the opportunity to compare laboratory toxicity data with field effects and should contribute to answer the two main questions of the Validation project.
During the course of the project, the development of a method for the performance of nematodes in toxicity tests in soil could not be established in the available time. Therefore the

research was focused on the particular value for studying effects on the organisation level of communities.
The experimental field plot in Amsterdam offered the opportunity for controlled treatments under semi-natural circumstances, since the indigenous soil nematodes could be used in all zinc treatments. As discussed in Chapter 1, various complications and uncertainties hamper a simple extrapolation of laboratory toxicity data to effects in the field situation. An experimental field approach permits the choice of factors taken into account. Comparison of laboratory and field data or investigation of the importance of environmental factors can be made in a stepwise manner.
With data from the experimental field plot, the hypothesis was tested whether ecotoxicological risk limits (e.g. HC5 or HC50) are associated with true (adverse) effects on community structure or function in the field, in this case expressed through effects on nematode species in particular. The effects can be expressed in different forms, e.g. through species number or diversity indices and other measures of community structure or function. In paragraph 7.3.2 effects are quantified with various possible parameters, and compared to risk limit concentrations calculated for the experimental soil type. The EC5 for the effect on community endpoints was considered to be the concentration level to compared to the field HC5. The current approach addresses a partial validation of the risk limit obtained by the extrapolation method, since it is based on only one taxonomic (but diverse) group.

Determination of effects in the field is a final check on the validity of an ecotoxicological risk limit. It should be noticed, that due to the way environmental quality objectives have been derived on one hand and the practical limitations of ecotoxicological research in the field on the other, every attempt for validation of risk limits stays incomplete. One way to address the question of toxicant levels, that cause effects in the field situation, is to sample pollution gradients. In the literature several classical cases can be found. The advantage of research along gradients is that there is a reasonable possibility that many environmental factors are comparable while the pollution increases gradually. The heavy metal pollution around Budel is one of the largest and best investigated in The Netherlands (see Chapter 2). Zinc is the major component in the (former) metal emissions. The composition of the nematode fauna was analysed at 10 sampling points within a range of 1.1 to 20 km of the zinc factory, and species occurrence (or community parameters) could be related to soil pollution by multiple regression. EC5-field values were compared to the site specific soil quality objectives.

In the Validation project most effort was spent on the research in the experimental field plot. The sampling programme lasted almost two years and ecotoxicological data were collected on both species and community level. As indicated before, emphasis is given in this chapter to effects on community characteristics.

7.3 Results

7.3.1 Laboratory toxicity tests with nematodes

Several toxicity tests on free-living nematodes have been developed in the past, mainly on aquatic and marine nematodes. Most of this work was performed in liquid media or on agar plates (Haight *et al.*, 1982; Howell, 1984; Samoiloff *et al.*, 1980; Bogaert *et al.*,1984; Vranken *et al.*, 1985, 1986, 1988, 1991). Recently, Kammenga *et al.* (1996) proposed a standardised test method for free living nematodes in soil. Further acceptance and incorporation in an international guideline has to take place yet.

Nematode research in the Validation project made a small contribution to the development of a toxicity test in soil. However, it was not possible to obtain test results on short notice in order to compare laboratory and field observations. Therefore data on zinc toxicity had to be collected from the literature. The work of Kammenga *et al.* (1994, 1996) would be most applicable in this context. Unfortunately experiments were carried out with another selection of toxicants: cadmium, copper and pentachlorophenol.

Acute toxicity data for nematodes and zinc are scarce. Haight *et al.* (1982) describe experiments with axenic cultured *Panagrellus silusiae* in (physiological) salt solutions. Different LC50 values are given for zinc, depending on the duration of the tests, ranging from LC50-72h = 20 mg Zn/l for juveniles to LC50-24h = 255 mg Zn/l for adults. Growth was not (significantly) inhibited in this case until 500 mg Zn/l. Haight *et al.* (1982) conclude that, compared to other well-known aquatic tests organisms like *Daphnia magna*, the nematode *P. silusiae* is relatively resistant to heavy metal poisoning. From an ecological point of view this is not surprising. The species is a typical fast growing r-strategist, belonging to c-p1 group[1] in the classification of Bongers (1990). Under the experimental circumstances used by Haight *et al.* (1982) the species completed its life-cycle in 96 hours. A typical opportunistic life style often combines with tolerance to severe environmental conditions. Mudry *et al.* (1982) delivered a possible mechanism for the relative high metal resistance of *P. silusiae*. It appeared that the proportion of nematodes feeding, measured by the pumping rate of the oesophagus, decreases significantly at longer duration of metal exposure. More effect concentrations of zinc are given in the above mentioned publications, they are not discussed in detail here.

It is difficult to summarise a range for observed zinc-EC50 values from the literature without differentiation to the concerning parameters (mortality, growth, fecundity) and exposure time. Moreover, it can be questioned whether or not toxicity data on marine animals are relevant for terrestrial nematodes. As a rough indication, EC50 values range from 0.38 to 255 mg Zn/l (in solution). Apart from certain conflicting results it is clear that fecundity and mortality are the most sensitive toxicity criteria (Vranken *et al.*, 1991).

The only soil toxicity test on the effect of zinc on nematodes is described by Korthals *et al.* (1996). These investigations were made in artificially contaminated arable soil (pH-KCl 4.1, 1.9% organic C, 4% clay) containing the indigenous nematode community. Soils were treated

[1] c-p refers to scaling of species on a range between colonizers (c) and persisters (p)

with zinc sulphate and incubated for 1 or 2 weeks. Both direct and indirect effects may have taken place by exposure to heavy metals and differences in the availability of food. On average 17 different taxa were found in the control samples. In total 36 genera were identified. EC50 values for zinc could be obtained for 10 taxa, and varied between 52 and 1538 mg Zn/kg soil. In this short-term experiment, the genera *Aporcelaimellus, Filenchus* and *Plectus* appeared to be most sensitive for zinc. As can be concluded from the lowest and highest EC50 values, differences between taxa may vary up to a factor 30. The intra-taxon differences in EC50 were always less than a factor 4.5.

7.3.2 Effects of zinc in the experimental field plot

In the case of free living nematodes, the experimental field plot was used as a partially controlled field situation, to study actual ecological hazards at the community level. As will appear from the gradient study in Budel (see 7.3.3 below), soil characteristics other than metals can play a dominant or unseparable role in the occurrence of species. Ecological effects of heavy metals can not be distinguished and quantified in that case.

To investigate the effects of gradual zinc concentrations on a community level (total abundance, diversity etc.), the development of the natural nematode fauna in the experimental field plot soils was followed. Before the start of the field plot experiment, a bulk of 6 m^3 soil was prepared (see Chapter 2), by sieving, mixing, drying, and the addition of zinc solutions. Because this soil pre-treatment might have detrimental effects on inhabiting nematodes, a sample of 10 kg was taken from the original bulk of soil material. The sample was stored at 4 °C and used to inoculate the compartments in the experimental field plot with the original nematode fauna.

Free living nematodes were sampled at 3, 10, 22 months after the compartments were filled with zinc contaminated soil in the nominal concentration series of: 0, 32, 56, 100, 180, 320, 560, 1000, 1800, and 3200 mg/kg. Sampling took place in September 1994, May 1995 and May 1996. At these dates, four small soil cores (Ø 1,7 cm, 10 cm deep) were removed from each of the 50 compartments with an auger. The soil cores (per compartment) were mixed and treated as one sample. Nematodes were extracted according to the standard 'Oostenbrink- and cotton wool filter method' (washing, sieving, active movement). Next, total numbers were counted in subsamples (5% or 10% of total). After counting, the entire sample was preserved by adding hot formaline (s' Jacob & Van Bezooijen, 1984). Per sample 150 randomly chosen individuals were identified with a Leitz reversed microscope (400x).

- Total numbers of nematodes

At the start of the field experiment (June 1994), the soil contained, in total, circa 600 nematodes per 100 gram fresh weight. Despite the treatments and manipulation prior to the beginning of the experiment the nematode abundance was still high enough for further community development and measurement of zinc effects. The inoculum appeared to be of little effect on the abundances or species composition.

In Figure 7.1 the total number of nematodes (individuals) is shown at different concentrations, after 3, 10 and 22 months exposure.

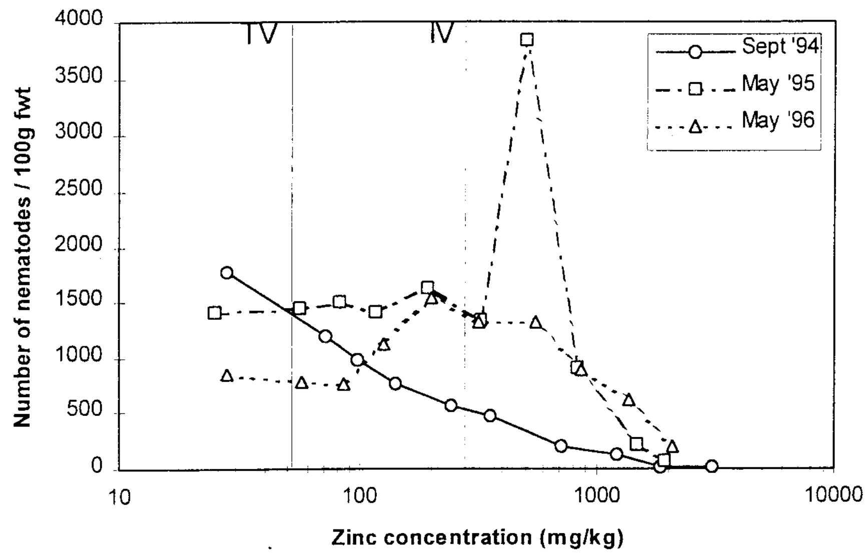

Figure 7.1. Nematode abundance in the experimental field plots after 3, 10 and 22 months exposure to different zinc concentrations (given as measured total Zn). For comparison with contemporary soil quality objectives, the pertinent values for the experimental field plot are added. TV= target value and IV= intervention value (see Chapter 2). Note that density at t=0 was 600 ind./100g fresh weight.

After the first three months, the total nematode abundance shows a strong dose related response to zinc treatment. In the control, numbers have grown from 600 to 1800/100 g. The zinc concentration in the control soil appeared to be 28 mg/kg. This must be regarded as the background concentration. Every additional application caused a negative effect. The 3 months-EC50 for the total number of nematodes is 1067 mg Zn/kg (95% conf. int. 854-1332). This calculation is based on the zinc concentrations measured in February 1995, because abiotic factors were not sampled September 1994. Further details and calculations based on other fractions are given in Schouten *et al.* (in prep.).
On the next two sampling dates total numbers of nematodes showed a different response pattern. In May 1995 the abundance at lower concentrations seemed to be recovered. An inhibition of the community size cannot be found anymore at lower concentrations. Nematodes at the nominal concentration of 560 mg/kg exhibit an high abundance, caused by the genera *Mesorhabditis* and *Aphelenchoides*. The reason for this peak is unclear. Possibly it is caused by indirect effects, like the availability of food (fungi) or absence of predators. The three highest concentrations clearly have a strong negative effect on nematode numbers. The 10 months-EC50 for nematode abundance increased to 1945 (1755-2155) mg total Zn/kg.
In May 1996 the situation seems to be stabilised. The peak density at 560 mg/kg has returned to a normal level. At the three higher concentrations community size is still reduced. An interesting effect can be found this time in the beginning of the concentration range. The

reduced number of nematodes probably reflects a nutritional exhaustion of the compartments. After all, these small ecosystems lack a supply of organic matter and nutrients, while decomposition processes are incomplete and disturbed. The foregoing assumption is supported by results from the microbiological research in this experimental field plot where lower number of micro-organisms were found at the lower concentrations (Rutgers, personal communication). The 22 months-EC50-abundance of May 1996 was 2862 (2353-3481) mg total zinc/kg, and thus higher than the previous sampling dates.

- *Number of nematode taxa*

Despite the fact that the total number of animals in a taxonomic group often is regarded as an insensitive characteristic, the nematode abundances give useful results about effects on (a part of) the communities in the experimental field plots. More detailed information can be obtained from the diversity (number of species, or better taxa in the case of nematodes) at different zinc concentrations. The effect parameter 'number-of-taxa' is also better comparable to extrapolated risk limits predicting the percentage of species that may encounter negative effects.

Results on the effect of zinc on the average number of nematode taxa are summarised in Figure 7.2. A comparison with the taxa in the mixed soil fractions at the beginning of the experiment is difficult to make because a different sampling technique was used and the average number of taxa cannot be calculated in the same way as was done for the experimental field plot. However, a detailed list of species found in 'pre-treatment soils' is given in Schouten *et al.* (in prep.). From this list it can be concluded that in total 22 taxa were present in the soil before artificial pollution with zinc. On average 14.6, and a maximum of 19 taxa were found in the control plots, 3 months after the start of the experiment.

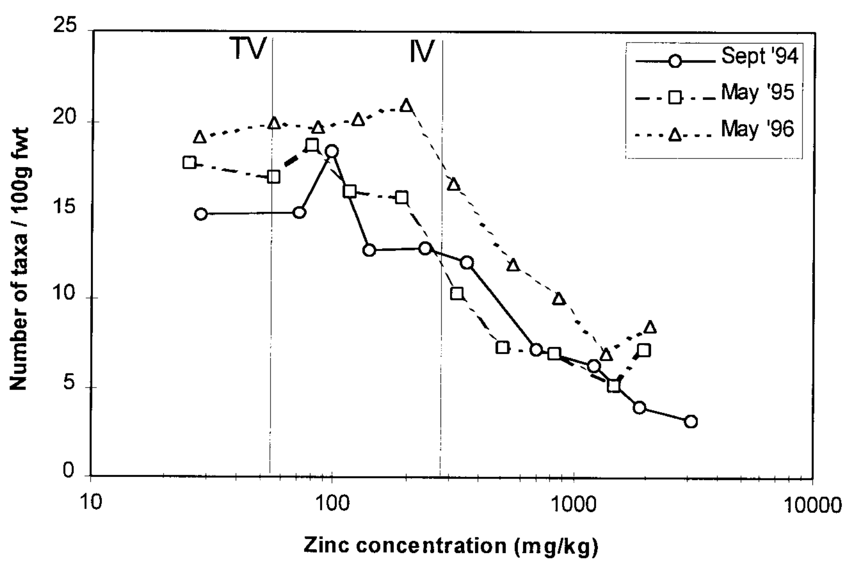

Figure 7.2. Average number of nematode taxa in experimental plots after 3, 10 and 22 months exposure to 10 zinc concentrations (given as measured total Zn). TV= target value, IV= intervention value.

A sigmoid-like response can be recognised in the number of taxa at all three sampling dates. More than found for the total abundance, the number of taxa show a remarkable constancy in the reaction pattern. During the experiment, there is a tendency for an increasing average number of taxa at most zinc concentrations.
The EC50 values for the number of taxa tend to show an increase with time: Sept. 1994: 574 (95% conf. int. 403-817) mg Zn/kg, May 1995: 434 (226-833) mg Zn/kg, May 1996: 934 (688-1271) mg Zn/kg. The average EC50 values on the three sampling dates do not differ significantly, as can be seen from the overlapping confidence intervals.

- Diversity index approach
EC50 values for the Shannon-Weaver diversity index were similar to the EC50s obtained with the species numbers. This can be expected because the index is based on the same data. The diversity index seems more sophisticated because the relative abundance of species is taken into account. However, it is also sensitive for rare species. More details and results on indices are given in Schouten *et al.* (in prep.).

- Reactions of particular species
In the Validation project, soil nematodes were especially used to study effects on community parameters. Nevertheless, detailed information about the reaction of different taxa is available. Taxa that obviously decline at zinc concentrations higher than 100 mg/kg are: *Anaplectus, Eucephalobus striatus*, and Qudsianematidae (probably *Eudorylaimus*). Most insensitive were *Acrobeloides* (only last sampling data), *Aphelenchoides, Ditylenchus, Plectus* and Rhabditidae.

7.3.3 Nematode communities in Budel field soils
Effects of chronic heavy metal exposure on species composition and community characteristics were studied along a gradient near the zinc factory at Budel. The former melting and production methods, between 1892 and 1973, caused a historic pollution gradient. Zinc is the dominant heavy metal in the soil, but significant amounts of lead, copper and cadmium do occur (Edelman *et al.*, 1985). The forest area around Budel is situated on poor sandy soils. As a consequence of prevailing wind directions, the pollution reaches out in north-east direction. Bulk samples were taken in this direction at 10 distances (1.1, 1.6, 2.6, 5, 6.6, 7.9, 9.6, 12, 15 and 20.7 km) from the factory in undisturbed Scotch pine stands. The zinc content ranged from 1787 mg/kg nearby to 11 mg/kg distant from the factory. The soil material was primarily collected as substrate for earthworm toxicity tests (Chapter 5). A well-mixed subsample of 300 gram was used to extract the indigenous free-living nematodes. Several characteristics of the nematode fauna were determined from the abundances and species composition (Alkemade *et al.*, 1996). Log-logistic regression models were not able to describe a relationship between the abundance of nematodes and soil zinc content. Therefore stepwise linear regressions were carried out between biotic parameters and total zinc in the soil. The influence of soil factors (pH, organic matter, water content, CN-ratio) was compensated for by defining them as cofactors in the regression models. This approach gave complications for the estimation of

effect measures like NOEC, EC50 or EC5, commonly resulting from logistic response models. It was chosen to define a field-EC5 as the concentration at which the abundance of a taxon (or parameter) has declined to 95% of its value in the undisturbed situation (or end of gradient, in this case 20.7 km from the factory). Meaningful results can only be obtained when biotic parameters decline at higher zinc levels. Field effect concentrations calculated in this way were compared to location specific Target Values.

Concentrations of the different heavy metals in the soil appeared to be highly correlated. Moreover, there was also a strong positive correlation ($R^2 = 0.90$) between acidity (pH) and the zinc content. This hampers straightforward conclusions on the separate effects zinc only.
In total, thirty-five different nematode taxa were found. The genera *Acrobeloides, Aphelenchoides, Cervidellus, Filenchus* and *Plectus* appeared in every sample. Only 11 of the 35 taxa were found at least 7 (out of 10) sampling points. Besides the community characteristics, regression analysis was carried out with the selection of 11 taxa. The results are summarised in Table 7.1, in which only significant models are shown.

Table 7.1: Coefficients of the descriptor variables of significant regression models for nematode taxa and community characteristics along a heavy metal gradient at Budel. Results are based on forward stepwise multiple regression with log-zinc content and soil characteristics (cofactors). Abundances of individual taxa and total nematode numbers were entered as log-transformed values. The % C was never selected in the models (no regression coefficients) and was left out of the table. H'= Shannon- Weaver index, PPI= plant parasite index.

Effect parameters	Const.	log (Zn)	pH	%H_2O	C/N	R^2	P
Community level							
log (number nematodes)	3.510			0.03	-0.021	0.74	0.01
Number of taxa	7.068	5.268				0.57	0.01
Diversity index (H')	0.560		0.109			0.39	0.05
PPI	0.887	-0.378	0.550			0.62	0.04
Number of bacterial feeding nematodes	4.471		-0.133		-0.037	0.76	0.01
Taxon level							
log (*Acrobeloides*)	3.931	-0.281		0.052	-0.052	0.78	0.02
log (*Cervidellus*)	1.425	0.939				0.42	0.04
log (*Filenchus sp.2*)	5.067		-0.363		-0.061	0.61	0.04
log (*Wilsonema*)	3.257	-1.171				0.78	0.001

In total, five out of nine community properties were described by significant multiple regression models. The number of taxa and (related) diversity index show positive coefficients for zinc and pH respectively. Both increase closer to the zinc factory. In contrast to results described in the previous paragraph (experimental field plot), a statistical association between zinc and the number of species (biodiversity) could not be demonstrated. Probably the pH effect has become a dominant factor in this situation of chronic mixed metal exposure with low bioavailability. The total number of nematodes was mainly related to moisture and CN-ratio. The number of bacterial feeding nematodes showed a negative response to increasing pH and CN-ratio. Only the plant parasite index (PPI) had a negative zinc-coefficient, but at the same time there is a (counter-acting) positive relation with higher pH-values. Therefore it is difficult to explain the PPI-model.

Looking at separate taxa, the occurrence of four individual taxa could be described by a significant regression model. *Acrobeloides* and *Wilsonema* decrease with higher zinc concentrations (negative Zn-coefficient). *Cervidellus* shows a positive reaction on zinc and *Filenchus* is negatively related to soil-pH. As a consequence of the high correlation between zinc content and pH, the response of taxa to zinc or pH might also be explained vice versa.

These results lead to the conclusion that only for two genera (*Acrobeloides*, *Wilsonema*) the abundance is negatively effected by zinc. When the negative coefficients of (increasing) pH are supposed to be effects of zinc too, a third genus (*Filenchus*) and the group of bacterial feeders can also be used to compare field effects with risk limits.

Field-EC5 values calculated from multiple linear regression models were checked with the Target Value, corrected for the composition (clay and organic matter) of Budel soils. So the zinc content was estimated on the point along the gradient where 95% of the number of individuals of the reference (20.7 km from factory) were found. Moreover, levels of the other heavy metals in the soil were calculated at the point were zinc reached its field-EC5. Results are given in Table 7.2

Table 7.2 Field-EC5 of zinc (mg total Zn /kg) for three nematode taxa and the group of bacterial feeders, calculated under the assumption that zinc (rather than pH) has caused the effects in these taxa. Also given: site specific Target Values (TV), Intervention Values (IV) and concentrations of other metals at the field-zinc-EC5 point. All concentrations in mg/kg (total metal)

Taxon		Associated metal concentration		
	EC_5 Zinc	Lead	Copper	Cadmium
Acrobeloides	32.1	34.3	4.6	0.3
Filenchus sp. 2	19.4	24.8	2.9	0.3
Wilsonema	13.4	19.6	2.0	0.2
Bacterial feeders	125	81.6	16.7	0.7
TV	60	55	18	0.5
IV	310	345	97	7.6

Table 7.2 suggests that the effects of zinc (EC5) on the three individual genera may already occur at concentrations below its local Target Value. The group of bacterial feeders represents a higher ecological organisation level. While effects and reactions of different species are averaged, consideration of this group as a whole may have yielded a less sensitive parameter.
Concentrations of other heavy metals at the zinc-EC5 point were calculated on the basis of the high mutual correlations between concentrations of the different metals (> 0.9). Therefore, the given concentrations are also an approximation of the EC5 of these metals. From the results in Table 2, it is obvious that all four heavy metals may cause effects below the TV. Except for zinc this is also true for the group of bacterial feeders. It is likely that the mix of metals will cause a combined toxic stress. In this case the field-EC5 of zinc is likely to be an underestimate of the true value, since (without the other metals) more zinc would be needed to cause the same response. For that reason, individual Target Values are not sufficient in a complex environmental pollution situation. In view of the fact that the effects of zinc (or other metals) could not be disentangled from the factor "soil pH", it must be concluded that the field study at Budel in fact did not support nor contradict the current risk limits.
Possibly the calculated field-EC5 values are low because of the dominant pH-effect along the gradient. This uncertainty or complication can however not be solved on the basis of field observations alone. One possible way to tackle this problem is to compare the pH preference of the concerning taxa. Field data from the Dutch Soil Quality Monitoring Network can be used to derive response models and optima for abiotic factors (Alkemade & van Esbroek, 1994). The available data indicate that *Acrobeloides, Filenchus* and *Wilsonema* occur most frequently at lower soil pH. In the pH range found at Budel, *Acrobeloides* has more or less an equal change of occurrence. From this point of view, it is most likely that the occurrence *Acrobeloides* is influenced by zinc (metals) alone. *Filenchus* and *Wilsonema* decline at the higher pH-values in the range. Nevertheless, experiments performed by Korthals (1997) indicate that *Filenchus* is quickly influenced by zinc too. In short-term (2 weeks) toxicity experiment the EC50 for zinc was found to be 141 mg/kg.
When effects on the three genera are truly caused by zinc, then three out of thirty-five taxa (9%) encounter negative effects. However, this conclusion has to be interpreted with care. In the first place, only eleven taxa appeared in numbers high enough to derive response models. So, for twenty-four genera no conclusions can be drawn. Secondly, there is a strong correlation between zinc (metals) and soil pH in the Budel gradient. The abundance of nematode numbers and taxa can also be affected by other environmental factors. Results of this field research clearly show the importance of including soil characteristics when ecological effects of pollution are to be judged. To distinguish between effects of toxicological and ecological nature, additional methods for risk assessment have to be developed.

7.3.4 Validation of risk limits with nematode data from the RIVM National Soil Quality Monitoring Network

The National Soil Quality Monitoring Network (in Dutch: LMB) consists of 200 locations, representing 10 combinations of the most common soil types and land-use. The aim is to give a global picture of the soil quality (on a national scale). The network was primarily designed for measurement of chemical /physical characteristics and pollution of the soil. The 200 locations are sampled in a five year cycle. Soil nematodes have many practical qualities for quick and large scale monitoring activities. They were therefore included as a first biotic component in the Soil Quality Network. Biological data can be combined with those on soil composition and levels of pollutants. By means of statistical (non-linear) regression techniques, empirical response models can be derived for one or more environmental factors.

Until now data are available for 112 locations. These locations are spread out over the entire country and include cattle farms, arable farming and forests, all on sandy soils. In order to compare ecological effects with risk limits, the occurrence (presence/absence) of nematode taxa was related to the concentrations of heavy metals (zinc, copper and lead) by multiple logistic regression. Soil characteristics like pH, clay and organic matter content were included in the regression models. Subsequently, for individual taxa the probability of appearance was calculated as function of heavy metal content and soil factors (Klepper *et al.*, 1997).

Significant multiple regression models were obtained for 62 taxa. Of those, 22 were influenced negatively by one or more heavy metals. The effect of a specific metal can be investigated by varying its concentration while all the other factors in the regression model are kept constant. The influence of a metal was judged to be negative when it reduces the probability of appearance to less than 50% of the average abundance. Figure 7.3 summarises the results of the model calculations.

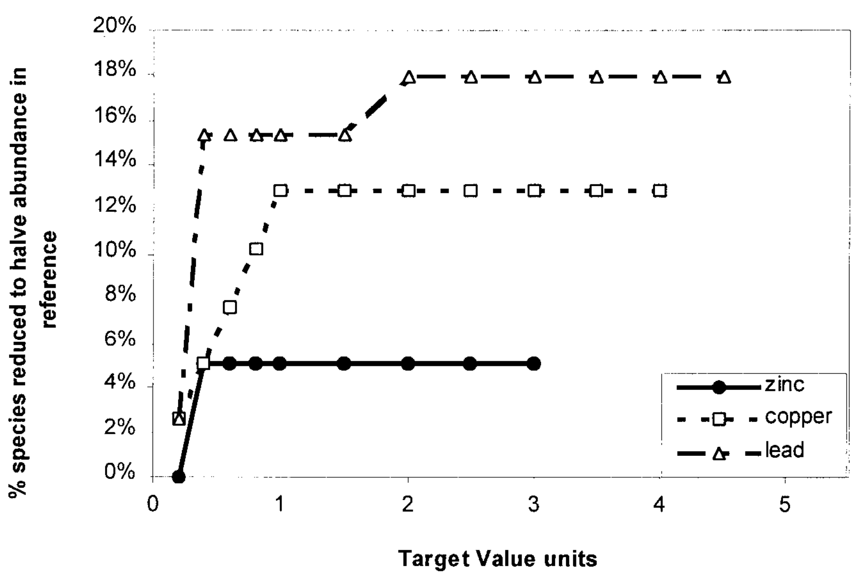

Figure 7.3. The fraction of nematodes with a strongly reduced probability of appearance (< 50% of the average) as a consequence of heavy metal concentrations, expressed in Target Value units.

In the set of field data, lead is the most important factor with a negative influence on the nematode fauna. At the Target Value, 11% of the species experience negative effects. Zinc is less harmful, at least within the low concentration range present in the LMB field samples. In this range, 4% of the taxa are influenced and the maximum is already reached at concentrations near the Target Value. It can be concluded that heavy metals influence the probability of appearance of nematodes in field soils recently collected in The Netherlands, even at concentration levels below the Target Values.

7.4 Discussion

- Standardised toxicity tests

Until now there are not enough results from standardised toxicity tests with soil nematodes to make a good comparison between laboratory and field effect concentrations. Species used in toxicity tests are often the better culturable (and probably insensitive) ones. Recent work of Kammenga *et al.* (1996) provided promising results for more relevant, moderately sensitive, soil inhabiting species. Within the Validation project, nematode effect studies contributed mainly to the second line of investigations: evaluation of the ecological significance of risk limits.

- Experimental field plot

In the experimental field plot effects of zinc could be examined without a part of the unavoidable complications of a natural ecosystem or historical pollution. The influence of zinc on nematodes was mainly worked out in terms of community characteristics. Effect concentrations for the number of species seem closely related to predicting the fraction of potentially endangered species with the extrapolation method.

Average EC5 values for total number of nematodes ranged from 110 to 1152 mg/kg (Schouten *et al.*, in prep.). EC5 for the number of taxa was found to vary between 77 and 106 mg/kg. These results were obtained for samplings after three different exposure periods. Most of the EC5 values lay close to, but above the (site specific) test field soil Target Value of 61 mg/kg. However, the lower part of the 95% confidence interval often stretches out beneath the TV.

The Intervention Value was calculated to be 312 mg/kg. EC50's for total nematodes, number of taxa, and the diversity index can be compared with this ecosystem risk limit (HC50) too. It appears that EC50 values of the three community parameters were always higher the Intervention Value of 312 mg/kg. Most close to the Intervention Value came an EC50 estimation for the Shannon Weaver diversity index after 10 months exposure. This EC50 value of 385 mg/kg total zinc was however quite distinct from comparable estimations on the other sampling dates. Reactions of individual species to zinc are available but have not been used to compute effect concentrations.

As was the case for standard toxicity tests, literature data comparable with the experimental field plot are scarce (e.g. duration, metal and circumstances). Korthals (1997) describes a one year container experiment including effects of vegetation (perennial ryegrass) and a 6 months pot experiment, both with combined effects of zinc and copper. However, Korthals used much lower concentrations (0-200 or 400 mg/kg) and soil pH was substantially lower (ca. 4.3 versus 5.5 to 7). The most direct comparison of results from this project and the work of Korthals (1997) can be made on the basis of EC50-$CaCl_2$ values for the total number of nematodes, and the number of taxa. In this case, experimental field plot results after 3 months exposure are used. Recalculated in extractable EC50s, a value of 225 (abundance) and 61 mg/kg (taxa) extractable zinc was found. Korthals (1997) gives EC50 values of 204 and 457 mg/kg respectively. For the total nematode abundance, results correspond quite well. The higher value of 457 mg/kg for the effect on taxa is probably due to a 55.5% zinc extraction obtained in the experiment of Korthals (1997). To make available data better comparable it would be of value to recalculate more results from both studies to the same units and effect measures. Sensitive taxa mentioned by Korthals (1997) are: *Acrobeles, Alaimus, Aporcelaimellus, Clarkus, Drilocephalobus, Eucephalobus, Prismatolaimus, Thonus*.

- Field sampling at Budel

The gradient study in Budel should have provided useful information to compare effect concentrations for different soil nematode species with risk limits. Strong correlation of soil characteristics with the pollution gradient near Budel hampered a simple and straightforward comparison. Moreover, the biological data were too limited or not detailed enough to estimate effects on the total species composition. This was caused by the dominance distribution of the taxa (many species in low frequency) and may also be influenced by effects of a single sampling. In general, repeated measurements show a reasonable constant species composition. However, the abundance of the individual taxa may fluctuate with temperature, moisture and availability of food (bacteria and fungi).

In a historical pollution gradient like the one at Budel, most sensitive species probably disappeared a long time ago. However, ageing of the pollution reduces bioavailability as was for instance shown in the earthworm experiments (see Chapter 5). Therefore recolonisation and re-establishment of populations must be considered. Moreover, also adaptation to high heavy metal concentrations may have taken place as was found for the microbial community (see Chapter 8). Part of the remaining uncertainty could be solved by an extended sampling programme, but the strong relation between soil characteristics and metal gradient will make it impossible to furnish a clear proof of heavy metals effect in the Budel gradient.

By rough estimation, 9% of the nematode genera along the gradient may have experienced negative effects of zinc. It is striking that species whose response could be described by multiple regression models at Budel all belong to the general occurring dominant nematodes in the soil. The most probable explanation is that significant models were obtained for the general species because they appeared in appropriate high numbers on every sample point along the gradient. Moreover, there is still a fair chance that the effect on population density was caused by the soil pH. Especially *Acrobeloides*, and to a lesser extent *Filenchus*, are supposed to be resistant to several kinds of environmental stress. Nevertheless their field-EC5 values for zinc were calculated to be low, 32.1 and 19.4 mg Zn/kg respectively.

An attempt was also made to compare effects on the same genera in the experimental field plot. The genus *Wilsonema* (a typical forest species) did not occur in test soil, so effect concentrations could not be compared in this case. Furthermore, the response of *Filenchus* in the experimental field plot did not follow a logistic response, and EC5 or EC50 values could not be calculated. As a rough estimation, the genus decreased obviously above 315 mg/kg total zinc after 22 months exposure. Numbers of *Acrobeloides* were already influenced in the lower concentration range at the first two sampling dates (3 and 10 months). Zinc-EC5 values were calculated to be circa 17 mg total Zn/kg on both sampling dates (EC50: 89 and 266 mg Zn/kg resp.). The response after 22 months, again could not be described by a logistic model. The genus had recovered at the lower concentrations and was reduced in numbers only above 860 mg/kg total zinc. Comparison between field results and the experimental field plot stays incomplete. The first two sampling dates seem to confirm the sensitivity of *Filenchus* and *Acrobeloides* for zinc. However, the effects changed with time, and may rather be caused by indirect than direct effects.
Experience obtained with the gradient sampling at Budel leads to the suggestion that field research should continue by investigating other suitable pollution gradients and comparing contaminated sites with an undisturbed reference. In this way a better and more detailed insight must be obtained in the effect concentrations attributable to heavy metals alone. Another approach that can be followed (and carried out practically) with soil nematodes is the check for development of Pollution Induced Community Tolerance (PICT-concept see Chapter 8). Development of PICT has been confirmed for marine nematode species (Millward & Grant, 1995). Use of the PICT method has the advantage that responses become independent of (variable) abiotic factors other than metals.

At the end of this overview, the basic validation question still remains to be answered: What do the foregoing results and discussion tell us about the relevance of soil quality objectives based on an extrapolation method for laboratory test results ?
- At first, the nematode fauna represents only a part of the organisms that occur in the soil. Free-living soil nematodes embody ecological diversity in the sense of different life history tactics and functional (feeding) groups. Both sensitive and extremely tolerant species occur. Nematodes offer many practical possibilities for environmental and policy oriented research. Despite these facts it is somewhat premature to postulate that the variation in nematode sensitivities covers the entire spectrum of soil inhabiting organisms. Factors like bioavailability, exposure routes and indirect effects cause complicated reactions in a polluted ecosystem. In that respect it is unrealistic to consider effects on nematode species as a random sample out of the entire soil ecosystem. However, by the lack of alternatives it could be a first approximation.
- Contrary to the experience with Budel data, the number-of-taxa in the experimental field plot appeared to be a very useful variable to follow effects on a community level. Both the Target Value and Intervention Value were sufficient to protect average community characteristics of the nematode fauna (abundance, taxa, diversity index) in this situation.

However, examining the effect on community characteristics in this way also provided relative insensitive response-variables, as is explained below.

- Effect concentrations for community characteristics (e.g. diversity) were used as a verification of the Hazardous Concentration values for ecosystems, extrapolated from laboratory test results. The two approaches are however quite different and in fact the outcome cannot be compared directly. Field community parameters appear to be relatively insensitive. This can be expected because many species contribute to a community characteristic. These species will represent a broad range of sensitivities for a certain kind of pollution. So the effect value of the community parameter will average out at a high level. A better solution may be found in an analogous approach as followed with the extrapolation of laboratory toxicity data. Instead of an average community sensitivity, ecotoxicological responses of species in the field could be used to estimate the distribution of NOEC's and to calculate a HC5 value. In this case input data for the extrapolation method would be based on realistic field observations, including all kinds of direct and indirect influences. However, field investigations often do not have the right sampling design to estimate NOEC's (repeated measurements at different concentrations). This can be an item in future ecotoxicological research.

- Field investigations at Budel showed no effects on the number of species in the community. Such an overall parameter may however, be influenced by variation in soil characteristics and the substitution of species. From observations on individual taxa it was estimated that 9% of the taxa may have encountered negative effects at the Target Value for zinc in that situation. Regarding the many uncertainties, like the coinciding pH gradient and the few species that could be used in this judgement, the data do not give an obvious clue towards rejection of the Target Value for zinc. The experience with this kind of validation research justifies a further development of the PICT method and an extension of field research at comparable polluted sites.

- The 112 sites in the national survey were not selected for reasons of soil pollution. Therefore, heavy metal concentrations were much lower than those in Budel and experimental field plot soils. Nevertheless effects were found even below the Target Value (TV). Zinc appeared to have a serious negative effect on 4% of the species at the LMB averaged (soil specific) TV of 87 mg/kg. The dataset contained zinc concentrations in the range up to eight times the TV. But no further zinc effects were found on top of the 4% that was reached below the average TV. In this case the analysis is based on reactions of individual species.

Taken into consideration that the Target Value should be a No-Effect-Concentration, but that the HC5 and TV lay close together, there are no indications that the intended protection level seriously deviates from the effects found in the field situation.

Several approaches (experimental field plot, field sampling, analysis of monitoring data) were used to investigate the validation question. It can be concluded that effects on nematode communities often support, and sometimes contradict the protection level that is aimed by the soil quality standards. However, considering the remaining uncertainties, effects do not seriously deviate from the risk limits. It is recommended to develop certain techniques (e.g. PICT) and extend research in pollution gradients.

7.5 Conclusions

1. Free-living soil nematodes are a very suitable group to compare risk limits with toxic effects on community characteristics like number-of-species or diversity indices. However, it must be realized that properties on the community level are somewhat insensitive compared to species-level endpoints. The effect value of the community will be an average of the responses of susceptible and resistant species.
2. Obvious effects on community endpoints were found in the experimental field plot. The number of taxa appeared to be a good (reproducible) and relatively sensitive characteristic. The EC5 and EC50 effect levels for this characteristic were very close to the soil-specific Taget Value and Intervention Value for zinc.
3. Field research at the pollution gradient near Budel did not yield obvious ecotoxicological effects on the nematode community. Conclusions on the effects of metals were hampered by a strong correlation between soil-pH and metal concentrations. Moreover, the biological data were too limited or not detailed enough to estimate effects on the community level. Three of the individual species (9% of the taxa in the community) encountered negative effects that may be caused by heavy metals in the soil.
4. Data from the RIVM Soil Quality Network indicate that zinc may have serious negative effects on 4% of the species at the average Target Value of 87 mg/kg calculated for the sampled soil series. The Target Value for each location is, however, associated to background concentrations in nature areas, and has no relationship to ecotoxicological risk limits. Nonetheless, in the true field situations from the Network, small exceedance of the Target Value is related to minor effects on nematode communities.
5. Until now there are not enough results from standardised toxicity tests with soil nematodes to make a good comparison between effect concentrations obtained in laboratory- and field research. There are however, recent promising steps towards the development of standardized test systems with nematodes.
6. It can be concluded that effects on nematode communities often support the protection levels that are aimed at by the soil quality standards, or the results are not unequivocally attributable to the supposed toxic agent. However, considering the remaining uncertainties, effects do not seriously deviate from the risk limits.
7. It is recommended to develop new techniques like PICT for soil nematodes and extend research in pollution gradients.

8. EFFECTS OF ZINC IN SOIL ON MICROBIAL MINERALIZATION AND MICROBIAL COMMUNITIES

P. van Beelen, J.W. Vonk & M. Rutgers

8.1 Introduction

Micro-organisms play an important role in the soil ecosystem, mainly because they contribute considerably to the mineralization of organic matter and the cycling of nutrients through the biosphere. The aim of the present part of the project was to gain insight in the effects of metal contamination on soil micro-organisms in the field, in particular for zinc, and, for validation purposes, to compare the results obtained with field-contaminated soils with the results of tests carried out with soils contaminated in the laboratory. Research was focused on the question whether it is necessary to establish a laboratory-to-field correction factor for extrapolation of laboratory data to the field situation.

This Chapter is divided into two parts. In the first part microbial tests in freshly contaminated soils are compared with tests with field-contaminated soils. In the second part ecological changes due to metal contamination are measured. The first part gives an indication of the field relevance of toxicity tests (aspect 1 of this project, see Chapter 1) while the second part gives information about the ecological significance of environmental quality objectives for micro-organisms (aspect 2).

8.2 Part 1: The representativity of laboratory toxicity data derived from mineralization tests for the situation in the field.

8.2.1 Approach

Toxicity tests with micro-organisms in soil are generally not carried out with a single species. Instead, the effects on microbial processes of a part or the whole microbial community are investigated. Yet, work with single microbial species can give important information on metal toxicity in soil microbial communities. Both approaches have been used in our experiments.

The approach in this chapter differs necessarily from the approach in other chapters because the toxicity of zinc to micro-organisms can not be established in a standard toxicity tests with standard soil. Such tests do not exist. Instead, comparisons were made between different conditions of testing, to assess their relative influence on toxicity.

We selected mineralization of organic substrates as the microbial process to be measured in the toxicity experiments, because it is an ecologically important process. Mineralization was studied at two levels:

1. Mineralization under growing conditions by addition of relatively large quantities of glutamate (Vonk and Matla, 1993).

2. Mineralization under non-growing conditions using low concentrations of acetate (Van Beelen *et al.*, 1991)

One of the restrictions of the "soil microbial process" approach is that it is very difficult to find an uncontaminated site which is acceptable as a reference, because this will in most cases differ considerably from the contaminated site in the composition of the microbial community and thus in the rate of microbial processes. In this respect the experimental field plots with artificially contaminated soil (see Chapter 2) did provide a useful experimental set-up to obtain data.

The soil in these plots was homogenised before the addition of zinc and the construction of the field plot. The initial microbial community was therefore more or less similar in each compartment of the field plot. A less controlled and more realistically contaminated site was investigated near Budel.

We carried out mineralization tests with soil obtained from this experimental field plot and from contaminated and non-contaminated sites in the Budel gradient. [^{14}C]acetate or (non-radioactive) glutamate were added to these soils, and the rate of evolution of $^{14}CO_2$ was followed (for details of the method see: Van Beelen and Notenboom, 1996). It appeared necessary to measure effects of pH on acetate mineralization in soil, because of its strong effects on the rate of mineralization. These pH effects could be quantified by using a single species test with *Pseudomonas putida*, a gram-negative soil bacterium. The effect of zinc chloride on the formation of $^{14}CO_2$ from acetate was subsequently studied in this single species test.

The effects of zinc on the mineralization in several soil types were compared with the effects found in soil from the aged experimental field plots.

8.2.2 Results

- Effects of contaminated Budel gradient soils.

Experiments with soil samples from contaminated and clean (reference) sites from Budel showed that acetate mineralization (i.e.: acetate consumption and CO_2 production of the indigenous microbial community) was highest in the soil with the highest contamination and lowest in the uncontaminated soil from the reference site. This result could be explained by inhibition of the acetate mineralization at the low pH of the reference soil (pH 3.2). The most contaminated soil had a pH of 5.7. Effects of contaminants (mainly zinc) were obscured by the counteracting effect of the pH. Also, the glutamate mineralization was relatively high in contaminated samples compared with uncontaminated samples (Figure 8.1).

The major effect of pH on both microbial function parameters thus rendered the Budel gradient approach on functions redundant for answering the question of field relevance of toxicity tests with micro-organisms. Different ways of research were hence chosen, addressing the role of pH in zinc toxicity in single species test

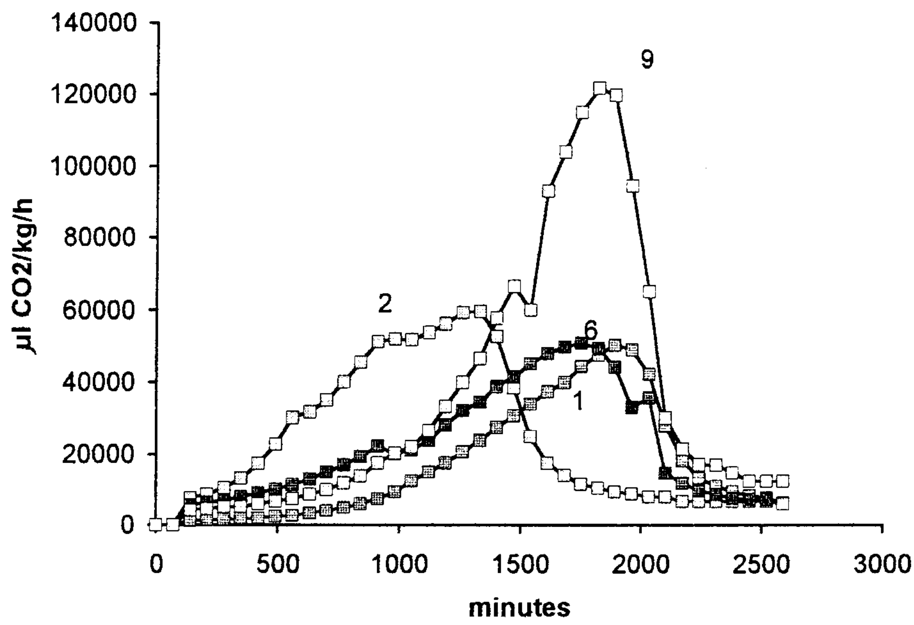

Figure 8.1. Formation of CO_2 from glutamate in Budel soils. The figures near the graphs correspond with the sample locations. 1, 2: contaminated; 6, 9: non-contaminated. The numbers correspond with sample locations mentioned in Chapter 2

- Single species tests with Pseudomonas putida

The effect of the pH on the toxicity of zinc for micro-organisms was tested in freshly contaminated OECD artificial soil. The set-up was very similar to the earthworm toxicity test (Chapter 5). A pure strain of *Pseudomonas putida* MT2 R62 was added to OECD artificial soil. About 1.5 . 10^5 c.f.u.'s were added to 10 g soil. The survival of these added bacteria among the numerous indigenous bacteria was monitored using plate counts on a selective medium with nalidic acid and rifampicin where only *P. putida* MT2 R62 can grow. In the absence of the added strain no growth occurred on the selective agar plates. In OECD soil with pH 7.3 the EC_{50} averaged around 1000 mg Zn/kg in different experiments. In OECD soil with pH 5.0 the EC_{50} was about 3 times higher.

It could be shown that zinc was less toxic to *P. putida* in buffer with low pH than at neutral pH, as reported previously (Van Beelen and Fleuren-Kemilä, 1997). This compensated for the lower sorption of zinc to soil at low pH (see Notenboom and Posthuma, 1994). In single species tests with Budel gradient soils the pH effect obscured also the effects of zinc on survival of *P. putida.* Survival was low in clean soil with pH 3.9 and was higher in contaminated soil with pH 4.7 and 160-340 mg Zn/kg.

- Glutamate mineralization in freshly contaminated soils

The effects of zinc added to several soil types on the mineralization of glutamate into CO_2 was studied. EC_{50} values were calculated from the cumulative CO_2 production during a certain

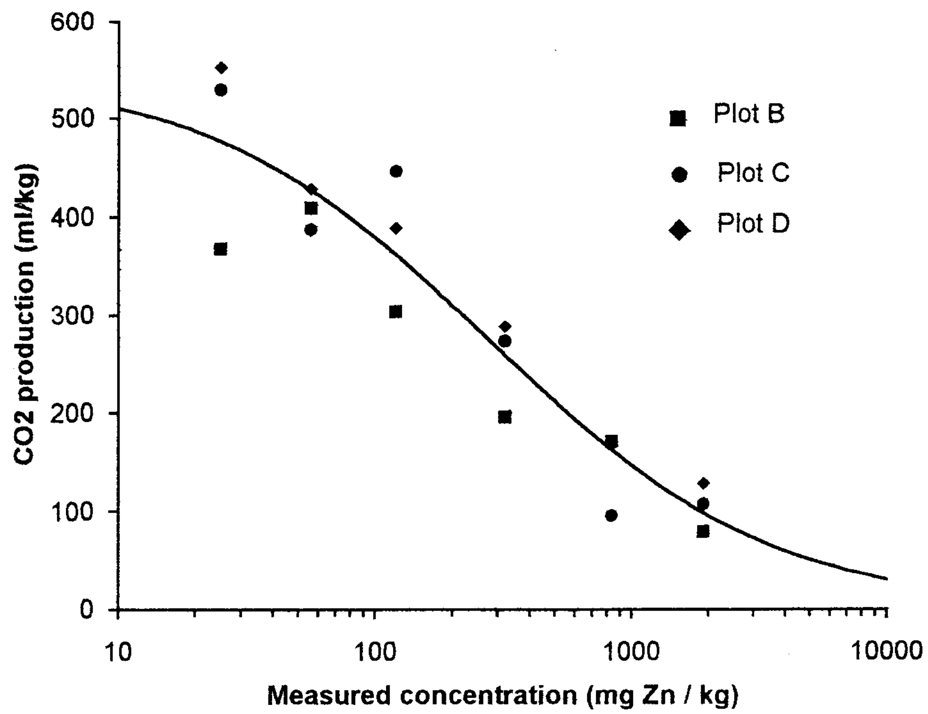

Figure 8.2. Concentration-effect relationship for the effect of zinc on glutamate mineralization in experimental field plot soil samples in 1995. The solid line represents the curve-fit according to a logistic model (Haanstra, 1985)

period (for details see Van Beelen and Notenboom, 1996). Results are given in Table 8.1. Budel reference soil was taken from the location with the largest distance from the factory.

- Glutamate mineralization in artificially contaminated field plot soils

Experimental field plot soils at the campus of the Vrije Universiteit (see Chapter 2) were sampled mid July 1994, shortly after the construction of the plots, and in March 1995. The rate of mineralization of glutamate (formation of CO_2) was determined in soil from three different plots (B, C and D). One sample per concentration from each plot was tested, to investigate whether there are differences between the plots. No significant differences between the different plots were observed. The EC_{50} values were calculated with the aid of all pooled results. The concentration-effect relationship for 1995 is given in Figure 8.2. No significant difference in toxicity of the soils was observed in the plots after 1 year based on measured total or $CaCl_2$ exchangeable zinc concentration (Table 8.1). Decreased toxicity based on total zinc concentrations during the first year might be explained by the loss of zinc by leaching and diminished availability.

- Acetate mineralization in artificially contaminated field plot soils

Experimental field plot soils were sampled in February 1995. Experiments on the mineralization of acetate (acetate consumption and CO_2 production) were carried out in moist soil under natural conditions and in soil slurry (which improves the distribution of acetate through the

Table 8.1. EC_{50} and NOEC values for effects of Zn and Cd on glutamate mineralization in freshly contaminated field soils and experimental field plot soils

Soil	NOEC (mg Zn/kg)	EC_{50} (mg Zn/kg)	
Wageningen humic sand	100 (n)	ca. 300 (n)	
Experimental field plot soil*	ND	239 (n)	
Budel reference soil (#11)	ND	270 (n)	
		Measured total concentration	$CaCl_2$ extractable concentration
Experimental field plot, July 1994**	71	203 (143-287)	6.8(4.4-11)
Experimental field plot, March 1995**	115	281 (100-792)	12***(7.9-18)

(n) = nominal concentration; ND = not determined; ( ) 95% confidence interval; *Before addition to plots;
**Pooled values for 3 plots;
*** Linear regression

Table 8.2. Comparison of the effect of zinc on the acetate mineralization in the experimental field plot soil (1995) under natural conditions, in a slurry and with added pure cultures of *Pseudomonas putida*

Test	CO_2 production		Acetate consumption	
	EC_{50} (mg/kg)	EC_{10} (mg/kg)	EC_{50} (mg/kg)	EC_{10} (mg/kg)
Moist field soil	264	236	297	262
Slurry	675	261	400	142
P. putida	680	363	477	376

soil). Additionally, single species tests were carried out with *P. putida* added to autoclave-sterilised field plot soil.
Table 8.2 shows the results of these experiments. An example of the concentration-effect relationship is given in Figure 8.3. This experiment with moist soil showed a rather high variability, which can be attributed to problems with mixing the acetate in with the soil. The difference between the slurry and the *P. putida* experiment in Table 8.2 therefore was statistically insignificant.

It can be concluded that the differences in sensitivity between the three tests are relatively small. The sensitivity of the mineralization tests in the soil slurry was about equal to the sensitivity of the test carried out with the experimental field plot soil. This indicates that the sensitivity measured in soil slurry is similar to that in the field.

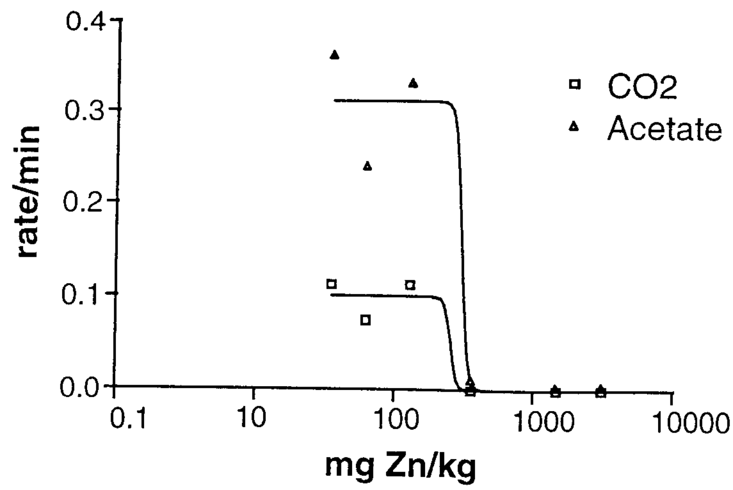

Figure 8.3. The effect of zinc on acetate consumption and CO_2 formation in moist soil sampled from the experimental field plots in February 1995

- Acetate mineralization: a dose effect curve from the experimental field plots sampled in January 1996.

In order to measure the Pollution Induced Community Tolerance (PICT, see section 8.2.1), a dilution method was developed to separate the micro-organisms from the soil.

Highly diluted soil suspensions were made with 30 mg of soil into 50 ml of Tris buffer (pH 8). Under these conditions sorption of added zinc to the soil particles was minimal. Therefore the sensitivity of microflora to added zinc could be measured without interference of sorption. ^{14}C - labelled acetate at a concentration of 1 mg/l was added to the diluted soil suspension. The micro-organisms in the soil suspension were able to convert the ^{14}C acetate into [^{14}C]carbon dioxide during the incubation.

In the diluted soil suspensions the carbon dioxide production was increasing at an exponential rate. The uncontaminated soil suspension showed the highest initial activity whereas the contaminated soil suspensions showed much lower initial activity. When the initial activity was plotted against the concentration of zinc at the different field plots a dose effect curve was obtained.

Figure 8.4 shows the effect of zinc present at the experimental field plots at the Vrije Universiteit on the initial activity of the micro-organisms in soil. On the X-axis of Figure 8.4 the amount of zinc added to the soil at the experimental field site at the Vrije Universiteit is plotted. Both the carbon dioxide production and the acetate consumption were inhibited in the soils with the higher zinc concentrations. The dose effect curve of figure 8.4 shows an EC_{50} of 55 mg/kg and an EC_{10} of 31 mg/kg.

- The representativity of laboratory toxicity data for the situation in the field.

In the literature there are reports on thick layers of undecomposed litter which accumulate in the vicinity of metal smelters . These thick layers of litter are formed because the degradation of litter to humus in these metal polluted soils is inhibited (Van Wensem, 1997). In addition to

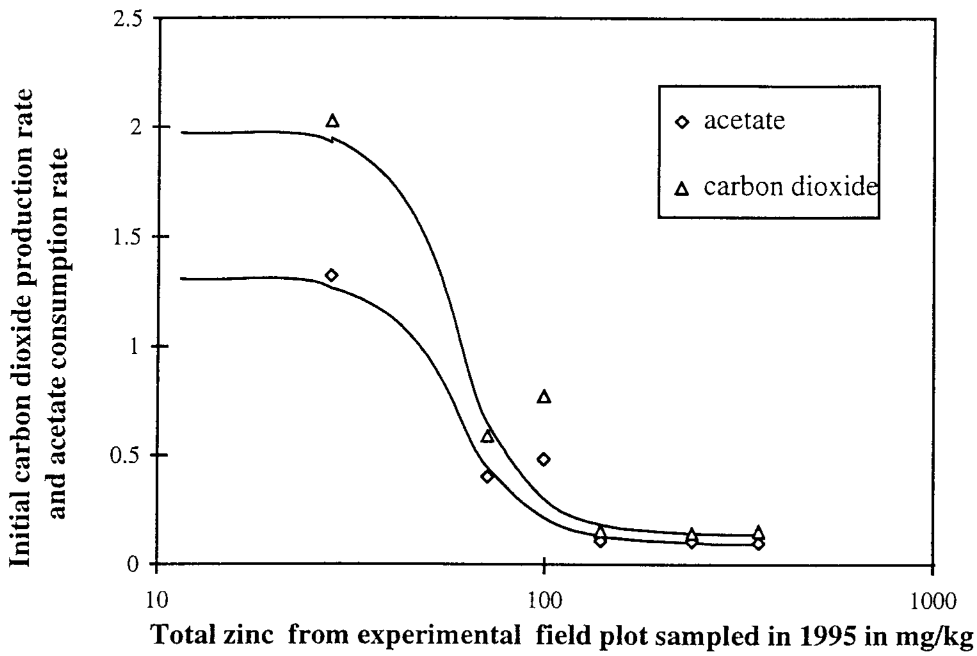

Figure 8.4. The effect of the zinc contamination in the Vrije Universiteit test fields on the initial mineralization rate of acetate in diluted soil suspensions

field observations, experiments were performed where organisms from the field are studied in the laboratory. The experiment with moist field soil, described in Table 8.2, is very close to the natural situation since only a small amount of water and a minute amount of radio-labelled acetate were added. The experiments with acetate mineralization in slurry and the experiments with glutamate mineralization were carried out in a complete soil and therefore also closely resembled the natural situation.

The comparison between the laboratory and the field can be complicated by differences in pH. Soil pH not only plays an important role in the sorption equilibria of metals but also in the uptake and toxicity of metals to organisms. In several studies an increased pH resulted in an increased metal toxicity (Vonk *et al.*, 1996; Hatch *et al.*, 1988; Bagy *et al.*, 1991). Also in our study an increased pH caused an increased toxicity of zinc for *P. putida* and for mineralization of acetate in soil. For this reason the effects of contaminants in Budel gradient soil could not be estimated, because the pH of the reference site was different from the pH of the contaminated sites.

The EC_{50} values for the effect of zinc on mineralization processes measured in soil samples freshly contaminated in the laboratory (including experimental field plot soil) corresponded very well with EC_{50} values found in the (aged) artificially contaminated experimental field plots. It should be noted that in both cases the measurements were carried out under controlled laboratory conditions. However, one may assume that a freshly taken soil sample from the field will not be altered very much after transport to the laboratory. Moreover, the conditions as far as moisture and temperature are concerned, were realistic. This means that for micro-organisms the EC_{50} for the toxicity of zinc for mineralization as determined in the laboratory is representative for this effect in the artificially contaminated experimental field plots.

Our results showed that ageing by leaching of readily available zinc slightly reduced its toxicity for micro-organisms. The contamination of Budel gradient sites #1 and #2 with zinc is sufficiently high to expect effects on the mineralization. Yet, a considerable mineralization potential was found at these sites. Apart from effects of pH and leaching as discussed above, this phenomenon could be explained assuming a replacement of sensitive species by tolerant ones as the result of long-term exposure, as has occurred at the Budel sites.

In summary, our results indicate that the mineralization in the freshly contaminated laboratory soil and in the soil from the experimental field plot show a good correlation. In soils from the Budel gradient complicating factors disturb the correlation.

8.3 Part 2: The detection of the toxic effects of environmental pollutants in the field by the measurement of the pollution induced community tolerance.

8.3.1 Introduction

Field observations on toxic effects of contaminants at the level of the whole community provide a means to validate environmental quality objectives (EQO's). Micro-organisms are useful indicators of soil contamination because of their important role in soil processes such as nutrient cycling and their high numbers present in the soil (also considering total biomass). From an ecotoxicological perspective, effects of contaminants on the microbial community in the field in terms of structure and function are undesirable and can thus be used to inspect the accurateness of the EQO's.

The measurement of toxic effects at a polluted field site is not straightforward because it is difficult to separate the toxic effects of pollutants from the other effects that also occur in the field. When we compare the most polluted field site near the zinc factory at Budel in the Netherlands with the less contaminated site, it is not easy to attribute ecological differences to the contamination alone. At the clean site the pH is lower which also has a pronounced effect on the local organisms (see section 8.2). The measurement of the Pollution Induced Community Tolerance (PICT) allows a more direct causal link of the ecological effects observed in the field with the contamination which is present in that same field.

The principle of the method to determine PICT for the detection of toxic effects in the field is depicted in Figure 8.5. Environmental samples are taken preferably from a gradient of contaminated field plots in order to collect several (microbial) communities. After extraction of the microbial community from the field sample, the micro-organisms are exposed to different concentrations of the predominant contaminant under controlled laboratory conditions. For each field sample, and thus for each microbial community exposed to different levels of contaminant

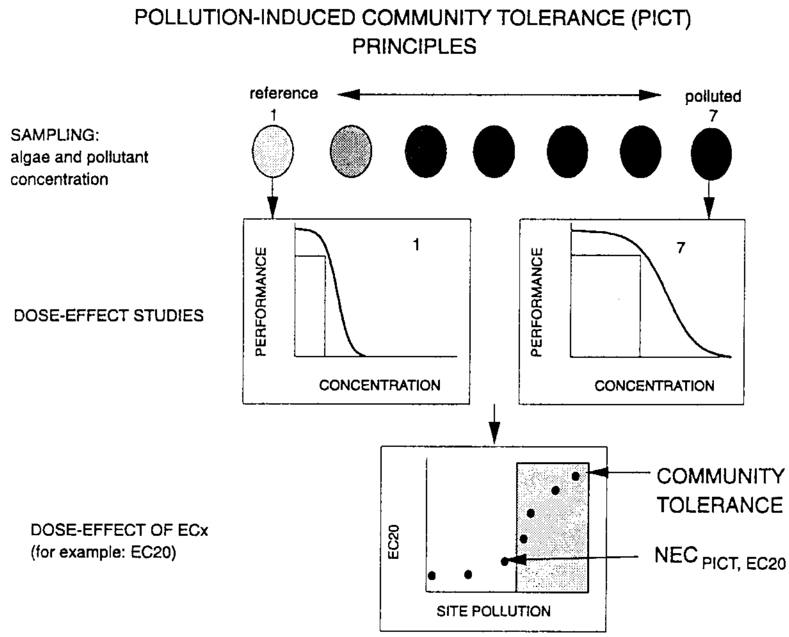

Figure 8.5. Schematic representation of the PICT concept (see text).

in the field, concentration versus effect relationships are established, yielding sensitivity values (e.g. EC_{50} values) of the microbial community. The sensitivities are then plotted as a function of field exposure values as is depicted in the lowest panel of Figure 8.6. When sensitivity of the exposed community has decreased compared to the control community, it is concluded that PICT is present. In turn, this can be considered a strong indication for real effects of the contaminant on the microbial community (Blanck et al 1988, Posthuma, 1997, Van Beelen and Doelman, 1997).

Various methods have been exploited to measure the PICT of micro-organisms in soil. In 1975, Jordan and le Chevalier used a plate count method in which the percentage of resistant micro-organisms at a polluted site were compared with the percentage of resistant micro-organisms at the clean site. Beside plate counts also a direct microscopic method was developed (Zelibor *et al*, 1987). The thymidine incorporation method was also used to measure the PICT (Bååth, 1992, Díaz-Raviña et al; 1994).

The plate count method and the microscopic technique suffer from a lack of accuracy, which means that only very pronounced effects can be measured. The thymidine incorporation method requires a technically difficult separation of the micro-organisms from soil. The use of a single parameter (like growth) means that false negative results can be easily obtained, because that parameter might not be very sensitive for the contamination at hand. We therefore have exploited new methods. We have used the mineralization of ^{14}C-labelled acetate as a tool to measure PICT and we have also used Biolog plates with 95 different tests.

- Outline of the Biolog method

The extraction of the microbial community from the soil samples and the method to establish concentration-effect relationships using Biolog plates have been described elsewhere (Rutgers *et al.*,accepted). In summary, the microbial community was extracted using a simple technique based on shaking the soil sample in an appropriate buffer and centrifugation of the debris.

Concentration versus effect relationships were produced in 96-well plates of Biolog (GP Microplate™; Biolog Inc., Hayward, CA, USA) using $ZnCl_2$ as the toxicant. Biolog plates are prefilled with a dried mineral salts medium, a tetrazolium dye, and 95 (1 blank) different substrates (carbohydrates, polymers, aromatic structures, amino acids, etc.). Degradation of the substrate and concomitant production of reducing power yields a purple colour. In environmental studies, the conversion of a certain substrate in the Biolog plate is considered to represent one activity of the microbial community; so 95 activities are tested. After inoculation of the microbial community in the plates, the rate of colour development in each well under different artificial Zinc levels was used to quantify the effect on microbial activities. Consequently, for all the positive (maximally 95) microbial activities concentration versus effect relationships could be established. EC_{50} values were calculated from the curves, i.e. the concentration of zinc in the Biolog plate causing 50% decrease in activity compared to the reference level. We thus obtained for each field sample maximally 95 EC_{50} values.

Two different contaminant gradients were sampled in order to apply the method for the validation of EQO's. The first one was the zinc gradient in the artificially contaminated experimental field plots at the Vrije Universiteit in Amsterdam. The second one was the zinc gradient around the zinc factory near Budel (see Chapter 2). Total zinc concentrations in the soil from both fields were used to quantify the exposure history of the community, since EQO's are presently expressed in this part.

8.3.2 Results

- Biolog: Experimental field plots, 1997

Soil samples were obtained from the experimental field plots at the Vrije Universiteit in Amsterdam with actual concentrations of 28, 85, 200, 558, 1367, and 2096 mg/kg dw soil in 1997. Concentration-effect relationships were determined using Zn as toxicant in the multiwell plates. EC_{50} values were determined for all functions tested with positive colour development (about 80). The results are depicted in Figure 8.6 for 9 typical examples.

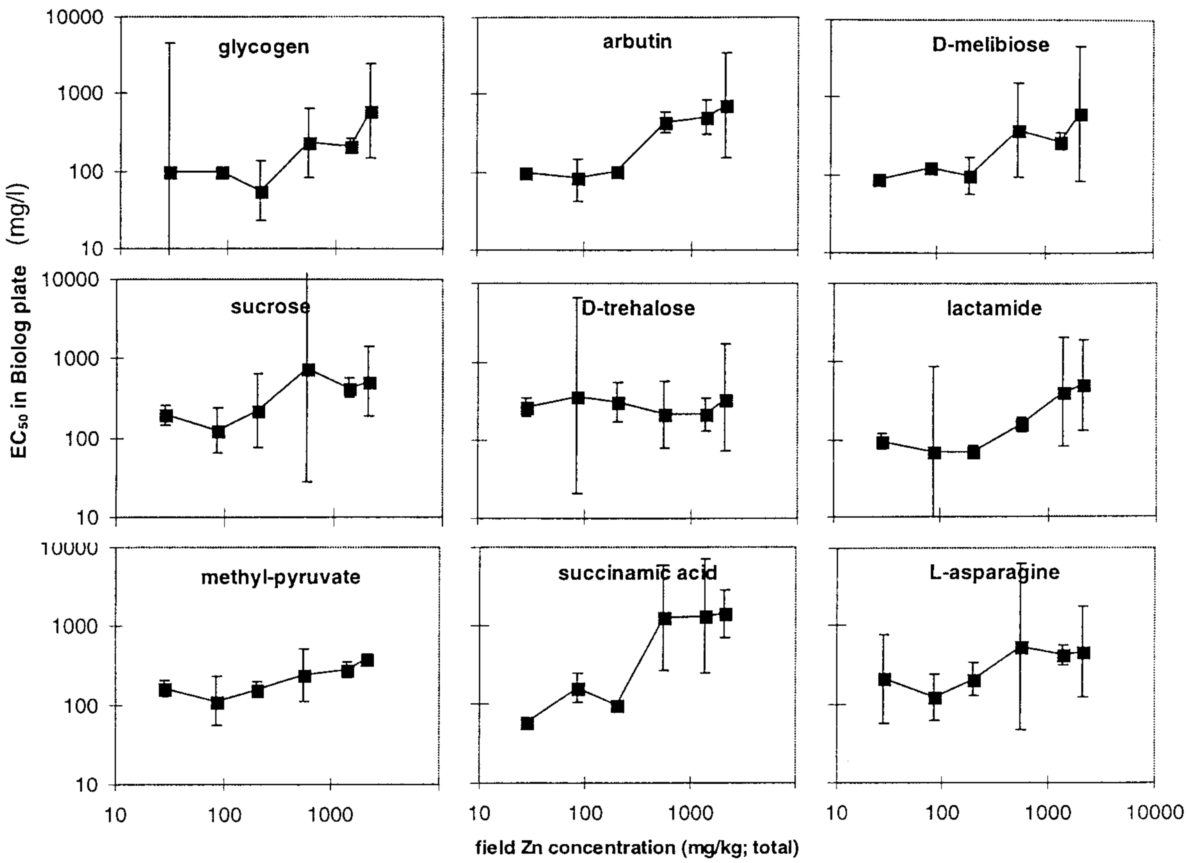

Figure 8.6. Sensitivities (EC_{50} values) of microbial activities (indicated in the panels) for artificial zinc exposure in GP Biolog plates as a function of the zinc concentration in field plots. Error bars indicate 95% confidence intervals.

Various EC_{50} patterns of the microbes from the field plots were distinguished. For instance, it was observed that the EC_{50} was almost invariant with the field zinc concentration for D-trehalose and methyl-pyruvate activity. Other EC_{50} values increased with increasing field zinc concentrations, for instance with arbutin, lactamide, and succinamic acid. The 95% confidence intervals were rather large in some cases. Two plausible mechanisms were responsible for this, i) the artificial zinc gradient in the Biolog plates covered only six concentrations so that sometimes top, or slope (bottom was set to zero, i.e. no activity) of the concentration versus effect curve was not really obvious, and ii) the rate of colour development was sometimes rather low. Bigger inoculum densities, and the application of a larger number of artificial exposure concentrations (zinc) in the Biolog plates should theoretically yield EC_{50} values with narrower confidence limits. Nevertheless, the trends in EC_{50} values as a function of the field zinc concentration were clear; in communities exposed to increased zinc concentrations there was either no obvious response or a decreased sensitivity. This was further substantiated by the EC_{50} values obtained from the curves describing the relationship between artificial zinc and the average microbial activity of all 95 substrates: the EC_{50} increased from 106 to 485 mg Zn/l (total concentration in the wells), concomitantly with the field zinc concentration. In addition, there was not a single relationship showing a decrease in EC_{50} with increasing field zinc concentrations. Therefore, it was concluded that many activities of the microbial communities in the zinc contaminated experimental field plots demonstrated increased tolerance for zinc, i.e. PICT was confirmed.

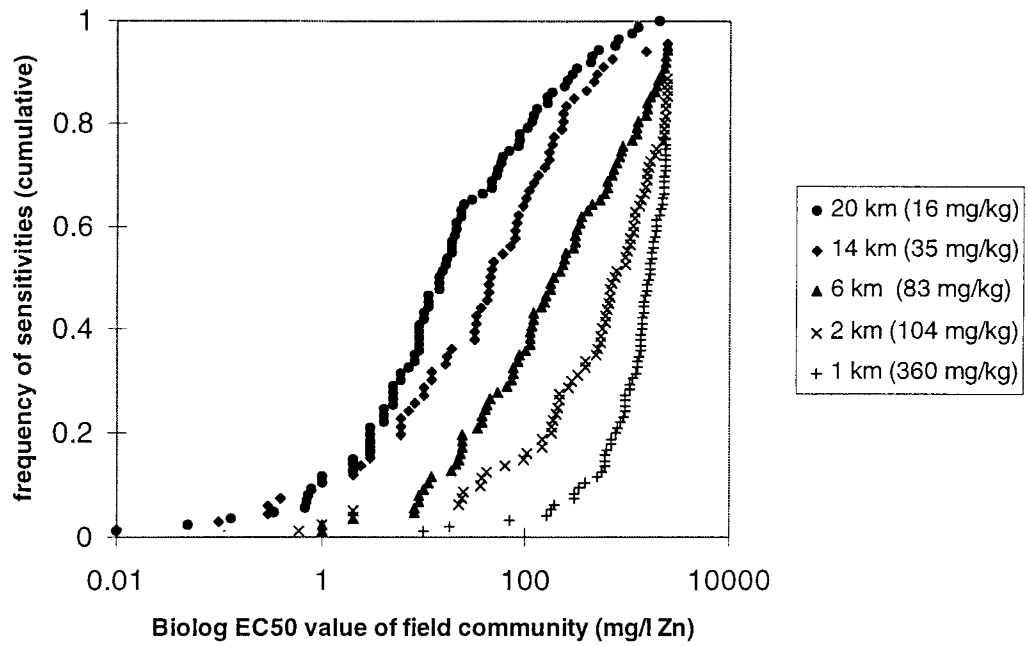

Figure 8.7. Cumulative frequency of Biolog EC_{50} of microbial activities around the zinc smelter near Budel. The figure shows Biolog EC_{50} values for each positive Biolog substrate (78 - 89) and 5 different field communities (●,◆,▲,×,+) with various levels of exposure. The distance to the zinc smelter (in km) and the contamination level are given (between brackets: total zinc concentration in mg zinc per kg DW soil).

Two conclusions can be drawn from the results presented in Figure 8.7. PICT was confirmed because i) the sensitivity of many activities of the microbial community decreased with increasing the field zinc concentration, and ii) in the reference soil with low zinc concentrations, microbial activities with different sensitivities were present while at high zinc concentrations near the zinc factory the number of sensitive activities was relatively reduced, possibly caused by elimination of some activities.

- Acetate mineralization: the measurement of PICT in the experimental field plots , January 1996.

The results given in section 8.2.2 on acetate mineralization in the experimental field plots showed that the microflora in the more polluted sites showed a very low initial activity. This direct effect rendered PICT measurement impossible, because without activity a tolerance measurement can not be performed. Therefore, in a second experiment, the slurries were pre-incubated for 48 hours in order to obtain an equal initial activity in all samples. The active suspension with soil from each different site was subjected to increasing zinc concentrations in the Tris-buffer.

In Figure 8.8 the sensitivity of the microflora from each plot (on the Y-axis) is correlated with the total concentration of zinc present in the field plots (on the X-axis). The acetate mineralization in soil suspensions from the cleaner sites showed an EC_{50} around 1 mg Zn/l Tris-buffer. The microflora from the more polluted sites with more than 100 mg of total zinc per kg soil, showed a decreased sensitivity for zinc. The microflora from the most polluted site showed an EC_{50} of over 20 mg Zn/l.

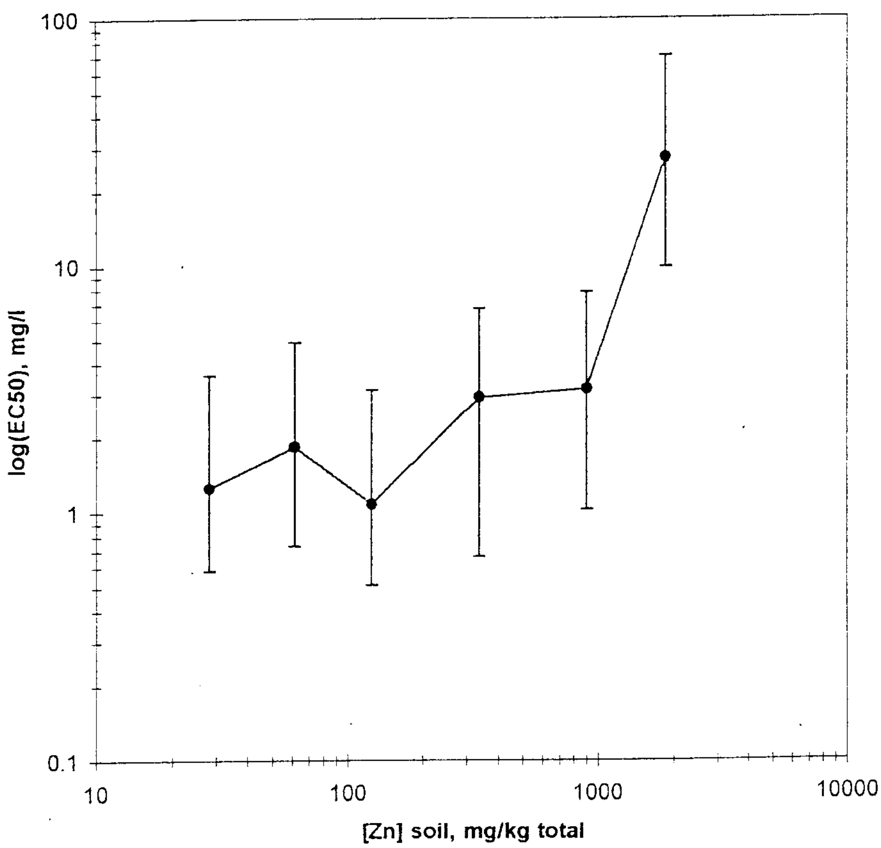

Figure 8.8. Pollution Induced Community Tolerance measured in diluted soil suspension, from the experimental field plots. The acetate (30ng/15ml Tris buffer) mineralization in the soils from the more contaminated plots showed a higher EC_{50} in mg Zn/l

Figure 8.8 indicates that PICT can only be measured in the microflora from the plots with more than 100 mg Zn/kg. This concentration is slightly higher than the EC_{50} of 55 mg Zn/kg that was measured in the dilute soil suspensions from the experimental field plot (Figure 8.4), in particular when the background concentration of 30 mg (indigenous) Zn/kg is subtracted from the former value, yielding an EC_{50} of 70 mg added Zn/kg for PICT. Although this PICT test seems a less sensitive indicator of zinc contamination compared to the acetate mineralization in the experimental field plot soil, it is more reliable because the latter results can be influenced by other factors such as nutritional state.

8.3.3 Comparison of results with Environmental Quality Objectives.

In both pilot studies using the field samples from the experimental field plots and from the zinc gradient at the zinc factory near Budel, PICT was confirmed, both with the acetate mineralization method and the Biolog method. This means that it is very likely that effects of chronic exposure to contamination with zinc at the levels present at the investigated sites has caused toxic effects on the microbial community. Doelman and colleagues (Doelman *et al.* 1994; Van Beelen and Doelman, 1997) have stressed the relevance of undisturbed microbial communities for a stable functioning of nutrient cycles and structure of the ecosystem. The occurrence of PICT in both marine periphyton (Molander and Blanck, 1992) and nematode communities (Millward and Grant, 1995) were accompanied by changes in species diversity. The Dutch environmental policy aims at the protection of the species diversity from the effects of pollutants (see Chapter 1). Therefore the occurrence of PICT is a clear sign of an undesirable effect of a pollutant. There are a few tests like the acetate mineralization (see section 8.3.2) or the short term CO_2 fixation (Molander and Blanck, 1992), which can be more sensitive than PICT and can therefore be used as an early warning system.

For both the Budel gradient soil and the experimental field plot soil the Target and Intervention Values are 60 and 300 mg Zn/kg (see Chapter 2). These site-specific EQO's were compared to the results presented in Figure 8.6, 8.7 and 8.8.

Our results show that a significant increase of the PICT was observed at concentrations slightly above the Target Value in both the Budel soil and the experimental field plots. In the case of the field plots the zinc sensitivity decreased for many activities of the microbial community with increasing field zinc concentrations. Severe loss of sensitivity was observed at the high zinc concentrations i.e. at the level of the Intervention Value.

In summary we were able to demonstrate field effects of zinc on the microbial community in artificially contaminated experimental field plots at the Vrije Universiteit of Amsterdam and in zinc contaminated soil from a gradient around a zinc factory near Budel. The field effects were obvious at the level of the Intervention Value, and started to occur at the Target Value.

8.4 Conclusions

1. Acetate mineralization and the survival of *Pseudomonas putida* were strongly inhibited by the low pH of some of the Budel gradient soils.
2. Contaminated Budel gradient soils showed no reduced potential for mineralization of acetate or glutamate compared to reference sites, possibly as a consequence of increased pH at the contaminated sites..
3. Evidence was obtained that dissolved zinc was less toxic for micro-organisms at a low pH.
4. The mineralization tests in the laboratory and in the experimental test field soil showed a good correlation. At the Budel site complicating factors disturb the correlation.
5. A concentration-effect relationship was found in the experimental field plots for the effect of zinc on mineralization of glutamate and acetate. The effect of zinc on glutamate mineralization decreased slightly after one year of ageing.
6. A Pollution Induced Community Tolerance (PICT) for the microbial community was present in the Budel gradient soils, as was indicted by a shift in the sensitivity to zinc of microbial substrate degrading activities.
7. PICT was also observed in the experimental field plots, based on shifts in sensitivity to zinc of microbial substrate degrading activities, as well as on sensitivity shifts for acetate mineralization at low concentrations.
8. The effects of zinc in the contaminated experimental field plot soils and Budel gradient soils start at the level of the Target Value and are obvious at the level of the Intervention Value

9. GENERAL DISCUSSION

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To evaluate the results of the Validation project, this chapter focusses on the following issues:
- the results of the experimental work discussed according to the Aspects 1 & 2 of validation as introduced in the General Introduction;
- the significance of the results for a wider range of organisms and substances;
- the order of magnitude of the differences between laboratory toxicity data and field effects of toxicants;
- an assessment of the obtained results for the meaning of generic risk limits obtained with the statistical extrapolation method, and for location-specific risk assessment.

The Conclusions and Recommendations are summarized in Chapter 10.

9.1 Aspect 1: Representativity of laboratory toxicity data for the field

When comparing the sensitivity of test species in laboratory and field soils, a number of aspects is of importance (Table 1.1). To guide a comparison along these aspects, Table 9.1 summarizes the results obtained with the different test species in different soils and under different conditions.

Table 9.1. EC_{50} values for the toxicity of zinc for different test species in freshly contaminated and aged soils, in tests performed in the laboratory or in the experimental field plot. EC_{50} values based on total (measured) soil concentrations. Toxicity endpoints were reproduction, except for *T. pratense* (shoot growth). Toxicity data from experimental field plot tests performed in 1994 were not used in this table, because of the large changes in zinc bioavailability which would require gross EC50-estimations. EFP-Experimental Field Plot.

	EC50 in mg Zn/kg dry soil based on the following tests:					
Test soil	*OECD*	*Budel reference*	*Budel gradient*	*EFP-soil*	*EFP-soil*	*EFP-soil*
Test conditions	*fresh, in lab.*	*fresh, in lab.*	*aged, in lab.*	*fresh, in lab.*	*aged, in lab.*	*aged, in field*
Trifolium pratense	356*	144	347	73-76*	663-890	340-556
Folsomia candida	473-626	185	>1787	184-266	2178	1491-1749
Eisenia andrei	429-921	n.t.	1790-2553	n.t.	n.t.	1277-1676
Enchytraeus crypticus	186-361	n.t.	205	262	n.t.	844

n.t. = not tested; * based on nominal concentrations

9.1.1 *Bioavailability*

For all organisms bioavailability has been shown to be a key issue for the extrapolation of laboratory toxicity data (expressed as EC_x or NOEC values on the basis of total soil concentrations) obtained with standardised substrates to effects in contaminated field soils. This key role relates to the experimental results that have been obtained (*par. 9.1.1.1-3*), and to the question how bioavailability can be taken into account for laboratory-to-field extrapolation *(9.1.1.4)*.

9.1.1.1 Comparison of different freshly contaminated soils.

Soil type may be of direct influence on the performance of test organisms (Crommentuijn *et al.*, 1997). From the assumption that the soil pore water represents a major uptake route of chemicals by soil inhabiting organisms, the sorption equilibrium between the solid and aqueous soil phase is regarded as the main process determining the bioavailability of chemicals (Van Gestel *et al.*, 1995). As was outlined in Chapter 2, the adsorption of heavy metals onto soil is dependent on many factors such as metal type, type and content of organic matter and clay, pH, cation exchange capacity and ionic strength of the pore water (Van Riemsdijk and Hiemstra, 1993). This implies that differences in toxicity may be partially explained by these differences in soil characteristics. Probably by coincidence, the soil type correction, which is in use in the Dutch soil protection policy (De Bruijn and Denneman, 1992, VROM, 1994a), reduces the difference in toxicity data (EC_{50} values on the basis of total soil concentrations) obtained using different freshly contaminated soils (for example for *Folsomia candida* the Coefficient of Variation reduced from 49% to 23%, Table 4.1; see also Tables 5.1 and 6.1). However, this is not the case when soils with different contamination histories are compared (e.g., Table 4.1). This demonstrates that the soil-type correction by no means fully accounts for differences in bioavailability. One major problem with the soil-type correction is that it does not take into account effects of soil pH, while this factor can greatly affect bioavailability.

The role of pH as a confounding factor is hard to unravel, since both the soil chemical behaviour of contaminants and the functioning of the organisms are influenced. This was demonstrated by Spurgeon and Hopkin (1996), who have studied the toxicity of zinc for the earthworm *Eisenia fetida* after incubation in OECD artificial soil in relation to pH and organic matter content. A comparable study was performed for the springtail *F. candida* by Crommentuijn *et al.* (1997), using cadmium as a contaminant. From the observation that pH influences the performance of the organisms in control conditions, the authors conclude that pH can directly influence the sensitivity of organisms to toxicants by acting as an additional stressor.

In contrast, effects of pH may also be leveled out. This is caused by the fact that soil is a three-phase system, which can simply be described as a solution containing abiotic and biotic phases. In the case of heavy metals, pH will affect (1) the equilibrium between the solution and the abiotic soil particles, generally resulting in increased solution concentrations at decreasing pH, and (2) the equilibrium between the solution and the organism, resulting in decreased organism concentrations at decreasing pH. This phenomenon was described by Nederlof *et al.* (1993) for the sorption of copper to soil and yeast cells, and may also have played a role for *Pseudomonas putida*, as described in Chapter 8. The degree of metal accumulation in an organism depends on the relative strength of both processes. Van Gestel *et al.* (1995) showed that the balance of both processes may also yield opposite results, since these authors demonstrated that zinc accumulation in earthworms increased with decreasing pH. Indications for a reduced metal uptake at increased soil solution pH were found for cadmium in *E. fetida* and *F. candida* (Vonk *et al.*, 1996).

9.1.1.2 Freshly contaminated versus aged soils

Several studies have shown that bioaccumulation and toxicity in earthworms of both heavy metals and organic chemicals is higher in recently spiked soils than in field soils or soils which after contamination have been equilibrated for a certain period of time (Spurgeon *et al.*, 1994; Spurgeon and Hopkin, 1995; Verma and Pillai, 1991). The reduced toxicity in field or equilibrated soils can be attributed to a reduced bioavailability, which can be approached by measuring the reduced water solubility of the chemical.

One of the reasons for the differences in zinc bioavailability between spiked soils in the laboratory and contaminated field soils is the chemical form of the initial contamination. The zinc contamination in the Budel area, which results from a secondary smelting process, is assumed to be emitted as relatively insoluble zinc oxides (Buchauer, 1973), whereas in the laboratory readily soluble zinc salts are used. A second, and maybe more important, reason for the reduced zinc bioavailability in field soils is the occurrence of ageing processes in the field situation. Ageing can be defined as the physico-chemical changes in contaminant binding to the solid soil fraction due to increased contact time, in combination with time and weather dependent changes in chemical form of the contamination; this definition excludes leaching, whereby the contaminant is lost from the system. Sorption of zinc onto soil involves an initial rapid process of binding to the outer surface of the soil particles, followed by a slow diffusion into the deeper part of the solid phase (Mann and Ritchie, 1994). Due to changes in other soil factors, such as pH, the sorption of zinc may change with time in addition. This was clearly shown by the data on the experimental field plot (Chapter 2).

Only for two organisms the effect of ageing could be demonstrated clearly, by comparing toxic effects in laboratory conditions between freshly contaminated experimental field plot soil, and field plot soil taken from the field plot after ageing under outdoor conditions (see Table 9.1). For *Trifolium pratense* and *F. candida*, ageing of the experimental field plot soil under outdoor conditions reduced toxicity (expressed as EC_{50} values on the basis of total soil concentrations) by a factor of approx. 10. For *F. candida*, this finding seems to be confirmed by the results of tests with freshly contaminated Budel reference soil and soil from the Budel gradient, while for *T. pratense* sensitivity between freshly spiked and Budel gradient soils differed only a factor of 2.2. For the other organisms, no such comparison is possible. For *Enchytraeus crypticus*, only a comparison between freshly contaminated experimental field plot soil tested in the laboratory and aged experimental field plot soil tested under field conditions can be made. When it is assumed that abiotic conditions play only a minor role (see below), the results show that ageing reduces toxicity (expressed as EC_{50} values on the basis of total soil concentrations) with a factor of 4.

As a result of the physico-chemical processes in the experimental field plot, EC_{50} values for plant growth and reproduction of Collembola in the outdoor tests showed an increase with time, indicating decreased toxicity. The decrease was largest between 1994 and 1995, although the

EC_{50} values for the tests in this period had to be interpolated from the zinc measurements made before and after the tests. The EC_{50} values obtained in the first year, shortly after preparation of the experimental field plot, were therefore omitted from Table 9.1. On the other hand, sensitivity of micro-organisms in the experimental field plot (e.g. measured as the glutamate mineralization rate) did not show such decrease with time (Table 8.1). This may be due to the effect of changed pH on the exposure of the microbial community, of the evolution of community tolerance (see below), or a combination of both.

It should finally be noted, that the processes addressed by the term 'ageing' not necessarily result in a decreased toxicity by reduction of the compound availability in the pore water, as was shown in the Amsterdam experimental field plot. The net result of may also be an increased toxicity, depending on the types of processes involved. An opposite effect of time was shown in a field plot after application of metal-containing sewage sludge (McGrath *et al.*, 1988): as a net response, metal sorption decreased (and toxicity increased), since pH decreased after the sludge application, and organic matter was broken down.

9.1.1.3 Artificial field plot versus real field pollution gradient.

Comparing zinc toxicity in the Budel gradient samples with effects observed in the experimental field plot is more complex. Although zinc sorption in the experimental field plot was much higher than in the Budel gradient samples, effects of zinc on the Collembola were more pronounced in the experimental field plot soil than in the Budel gradient samples (Table 9.1). This indicates that the speciation of zinc in the water fraction may be different between the soils. Preliminary results of model calculations indicate that in the experimental field soil zinc is mainly present in the free ionic form (W. Peijnenburg, personal communication), which is supposed to be taken up most easily by organisms. The addition of $CaCO_3$ to adjust pH in the toxicity experiments performed with Budel gradient samples may have caused a greater part of zinc to be present as inorganic species, such as zinc carbonate. The presence of inorganic zinc species may explain the reduced uptake and toxicity of zinc in these samples. For *T. pratense* and the earthworm *E. andrei*, the difference in sensitivity between experimental field plot soil and Budel gradient samples appeared to be small, whereas the enchytraeids were a factor of 4 more sensitive in Budel gradient soils than in the experimental field plot. This may suggest different routes of uptake for the different test organisms or different sensitivities for soil pH.

pH may well explain the differences among plant species in the phytotoxicity tests in Budel gradient soils. The soils had to be limed to a pH of appr. 7.6 in the case of *Sinapis alba*, and at that pH this plant species showed hardly any effect on soils from the Budel gradient (see Chapter 3). This contrasts to *T. pratense,* which is tolerant to lower pH and which showed marked heavy metal effects in gradient soils.

9.1.1.4 Methods for estimating bioavailability.

The proper estimation of exposure concentrations is essential for a comparison of data, in particular for practical use in risk assessment procedures. As outlined in Chapter 2, several approaches are theoretically possible to bridge the gap between soil chemistry and ecotoxicology:

1. A mechanistic approach has recently been presented by De Rooij and Smits (1997). Assuming that free ion activity in the soil solution determines the bioaccumulation and toxicity of heavy metals, they develop models that predict the free ion activity of heavy metals in soil solutions. Crucial for the ecological relevance of this methodology is a relationship between predicted ion concentrations and effects on biota. Before meticulously characterising the soil solution chemically, the applicability of free ion concentrations for the prediction of heavy metal toxicity should be investigated. Vonk *et al.* (1996) have shown that the free cadmium activity in soil pore water not fully explains the uptake and toxicity of this metal by *F. candida*. The results of Nederlof *et al.* (1993) and Temminghoff *et al.* (1995), further elaborated on by Plette (1997), indicate that pH and calcium content are of major influence on both heavy metal sorption onto soil and bioaccumulation, since H^+ and Ca^{2+} effectively compete for binding sites on soil particles and biota. The influence of pH on zinc toxicity for micro-organisms was confirmed in the present study (Chapter 8), the data obtained for earthworms suggest a comparable mechanism.
2. Another approach is to establish empiricial relationships between soil characteristics and bioacumulation or toxicity of heavy metals.
   a. Van Gestel *et al.* (1995) reviewed literature data for soil invertebrates. Relatively few studies were available and most of the literature was restricted to earthworm studies on cadmium, lead and copper, and on Collembola studies on cadmium. Organic matter content, pH and cation exchange capacity were identified as important soil characteristics governing heavy metal accumulation, but the influence of these factors on toxicity could not be quantified on the basis of the available literature. A major problem using literature data is that most studies were initially not designed for this purpose and that a detailed description of relevant soil parameters is lacking. Moreover, in experimentally contaminated soils, the pH is often dependent on the contaminant concentration and the relative importance of this factor therefore cannot be evaluated.
   b. Crommentuijn *et al.* (1997) state that artificial soil represents a suitable tool to study the influence of soil characteristics because the substrate can easily be manipulated with respect to organic matter content or pH. This was also shown by Spurgeon and Hopkin (1996). It should be kept in mind that the use of different amounts of $CaCO_3$ to adjust soil pH can lead to unexpected results because Ca^{2+} competes with heavy metals for sorption sites on both biota and soil particles (Kessels *et al.*, 1985; Kiewiet and Ma, 1991; Pellegrini *et al.*, 1993; Plette, 1997). Furthermore, addition of carbonate may induce changes in heavy metal speciation and result in the formation of less soluble zinc species.
   c. These shortcomings were avoided by Janssen *et al.* (1997ab) and Posthuma *et al.* (in press), who established empirical relationships between soil (chemical) characteristics and bioaccumulation of zinc by the oligochaete worms *E. andrei* and *E. crypticus* in moderately metal-contaminated field soils. They used a large series of natural soil

characteristics to investigate whether heavy metal partitioning over the solid and aqueous soil phase could be described as a function of soil characteristics. Moreover, they investigated whether zinc uptake by *E. andrei* and *E. crypticus* could be described by similar formulae. The concentration of zinc in the pore water was mainly dependent on pH. The same was found for the uptake of the metal by *E. andrei* and *E. crypticus*, with aluminium oxide and clay content as additional, but less important factors. The similarity of the relationships with respect to the effect of acidity implies that both abiotic partitioning and uptake by biota are governed by it. If these findings are confirmed by data from other soils and chemical elements, empirical equations may be used to correct total metal concentrations for pH (and other soil factor) dependent differences in bioavailability.

3. A third option is to use operationally defined parameters to indicate differences in bioavailability between soils. Mild extractants can be used to simulate the bioavailable fraction of heavy metals in soils (Houba *et al.*, 1996; LeBourg *et al.*, 1996; Spurgeon and Hopkin, 1996). In the present study, exchangeable or water soluble concentrations were used to correct for differences in bioavailability. For most test organisms, it can be concluded that a better agreement of effect concentrations in different soils can be obtained by expressing effect levels on the basis of water soluble or 0.01 M $CaCl_2$ exchangeable concentrations. In Table 9.2, EC_{50} values for the effect of zinc on the different test organisms in the different soils are expressed on the basis of 0.01 M $CaCl_2$ exchangeable soil concentrations. Comparing tables 9.1 and 9.2 it may be concluded that especially for earthworms differences between soils are strongly reduced when EC_{50}'s are based on $CaCl_2$ exchangeable concentrations, since the range of variation reduced from 429 - 2553 to 133 - 307 mg Zn/kg soil. For the other organisms, differences remain large, albeit between laboratory and field soils reduced. When water soluble concentrations are considered, especially for *F. candida* the situation significantly improves and differences between laboratory and field soils become much smaller. Due to some outliers, for *F. candida* EC_{50} values on the basis of water soluble zinc levels may show a variation of a factor of 5-10, but in general these values are between 10 and 25 mg Zn/kg dry soil.

Table 9.2. EC_{50} values for the effect of zinc on the growth (plants) and reproduction of different test species in freshly contaminated and aged soils, in tests performed in the laboratory or in the experimental field plot. EC_{50} values based on 0.01 M $CaCl_2$ exchangeable soil concentrations. Data obtained from tests in 1994 in the experimental field plot under outdoor conditions were not used in this table. EFP = Experimental Field Plot.

	EC50 in mg Zn/kg dry soil based on the following tests:					
Test soil	*OECD*	*Budel reference*	*Budel gradient*	*EFP-soil*	*EFP-soil*	*EFP-soil*
Test conditions	*fresh, in lab.*	*fresh, in lab.*	*aged, in lab.*	*fresh, in lab.*	*aged, in lab.*	*aged, in field*
Trifolium pratense	n.d.	144	121	n.t.	70-112	11-45
Folsomia candida	62.2	48.6	>313	83.1-125	268-332	255-359
Eisenia andrei	133-172	n.t.	300-336	n.t.	n.t.	219-307
Enchytraeus crypticus	18-144	n.t.	123	149	n.t.	102

From these findings, it can be concluded that the use of water soluble or $CaCl_2$ exchangeable zinc concentrations reduced the differences between soils for earthworms, springtails and likely for plants. For enchytraeids, the collected data show a large difference among repeated tests in freshly spiked OECD artificial soil, so that the same conclusion may not be drawn at present for this species. The precise cause of the variation could not be established. Differences in uptake strategies may be responsible for the observed pattern, but definite conclusions can not be drawn until more experimental data are available. Water soluble or 0.01 $CaCl_2$ exchangeable concentrations may provide useful information about the potential bioavailability of zinc for different test organisms. The predictive value of one or the other extraction method seems, however, to depend on the test species, because the speciation of the metal in the soil solution can not properly be predicted from exchangeable concentrations. But as long as it is not possible to properly interpret speciation differences between soils, application of mild extraction techniques may be a feasible option to assess bioavailability of metals in soils.

4. It is advocated by several authors to base risk limits on internal concentrations of chemicals in the test organisms. They argue that internal concentrations may integrate exposure to fluctuating concentrations over time and may provide a good insight in the bioavailability of pollutants (Van Wensem *et al.*, 1994; Van Straalen, 1996). In Table 9.3, EC_{50} values for the effect of zinc on the basis of internal concentrations are summarized for the various test organisms and soils used. From this Table, it appears that internal EC_{50} values are more or less similar for the different test animals used. All values are in the order of 100-370 mg Zn/kg dry body weight, and differences in internal effect concentrations obtained on different soils are small. The largest difference was obtained for *F. candida,* between freshly contaminated soil and soils that were aged, or experimentally percolated after spiking with zinc. This difference may, however, be attributed to the toxicity of the chloride ion in the freshly contaminated soils without percolation pretreatment. For the other test organisms (earthworms and enchytraeids), no experiments were made on freshly contaminated soils that were percolated before the test, so that conclusions about toxicity of the anion can be drawn.

Table 9.3. EC_{50} values for the toxicity of zinc for different test species in freshly contaminated and aged soils, in tests performed in the laboratory or in the experimental field plot. EC_{50} values based on internal concentrations in the test organisms for reproduction as toxicity endpoint, except for *T. pratense*. EFP = Experimental Field Plot.

	EC50 in mg Zn/kg dry soil based on the following tests:					
Test soil	*OECD*	*Budel reference*	*Budel gradient*	*EFP-soil*	*EFP-soil*	*EFP-soil*
Test conditions	*fresh, in lab.*	*fresh, in lab.*	*aged, in lab.*	*fresh, in lab.*	*aged, in lab.*	*aged, in field*
Trifolium pratense						
shoots/shoot growth	1020	1600	1460	1060	-	200-569
roots/root growth	5300	3200	3700	6600	-	-
Folsomia candida	96.5-150	-	-	146-147	328	-
Eisenia andrei	150-300	-	>130	-	-	305-356
Enchytraeus crypticus	372	-	-	-	-	-

- = not tested; *after percolation of the test soils

For a regulated element, such as zinc, the use of internal concentrations may not necessarily reflect increases or decreases in bioavailability. In addition, elevated soil concentrations may already affect the performance of the test organism without leading to increased body concentrations. The internal EC_{50} values found for Collembola and earthworms only slightly exceed the regulated level (100-200 mg Zn/kg dry body weight). Van Gestel *et al.* (1993) and Smit (1997; Chapter 4) already observed effects on the reproduction of *E. andrei* and *F. candida* when internal concentrations were still in the range of regulated levels. Apparently, regulating the internal zinc levels took so much energy that the expenditure of resources for reproduction diminished.
In most tests, the internal effect concentrations for *T. pratense* were much higher than those for the soil animals tested. Effect concentrations in the plant roots generally were a factor of 2-6 higher than those in the shoots, probably due to the binding of zinc to the root tissue. Internal EC_{50} values obtained in the outdoor tests in the experimental field plot were a factor of 2-8 lower than those from the laboratory tests. As indicated in Chapter 3, differences in growing conditions and growth stage may have caused these differences. Considering the importance of such conditions, it may be questioned whether internal effect concentrations may be a useful tool to assess the phytotoxicity of zinc. When growing conditions and growth stage are more or less similar, internal effect concentrations may be useful, as indicated by the similarity of internal EC_{50} values for all indoor tests: for both shoots and roots differences were less than a factor of 2.

9.1.2 Multiple substances vs single contaminants.

A comparison of results obtained in laboratory tests, experimental field plot, and the Budel gradient, may also shed some light on the effects of mixtures of metals. Unfortunately, insufficient data are available to make such a comparison. When it is, however, assumed that variable abiotic conditions play a minor role (see below), data from laboratory tests and the experimental field plot can be compared with results obtained in Budel gradient soils (see Table 9.1). This comparison shows that differences are less than a factor of 2, suggesting that zinc was the major toxic component in the Budel gradient soils.
Within this project mixture toxicity was addressed for earthworms, springtails and enchytraeids; TNO performed mixture toxicity experiments with plants in related projects. With respect to the soil chemical aspects of mixture toxicity, bioavailability of heavy metals is altered considerably when additional metals are present (Chapters 2 and 4). However, this had only little effect on the uptake of the metals in the test organisms. Hence, the type of joint action of heavy metals not only depends on the metals used, but also on the toxicity endpoint and level of toxicity considered. The experimental data suggest that it is unlikely that there is a general mechanism of joint action of heavy metals for these organisms. Unless strong evidence exists for a synergistic effect of chemicals, concentration addition seems the best option to evaluate the potential risks of exposure to multiple substances.

9.1.3 Uncontrolled conditions vs controlled conditions

Comparison of laboratory and field tests with soil from the experimental field shows the different responses due to abiotic (climatic) conditions (e.g. temperature, moisture content, air humidity). From Table 9.1, it can be concluded that such a comparison can only be made for plants and Collembola. For *E. andrei* the comparison between two years with different exposure conditions showed only a slight effect on sensitivity (Chapter 5), but a comparison with effect levels established under constant conditions in the same (aged) soil can not be made. The plants seem to be somewhat more sensitive under outdoor conditions. In 1994 (Table 3.5), EC_{50} was low (127 mg Zn/kg dry soil), which may be explained from the poor weather conditions and high bioavailability (no leaching of zinc had taken place yet when these experiments were started), whereas in 1995 and 1996 differences may be attributed to weather conditions only. For the collembolans, high sensitivity in the 1994 field plot (EC_{50} = 901-979 mg Zn/kg dry soil) can also be ascribed to the fairly high bioavailability of zinc at the time of the experiment. At later times, field sensitivity is almost equal to the response in laboratory tests, suggesting that abiotic factors (climate) do not affect sensitivity to a large extent. For soil moisture content, this was already demonstrated by Van Gestel and Van Diepen (1997), while for fluctuating temperatures similar conclusions were drawn by Smit and Van Gestel (1997).

When differences in bioavailability are taken into account, results from the outdoor toxicity experiments were in agreement with laboratory toxicity data. This indicates that for the organisms tested, exposure under uncontrolled conditions does not induce large changes in sensitivity. It must be mentioned that in this case, uncontrolled conditions were in fact fluctuating climatic conditions, which means that there was an alternating cycle of optimal and adverse circumstances. It can be assumed that climatic conditions that may enhance zinc toxicity were alternated by conditions that may reduce the effects of zinc. It should also be noted, that all outdoor tests were performed during summer and autumn, so organisms were not exposed to real harsh conditions (except for the high temperature exposure of *T. pratense* in 1994). It is reasonable that for mild fluctuations of the Dutch climate the use of average moderate temperature and humidity conditions does not lead to large mis-interpretations regarding the direct effects of toxicants on species.

In addition to temperature and soil moisture or air humidity, other environmental conditions, such as food availability, may affect sensitivity. Postma *et al.* (1994) have shown that the occurrence of multiple stressors can sometimes have complicated effects. In experiments with *Chironomus riparius*, exposure to cadmium and food deprivation caused a significant reduction of the population growth rate when treatments were applied separately. Exposure of food-limited animals to cadmium caused a significant mortality of the larvae which consequently resulted in an improvement of the feeding situation of the remaining animals. Results on *F. candida* (Chapter 4; Smit *et al.*, 1998a) indicate that food shortage has a major influence on population development, the interaction between food supply and zinc toxicity is small as far as short term exposure (less than a generation time) is concerned. Long term effects, with exposure times beyond the period common for standard toxicity experiments, remain to be established.

9.1.4 Heterogeneity

Despite the thorough homogenisation of the soil and added contaminants during the construction of the experimental field plot, small scale differences in soil related factors could not be prevented. This could have caused the considerable variation between replicates observed in the *F. candida* outdoor toxicity tests. The field experiments performed with *T. pratense* in 1994 demonstrated the influence of spatial differences in radiation and temperature on the applicability of an outdoor toxicity study and stress the importance of a careful test design.
In the studies on the Budel gradient soils, heterogeneity will have played a much greater role. Marinussen (1996) demonstrated that polluted sites may show a large spatial variation in environmental concentrations of polluting chemicals. This will also have been the case in the Budel area. When considering the field observations on enchytraeids and nematodes the variation of metal distribution will have been increased by differences in vegetation and soil characteristics: see 9.2.
The above mentioned considerations are even more important when field observations are used for location-specific risk assessment to decide about clean-up or remediation of contaminated areas: the absence of a species is not necessarily caused by the contamination and its presence is no guarantee that no ecological damage has occurred. The situation is further complicated by the fact that in these cases no clear reference site can be specified. For this specific goal, an alternative approach of in situ exposure of caged test organisms or performance of laboratory bioassays may be considered. These assays should preferably be performed using test organisms that do not require adjustment of soil characteristics such as pH prior to the experiment. In turn, this requires thorough knowledge of the autecology of the species. Unfortunately, the range of species for which a standardized test system is currently available is rather limited in view of the variability in soil characteristics encountered in the field. The tests investigated in the Validation project, for example, all involve species with an intermediate pH-optimum.

9.1.5 Population fitness

The single-species tests performed within the framework of the Validation project mainly concentrated on effects on the survival, growth and reproduction within a single generation of the test organisms. Applying population growth models, an approximation of possible effects on the population dynamics of the test organism can be made. Such an approximation however, requires a thorough knowledge on the life-history of the organism in both laboratory and field (Van Straalen *et al.*, 1989), the plasticity of its life cycle and the possible trade off between life cycle components (see e.g. Kammenga, 1995, Calow *et al.* 1997). The standardized short-term test methods followed in the Validation project, however, cannot provide insight in the potential cumulation of effects due to exposure over several generations.
Multi-generation experiments with the midge *C. riparius* by Postma and Davids (1995) have revealed that exposure to cadmium at the NOEC for an individual sublethal endpoint caused effects on the population growth rate after several generations in a long-term experiment. Calculations of the intrinsic rate of population increase for *F. candida* on the basis of laboratory life table data, indicate that the effects of zinc on reproduction, which is used as the toxicity

endpoint in the standardised test, can theoretically induce large changes in long-term population development (Smit, 1997). Experimental confirmation on these long-term effects of toxicants on *F. candida* as well as on other organisms seems relevant.
Other aspects related to this item are *adaptation, cost of tolerance, species interaction and community tolerance.* These aspects will mainly be dealt with in 9.2.

9.1.6 Test species

The test species used in the Validation project were mainly chosen because of their use in more or less standardized test protocols. Generally, these organisms are not very common in field soils. It may be questioned whether results obtained with these species are relevant for related field-inhabiting species, or even for unrelated taxa for which no standardized test is in use. For taxonomically related species, the question whether laboratory cultured organisms are equally sensitive compared to their 'wild' field-inhabiting relatives is difficult to answer, as several factors are involved. The idea that extrapolation is possible due to taxonomic relatedness is based on the idea that structural and physiological features to cope with chemical exposure are likely similar, rendering the field species similarly sensitive as the test species. In a study on cadmium accumulation and toxicity, Crommentuijn *et al.* (1994) have shown that taxonomically related species have a comparable accumulation pattern which is reflected in similarly changing LC_{50} values over time. However, when based on internal body concentrations, the LC_{50} values show large differences between related species. As mentioned above, internal effect levels for zinc did not differ to a great extent for the soil invertebrates tested in the Validation project (earthworms, enchytraeids, Collembola; Table 9.3). This may be due to the fact that zinc is a regulated element with effect concentrations being close to the regulation level. It should be realized that the measurement of lethal body concentrations, implying the analysis of dead organisms, is much more difficult and less reliable than the assessment of internal concentrations for effects on sublethal endpoints.
Extrapolation beyond the level of related taxa is even more difficult. Probably, it is not useful to try generalizing toxicity data from the test species to related or unrelated species, in the present context. The extrapolation method as such aims at predicting Hazardous Concentrations beyond the species tested, which should give a general protection level for related as well as unrelated species. It is obvious that the use of a broad spectrum of species types (and microbial processes) in the extrapolation procedure will generally yield more robust results than a procedure in which only few data are used. This rule of thumb has been adopted already in an early phase (e.g., Van De Meent, 1990) by requiring input data from various species groups for the statistical extrapolation method.
The question on representatitivity of test species has also been addressed for aquatic toxicity testing, where more data are generally available. Earlier research (Slooff *et al.*, 1986) and recent observations (Vaal *et al.*, 1997ab) have shown that the sensitivity difference among species could span five orders of magnitude, and that this range was only slightly smaller for taxonomically related species. Vaal (1997a), moreover, concluded that the range in variation in toxicological data (concerning 26 aquatic species and the acute effects of 21 compounds) was

mostly due to differences among compounds, and to a lesser extent to differences among species. Vaal *et al.* (1997b) showed that one should take into account the mode-of-action of a compound before a prediction of risks at the ecosystem level can be made: in comparison to narcotic compounds, more tests are needed for reactive and non-polar compounds to derive Hazardous Concentrations with a similar uncertainty level. In general, it can only be concluded that differences in sensitivity between species are dependent on the chemical tested and the endpoint measured. It can not be concluded that the species used in laboratory tests are systematically more or less sensitive than field-inhabiting species. In this respect, it is useful to note that in the current practice of deriving Hazardous Concentrations investigations are made into the shape of the sensitivity distribution, i.e. whether the collected toxicity data can be accurately described using a log-logistic model (see, e.g. Crommentuijn *et al.*, 1997). Considering location-specific risk assessment, however, one may be more interested in toxic effects of the local chemical contamination on representatives of the local community. The rule of thumb used for derivation of generic risk limits may not be applicable to such cases. This may bias the choice of test species, e.g., for the design of bioassay studies.

9.1.7 Extrapolation to other metals

Ageing and reduced bioavailability after long-term contact with soil have also been reported for some other metals. Mann and Ritchie (1994) reported that cadmium added to soils transformed with time to less soluble forms, the extent depending upon soil type. In addition, both pH and the rate of cadmium addition affected the rate of transformation. In a sandy soil, soluble (=0.005 M KCl extractable) cadmium decreased with time whereas exchangeable (0.1 M $BaCl_2$ extractable) cadmium increased. In a podzolic soil, there was no change of cadmium forms with time at pH < 5, while at higher pH exchangeable cadmium decreased with increasing amounts of cadmium bound to organic matter. Bogomolov *et al.* (1996) reported a decreased $CaCl_2$ exchangeability of copper with time; the extent to which the exchangeability changed ranged from an increase with 16% at 100 mg Cu/kg dry soil to a 45% decrease at 200 mg/kg after 40 days incubation, indicating that soil concentration and probably also other factors may play a role. Korthals *et al.* (1996b) found an increased sorption of cadmium, copper, nickel and zinc to soils with time and developed a regression model to describe sorption of these metals to soil in time.

Sheppard and Evenden (1992) determined the bioavailability of uranium for *Lumbricus terrestris*, one and two years after spiking of soil. BCF values were much lower in the tests performed in the second year compared to the first year: in garden soil and limed sand average BCF values reduced over time.

It should further be noted that bioavailability in the field may not be constant. Forge *et al.* (1993), applying bioassays with *Colpoda steinii* on pore water obtained by centrifugation, observed higher levels of zinc, copper and nickel in pore water in summer than in winter. This was not found for cadmium and chromium, while for lead the situation seemed to be just opposite.

The reduction of bioavailability over time seems to be a common phenomenon, but two aspects need closer examination. First, the effect of soil acidity on bioavailability seems to be paramount, and the bioavailability in aged field soil at low pH may be higher than in spiked laboratory soil at medium pH. The quantitative importance of the counteracting effects of pH and ageing on metal solubility determine whether true risks occur at sites where the current extrapolation method suggests that risk limits are exceeded. Second, it has been argued that the freshly contaminated substrates in the laboratory tests mimic the immission situation in the field, in which the soil receives 'fresh contamination'. In this argument, risk assessments based on the extrapolation of laboratory toxicity data may be useful to address immission problems, whereas the effects of aged contamination on site would require correction for the effects of time on bioavailability in location-specific risk assessment procedures only.

In conclusion, also for other metals bioavailability may be overestimated in laboratory toxicity tests compared to freshly contaminated soils. The extent to which ageing and the presence of other chemical forms will affect exposure and toxicity under field conditions will, however, be hard to predict and can not be quantified on the basis of the available experimental and literature data. Probably, this will differ for each metal and soil combination.

9.1.8 Extrapolation to other chemicals

Hamers *et al.* (1996) compared literature data on the toxicity of pesticides for soil organisms in laboratory and field studies. They found a similar general trend of increasing absolute and relative toxicities of pesticides in laboratory and field studies. This supports the hypothesis that the response of organisms in laboratory and field conditions does not differ. The correlations were, however, weak and laboratory toxicity data did not allow for realistic prediction of effects to be expected in the field. This weak correlation could mainly be attributed to the poor database that these field studies provided after a critical methodological assessment. There was a large heterogeneity in field studies (species, weather conditions, pesticide treatment, sampling effort etc.) and a lack of field studies with multiple pesticide treatment rates from which proper dose-response relationships could be derived. Moreover, the lack of a proper assessment of exposure hampered the interpretation of these field studies.

Van Gestel (1992) concluded that laboratory toxicity data for earthworms were in agreement with field effects, especially when long-term effects were compared with effects on reproduction. This conclusion is supported by Heimbach (1992), who compared the outcome of standardized laboratory toxicity tests on *E. fetida* with effects of pesticides on earthworm populations in a more or less standardized field test.

Also other authors have drawn the conclusion that extrapolation of laboratory to field data is mainly hampered by uncertainty about exposure. Crossland (1994) argues that extrapolation from laboratory or mesocosm to the field is mainly hampered by differences in the behaviour of chemicals; toxicity in laboratory and field is mostly similar provided that exposure is the same. For organic chemicals, also ageing is an important factor that may lead to a reduced bioavailability with increased exposure time (Alexander, 1995; Hatzinger and Alexander, 1995). For polycyclic aromatic compounds and atrazine, ageing reduced bioavailability for uptake in earthworms and biodegradability in soils (Kelsey and Alexander, 1997; White *et al.*, 1997). It remains difficult, however, to quantify the effect of ageing on bioavailability, as it may depend on physico-chemical properties of both the chemical and the soil. This conclusion is confirmed by the results of the Validation project: for all single species tests, bioavailability as major factor hampered a straight-forward comparison of sensitivities in laboratory and field experiments.

9.1.9 Derivation of ecotoxicological risk limits on the basis of the present Validation project.

Risk limits for chemicals in different environmental compartments are derived on the basis of laboratory toxicity data, applying extrapolation methods, separately for 'species' and 'functions': toxicity data from at least four different taxa or functions should be available before HC_5 and HC_{50} values are derived (see Chapter 1). Basis for the extrapolation method are NOEC (or EC_{10}) values. In the Validation project, NOEC and/or EC_{10} values have been obtained for five different test species in OECD artificial soil, or freshly contaminated or aged experimental field plot soils. In addition, three NOECs were available for effects of zinc on microbial functions (acetate and glutamate mineralization activity of microbial communities). In view of the criterion on the number of toxicity data, project-specific HC_5 and HC_{50} values were calculated only for 'species', not for 'processes'. Although HC-values could not be calculated for 'processes', a direct comparison of toxicity data for 'processes' and 'species' is possible; this shows that the microbial processes react consistently more sensitive to zinc then the studied species (compare Table 8.1/8.2 with Table 9.4), which confirms earlier findings (Van Beelen and Doelman, 1997).

In Table 9.4, EC_{10} and/or NOEC values obtained from the different studies are summarized, and HC_5 and HC_{50} values are calculated using the model of Aldenberg and Slob (1993). In the derivation, the geometric mean was used for a species when more than one entry was available. For the conversion of toxicity values from the previous chapters to the standard soil (containing 25% clay and 10% organic matter), the soil-type correction described by VROM (1994a) was used. For that purpose, OECD artificial soil was considered to contain 14% clay and 10% organic matter (Smit, 1997; Chapter 4). Experimental field plot soil used in the different laboratory studies had on average 2.4% clay and 2.2% organic matter (see Chapter 2), while in the experimental field plot this soil contained 2.9% clay and 2.0% organic matter (see Chapters 4-6).

Currently used risk limits can be compared to newly derived values of various origin. It should be realized that the current Target Value (TV) has been set to the background concentration level of zinc in the Netherlands (see Table 1.2).

- Firstly (level 1), newly derived HC_5 and HC_{50} values were obtained using total effect concentrations from the experimental field plot, obtained after ageing. These Hazardous Concentrations were higher than the values obtained with freshly spiked soils (level 4), probably as a result of the ageing processes in the experimental field plot. Note that the HC_5 and HC_{50} values are also higher than the current TV and IV.

Table 9.4. EC_{10} (or NOEC) values for the effect of zinc on the growth (plants) or reproduction of test organisms in freshly contaminated and aged soils. When more than one value was available, the geometric mean of the available data is given in this table; when both an EC_{10} and an NOEC were available, the EC_{10} is used. HC_5 and HC_{50} values are calculated applying the method of Aldenberg and Slob (1993). All values are in mg Zn/kg dry soil. Total concentrations were recalculated to values for the standard soil applying the soil-type correction given by VROM (1994a). TV=Target Value, IV=Intervention Value. EFP=experimental field plot.

Conc. based on	Soil type	*T. pratense* EC10	*F. candida* EC10	*E. andrei* EC10	*E. crypticus* EC10	*P. putida* EC10	HC5 standard soil	HC50 standard soil
Values obtained within the Validation project								
(1) total, aged	EFP, aged	731#	2403	1754	1845	853	523	1371
(2) 0.01 M $CaCl_2$	OECD, fresh	-	31.2	72	20.6			
	EFP, fresh	-	55	-	9.1			
	EFP, aged	9.95#	149	96.9	9.3			
	geom. mean	*9.95*	*63.5*	*83.5*	*12*		3.39	28.2
(3) water soluble	OECD, fresh	-	4.5	-	2.6			
	EFP, fresh	-	5.89	-	-			
	EFP, aged	2.65#	9.76	6.79	7			
	geom. mean	*2.65*	*6.37*	*6.79*	*4.27*		2.04	4.7
(4) total, fresh	OECD, fresh	171*	429	347@	169			
	EFP, fresh	69*	310	-	546			
	geom. mean	*109*	*365*	*347*	*304*		85.1	254
Literature data								
Current TV & IV	VROM, 1994a						140**	720***
HC-X 'species'	Table 1.2						132**	385***
HC-X 'process'	Table 1.2						16**	207***

*nominal values; #NOEC's; ** calculated for standard soil; *** preliminary calculations for standard soil based on data of Crommentuijn *et al.* (1997) @based on a measured EC_{10} of 220 mg/kg and a nominal NOEC of 320 mg/kg, corresponding with 288 and 419 mg Zn/kg, respectively in standard soil.

- Secondly (level 2 and 3), HC_5 and HC_{50} values were derived using water soluble and 0.01 M $CaCl_2$ exchangeable zinc concentrations, irrespective of aged or fresh contamination and soil type. This is likely to be an alternative way (compared to level 1) to correct for bioavailability differences among soils, as shown in section 9.1.1. The newly derived Hazardous Concentrations are thus expressed in the same units (soluble or exchangeable zinc).
- Thirdly, the comparison can be made on the basis of total concentrations in freshly contaminated soils (level 4). The HC_{50} newly derived from the data of the Validation project is a factor of 3 lower than the value of Denneman and Van Gestel (1990), which is 720 mg Zn/kg soil. This difference is probably attributable to the use of different species in both test series. In addition, the sensitivity of species may also have been influenced by uncontrolled experimental variation, e.g. due to pH differences. Further, the Hazardous Concentrations for 'species' calculated on the basis of Crommentuijn *et al.* (1997) differ by less than a factor of

two from the values obtained for 'species' within the Validation project. The HC_{50} values based on freshly spiked soils thus differed within one order of magnitude for three independent data series. This difference is small when all uncertainties of laboratory-to-field extrapolation are considered. In this context, the extrapolation method apparently yields relatively robust results.

In the next paragraph, the data summarized in Table 9.4 are compared to toxic effects of zinc on community endpoints, both for the newly derived data (level 1 - 4), and for the current Target and Intervention Value of zinc. In the former comparisons, the validation of Hazardous Concentrations is undertaken by comparing *expected* effects (based on the expectation formulated by the extrapolated Hazardous Concentrations at the 5 and 50% level) with *observed* effects on community endpoints. In these comparisons, for level 1-4 in the above table, it is a unique feature that the predicted and the observed effects have been derived (1) under similar soil and exposure conditions, or (2) using the same way of correcting for bioavailability differences. This offers the unique opportunity to study the predictive value of extrapolated Hazardous Concentrations for a chemical, in this case zinc, with a minimized number of uncertainties.

9.2 Aspect 2: Validity of Hazardous concentrations for Soils

In contrast to the studies related to aspect 1 of the Validation project, aspect 2 of the project was studied with observational rather than experimental approaches. Experimental control was mostly lacking, causing methodological limitations. Due to this, the relative importance of the factors that influence laboratory-to-field extrapolation (Table 1.1) cannot be quantified and statistical hypothesis testing methods cannot be applied (Suter II, 1996). The discussion on the validity of risk limits is therefore restricted to gross comparisons of predicted and observed effects, in particular focusing on the 5 and 50% effect levels.

It should be kept in mind that the sensitivity distribution obtained by the extrapolation method may deviate from a log-logistic distribution. As mentioned in the General Introduction, this has not been a subject of study in the Validation project. However, it is current practice to test statistically whether the collected toxicity data fit to the assumed model. Recent investigations have shown that the sensitivity distribution of toxicity data for metals is usually not significantly different from the assumed distribution (Crommentuijn *et al.*, 1997). For metals (see discussion below) this knowledge has been used as a core assumption to address the validity of risk levels. For other compounds, if deviations from the assumed model occur, the number of toxicity data required to derive 'safe' concentrations is higher (Vaal *et al.*, 1997b), which should be taken into account when studying the validity of quality criteria for such compounds.

In this part of the study, the central question is if the concentration level at which field effects on community endpoints occur can be understood on the basis of laboratory toxicity data and field characteristics. In combination with the previous part of the study (section 9.1) this may help to identify the order of magnitude of differences between laboratory and field, and its causes, but may not fully close the gap between laboratory and field.

To allow for comparisons of predicted and observed effects, choices were made on:

1. the parameter to quantify exposure of the community (X-axis, exposure);
2. the toxicity endpoint at the community level (Y-axis, effect).

Exposure was mostly quantified using total soil concentrations. Only for the data on zinc collected within the Validation project it was possible to quantify exposure after approximate corrections for bioavailability (see Table 9.4, level 1-3). Toxicity endpoints at the community level were the diversity of enchytraeids and nematodes in the Budel field gradient and the experimental field plot, and the occurrence of microbial PICT in these soils (Section 9.2.1). Next to these examples from the Validation project, predicted and observed effects were compared using some selected literature data for other contaminants (Section 9.2.2).

Comparison of predicted and observed effects requires a criterion to judge (dis)similarity. In view of the many sources of uncertainty in laboratory-to-field extrapolation (Table 1.1), and the magnitudes of their influence upon these comparisons, a factor 10 as bandwidth appears scientifically justifiable. It is noted that this bandwidth may be large from the perspective of the required precision of risk limits to judge situations; it should be kept in mind, however, that (1) the factor pertains to a first-tier assessment (second-tier assesssment can be applied to avoid 'false positives'), and (2) that this value is the lowest of often used assessment factors (usually 10, 100, 1000, see e.g. ECETOC, 1997). Throughout this chapter, similarity between predicted and observed effects is indicated when differences are less than a factor of 10:

- within a range of this magnitude around the HC_5-level, the community endpoint should have a performance similar to that of the reference;
- similarly, at the HC_{50}-level toxic effects on the community endpoint should be obvious.

Dissimilarity is identified for the opposite cases, i.e. if extrapolated Hazardous Concentrations would yield obviously over- or underprotective risk limits.

9.2.1 Evaluation of risk limits and Hazardous Concentrations for zinc

9.2.1.1 Experimental field plot data.

The community endpoints studied in the experimental field plot can be compared to HC-values with or without bioavailability correction. The data from the 1996 and 1997 observations on nematodes and microbial PICT, respectively, in the experimental field plot were used for the comparison of predicted and observed effect levels with bioavailability correction (levels 1-3 in Table 9.4). Comparisons without bioavailability corrections (level 4 of Table 9.4) make use of the nematode data from 1994, since both the input data for extrapolation and the response data thus bear on recently added zinc. For PICT, no broad assays on a series of processes were made before 1997.

For the nematode data, it should be kept in mind that the effects that are shown for the community endpoint (number of taxa) do not necessarily imply reduction in numbers only. The data from May 1995 demonstrate that two genera had extraordinarily increased numbers in the

560 mg Zn/kg soil treatment. For these genera, possibly the loss of other taxa in the period preceeding their relative dominance has created a different competitive balance that promoted their population growth. Integrative parameters, such as Shannon's diversity index, may lack to show important structural changes. Numerically, a balance of in- and decreasing abundances of genera may occur, leaving the index unchanged, with the incorrect interpretation of 'no adverse ecological effects'. The same masking effect of opposing responses in underlying endpoints may occur in the PICT evaluation (see Chapter 8): for example, the zinc tolerance of succinamic acid metabolisation evolves at a lower ambient zinc concentration than the average response over all metabolic functions. Evidence for toxic effects on community-level endpoints like diversity indices for structure or function are thus likely underestimating true effects at lower levels of integration.

- Assessment on the basis of data from the Validation project *with* bioavailability correction. Hazardous Concentrations for zinc could be calculated from the data collected within the Validation project, in three ways: based on (a) aged (total)-, (b) exchangeable- and (c) water-soluble zinc concentrations. Each of the ways of calculation boils down to reducing uncertainties related to bioavailability differences among soils and test systems (see Section 9.1). The Hazardous Concentrations are taken from Table 9.4 (levels 1-3), and were plotted after soil type correction towards the experimental field plot soil following VROM (1994a). The observed community toxicity endpoints were scaled using the same measures of exposure. The results are shown in Figure 9.1.

First, looking at *total aged* zinc concentrations, long-term effects on community endpoints were insignificant below the HC_5, or only a slight tendency of change was present. This way to calculate the HC_5 apparently yields a result which offers reasonable protection for the studied integrative endpoints. Note that the detailed analyses on nematodes in Chapter 7 have shown that adverse effects for some separate species may occur before community structure responds. Considering the HC_{50} it is evident that this exposure level is associated to severe structural and functional community effects.

The use of *$CaCl_2$-exchangeable* and *water-soluble* zinc effect concentrations as other realistic methods of bioavailability correction yielded the four additional graphs of Figure 9.1 (middle and right). Hazardous Concentrations based on the exchangeable- and total-aged zinc concentrations showed a very similar pattern, i.e. the HC_5 appeared to offer reasonable protection for the studied endpoints, and the HC_{50} was associated to severe effects. Hazardous Concentrations based on the water soluble fraction of zinc, however, performed worse; this HC_5 offers too little protection for the studied endpoints. It should be noted that only few data were used to derive the latter values, so that conclusions are uncertain for this measure of exposure.

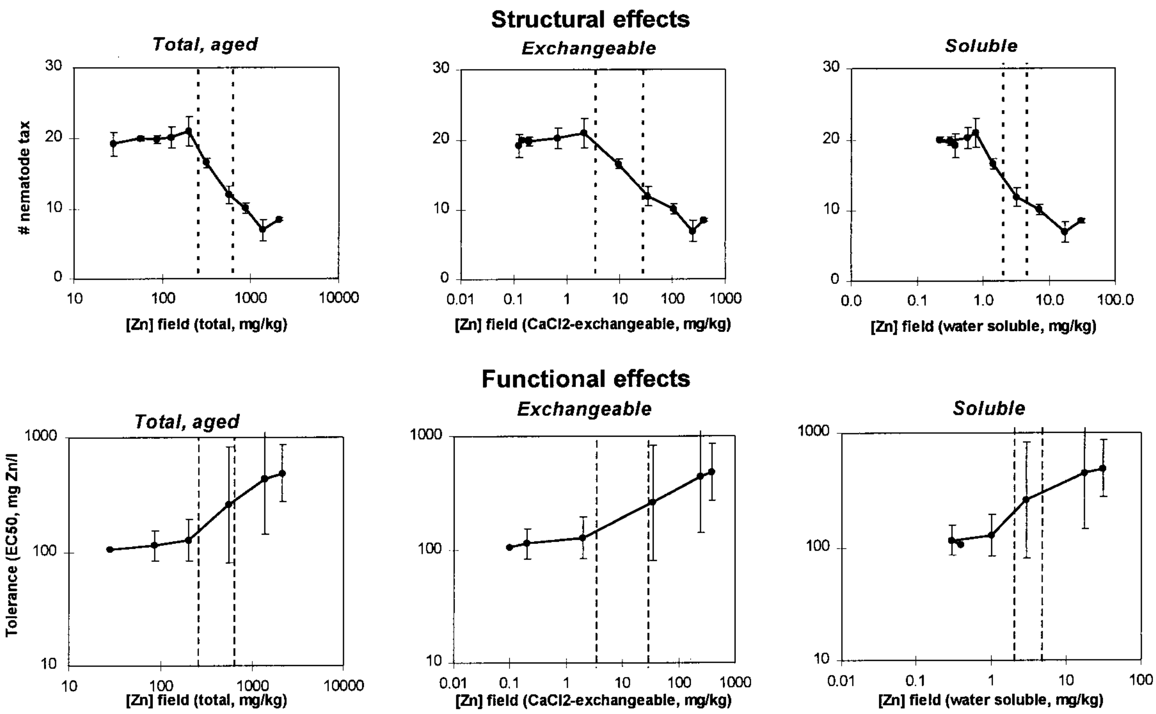

Figure 9.1. Comparison of Hazardous Concentrations extrapolated from toxicity data collected in the Validation project with toxic effects on community endpoints, using aged total (left), $CaCl_2$-exchangeable (middle) and water-soluble (right) zinc effect-concentrations from experimental field plot experiments (Table 9.4, level 1-3). Top row: structural effects expressed by the number of nematode taxa (data from 1996 only); bottom row: microbial functions using PICT. Broken vertical lines indicate project-specific soil-type corrected HC_5 (left) and HC_{50} (right) values derived from single-species toxicity tests. Note that the functional effects are thus judged using HC-values derived from 'species' toxicity data, although 'processes' are more sensitive for zinc (Van Beelen and Doelman, 1997).

- Assessment without bioavailability correction. The comparisons made above can be repeated by comparing toxic effects on community endpoints with HC-values derived on the basis of toxicity tests with freshly spiked soils (level 4 in Table 9.4), with HC-data from the recent report of Crommentuijn *et al.* (1997, separately for 'species' and 'processes'), and to current environmental quality objectives (TV and IV). Figure 9.2 shows the result of these comparisons. The observations suggests that the community NOEC for structural effects is accurately predicted by the HC_5 for 'species' derived from the data of the Validation project, whereas community effects appear evident when local concentrations exceed the project-derived HC_{50}; furthermore, the project-specific values are close to the values from Crommentuijn *et al.* (1997; upper left panel).

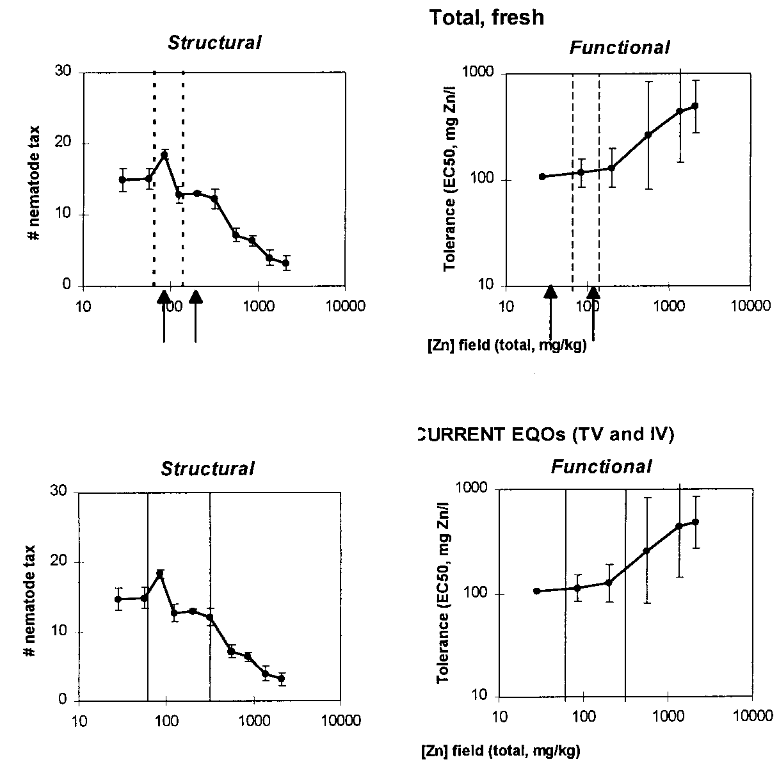

Figure 9.2. Comparison of predicted and observed toxic effects of zinc in the experimental field plot using structural (left) and functional (right) toxicity community endpoints. The number of nematode taxa in 1994 was used as structural endpoint, whereas the functional endpoint refers to the average EC50 for microbial degradation of different substrates (PICT). Vertical broken lines indicate newly derived Hazardous Concentrations (HC_5 and HC_{50}) on the basis of single-species toxicity data collected within the Validation project in freshly contaminated soils (Table 9.4, level 4). Arrows indicate the HC_5 and HC_{50} derived from Crommentuijn *et al.* (1997), separately for 'species' (structure plot) and 'processes' (function plot). Vertical solid lines indicate current Target Values (left within figure) and Intervention Values (right within figure) of zinc for the experimental field plot (from Table 2.7). All concentrations are based on total zinc concentrations in the soil.

Although the structural community effect consists of an evident species reduction at the HC_{50} level (and higher), it should be noted that the extrapolation method does not predict that the response should equal 50 percent of the reference performance. Strictly, it 'generically' predicts that 50% of the potentially exposed species is exposed beyond its chronic NOEC for a sublethal individual endpoint. This may, but does not need to imply disappearance of those species. Therefore, it is likely that community endpoints of a 'generic community' are affected by less than 50%. Specific communities may show stronger responses than 50% if their 'specific' sensitivity is higher than the 'generic' sensitivity. For functional effects, the comparisons should be interpreted with care, since (1) the HC-values are derived from 'species' toxicity data and thus refer to structural effects, and (2) the effect-curve has been measured in 1997, when part of the total zinc concentration was already sorbed to the soil, which means that the effect-curve is shifted to the right compared to the calculated 'fresh' HC-values. Still, within the bandwidth of

ten, the average performance of functions at the HC_5 level is similar to the reference, and beyond the HC_{50}-level the averaged tolerance of various metablic functions is increasing.
Comparison of the effects to the current Environmental Quality Objectives (lower panels of Figure 9.2) yields the conclusion that effects are low or negligible at the Target Value, and clearly visible at the Intervention Value, although the Target Value has strictly speaking no ecotoxicological basis.

-(Dis)similarity. Looking at Figure 9.1 and 9.2, the differences between predicted and observed effects were, in all cases, accurate within the bandwidth of the factor of 10 motivated above. In view of this, the question arises whether this degree of similarity between predicted and observed effects is a consequence of inherent correctness of the extrapolation method, or just coincidental. Data lack to discriminate between these possible causes. However, a thought experiment may help to clarify the reasoning. If the chosen endpoints (PICT and nematode diversity) are 'generically' sensitive (i.e., representative for other groups), then bioavailability correction by using aged-total- or $CaCl_2$ exchangeable zinc concentrations not only reduces the uncertainty associated with bioavailability differences in laboratory-to-field extrapolation (section 9.1), but it also yields Hazardous Concentrations that accurately discriminate between absence and presence of community-level effects. If the chosen endpoints, however, represent the most sensitive species groups among the groups that occur in field soils, then the concentration-effect curves for all the other (less sensitive) groups would be shifted to the right in the figures, while the predicted effect levels will remain unchanged (the laboratory toxicity data are not influenced by the choice of field endpoints). If those less-sensitive groups would have been chosen for the comparisons, this shift to the right of the response curves would have led to the conclusion that all Hazardous Concentrations are 'overprotective', or that water-soluble zinc concentrations provide a good correlation with no- or severe effects at the HC_5 and HC_{50} level. In the opposite thought experiment, the accidental choice of *more* sensitive community endpoints would have led to the conclusion that even bioavailability correction does not offer sufficient protection at the HC_5 level.
These thought experiments show that the validation of Hazardous Concentrations depends on the choice of endpoints. Apparently, the choice of community endpoints is of utmost importance to judge the validity of Hazardous Concentrations. In this context, the domain of ecotoxicology (see Figure 1.1) currently yields Hazardous Concentrations on the basis of laboratory experiments (viz. the curve relating soil concentration to predicted generic risks), and the regulatory domain weighs the ecotoxicity data with other data, usually in an iterative process. The thought experiments demonstrate that the choice between possible community endpoints should be addressed in the iterative process, in particular when ecotoxicological risk limits are questioned. The ecological relevance of the community endpoints studied in the Validation project is further discussed in Section 9.3.2, within the framework of soil protection issues.

9.2.1.2 Budel gradient samples.
- Invertebrates. Large variation in the composition of the nematode as well as the enchytraeid community structure along the Budel gradient was demonstrated (Chapters 6 and 7). For the enchytraeids, only 15 percent of the biological variation was statistically attributable to the measured environmental variables: metal concentrations (zinc, copper, lead and cadmium), soil acidity, soil moisture and organic matter contents. This means that 85% of the variability observed for this species group remained unexplained. For the nematodes, multivariate regression analysis showed that the observed species-, genera- or diversity variation, was asscoiated with zinc, but next to the metal also with soil acidity, soil moisture and organic matter content. In this species group, probably due to the (much) larger numbers of individuals and species, the explained variance was much larger (R^2 values between 0.39 and 0.76 for diversity measures).
For the validation of Hazardous Concentrations, however, the effects of zinc should be disentangled from the confounding effects of other factors, and the effects of the other covarying metals should be addressed (for method: see Gusum-case below). In the case of the Budel gradient soils, however, soil acidity strictly covaried with the degree of metal contamination. It is therefore not possible to judge the validity of risk limits for zinc only with the enchytraeid and nematode data from the Budel field gradient. Conversely, the data can not be used to reject the current risk limits of zinc.
- Microbial community. The covariation of zinc with acidity or other confounding factors was controlled in the experimental procedure to establish PICT. This is due to the use of the 'internal control' for each sampling site in the artificial exposure experiments performed in the laboratory (see Chapter 8). Zinc sensitivity decreased steadily at increasing ambient zinc concentrations (despite large standard errors due to low replication, Figure 8.6). Note that the data show that PICT in the local microbial communities tended to increase in each of the sampled soils in comparison to the most distant Budel soil that was chosen as reference. This increase included the first sample station next to the reference soil, and reinforces earlier findings that microbial functions are relatively sensitive to zinc in comparison to soil invertebrates. Looking at validation, the data suggest that the current target and intervention values correctly distinguished between no (or not-measurable) effects and evident increase of tolerance. An evaluation on the basis of bioavailability-corrected HC_5 and HC_{50}s (Table 9.4, levels 2 and 3) could not be made, since the $CaCl_2$- exchangeable and water-soluble zinc concentrations of the Budel gradient samples used for PICT measurements have not been determined.
Comparison of the PICT data from the Budel field gradient with the data from the experimental field plot in 1996 shows that decrease in sensitivity in Budel was largest by far. After a 100 years exposure history, zinc tolerance along the gradient increased with a factor of 100, with average EC_{50} values of approximately 10 and 1000 mg Zn/L (artificial exposure concentrations) for the reference soil and the most polluted Budel soil, respectively. In the experimental field plot zinc tolerance increased by a factor of approx. 4 only (see Chapter 8 and Rutgers *et al.* accepted). This difference in degree of tolerance development may be due to differences in exposure duration, co-tolerance caused by other metals in the Budel field soils, or differences in

bioavailability of zinc. Although the precise cause of the difference cannot be identified from the present data, it can be concluded that PICT measurements may be a valuable tool for location-specific risk assessment, which is mostly due to the reduced influence of confounding factors upon bioassay results (see also Bååth *et al.*, 1998).

9.2.2 Validation of Hazardous Concentrations for other contaminants

Are the conclusions reached for zinc also relevant for other chemicals? Are data available that show that Hazardous Concentrations at the 5 and 50% level do *not* relate to the absence and presence of toxic effects at community endpoints as defined above, and due to what uncertainties?

Among the possible study designs applicable for validation studies, the gradient approach as applied to the Budel soils suffices better than analyses of separate sites. Gradient-approaches (1) minimize (but not fully exclude) the influences of confounding factors, and (2) allow for the construction of 'field concentration-(community)-effect curves'. Ideally, field studies should fit to the criteria of Table 9.5. At present, all current field-effect data found in the literature have an imperfect study design for the present purposes, probably since the studies were designed for less complex purposes. Adequate studies should resemble (or improve) the approach used to evaluate the Hazardous Concentrations of zinc, but such studies are unknown in literature. Therefore, some selected examples are outlined below, to judge the validity of Hazardous Concentrations for some other metals. The chosen examples allow for clear conclusions on the (dis)similarity of predicted and observed effects.

Table 9.5. Prerequisites for ideal field studies when used for Validation purposes, either for validation of toxicity data with endpoints at the species level, or for the validation of Hazardous Concentrations with endpoints at the community level (adapted from Posthuma 1997).

Aspect	Prerequisite
Sampling strategy	- a gradient approach is preferred over site-by-site approach - the number of samples should exceed the usual number of laboratory studies - the reference situation should be well defined, considering natural variation through replicated sampling; - preliminary information should be obtained from 'range-finding' sampling - sample density should be high at concentration levels of concern, i.e. near HC_5 or HC_{50} - concentrations of the contaminant and biotic responses should be measured for all samples, next to measurements of confounding factors
Choice of endpoints	- species that disperse on a scale (much) smaller than the scale of the gradient should be incorporated only - the endpoints should be insensitive to environmental variables other than the pollutant (spatial and temporal heterogeneity)
Validation of toxicity data or risk limits	- concentrations are expressed in units that are closely related to true availability

9.2.2.1 Effects on structure parameters

- The Kastad-lead case. The study fitting best to the above methodological criteria is that of Hågvar and Abrahamsen (1990) on the toxic effects of lead on the invertebrate fauna in a

Norwegian primary forest. The local gradient was sampled intensively (n=98), and invertebrate diversity and total lead concentrations were determined. Recently derived Hazardous Concentrations for lead (for 'species', Crommentuijn *et al.*, 1997), extrapolated using a large number of toxicity data, are compared to structural community effects, in Table 9.6 expressed as number of invertebrate species per sample. A summary of the data is presented by Posthuma (1997). The HC_5 for 'species' is only slightly lower than the field-EC5 for the endpoint 'loss of species'. Concentrations only slightly exceeding the 5%-Hazardous Concentration thus apparently cause a loss of 5 percent of the species. It is however, likely that most peripheral sample does not represent the local reference situation. Therefore, the field-EC5 for species loss may be an inaccurately estimate. A discrepancy (factor 30) was found between the HC_{50} and EC_{50} for species loss. This difference is likely attributable to the factors 'bioavailability' (Låg *et al.* 1970) and the implicit notion of the extrapolation method that, at the HC_{50}, a 'generic' community is exposed for 50% of the species at a *sublethal* level. It was evident, however, that a reduction of invertebrate diversity was present at the HC_{50} level. Further evaluation of Hazardous Concentrations in the Kastad case requires accurate information to transpose total- into estimates for bioavailable concentrations.

Table 9.6. Comparison between Hazardous Concentrations for lead (for standard soil, source: Crommentuijn *et al.*, 1997) and structural community-level effects on soil invertebrates inhabiting a site long-term contaminated by lead only. Field data from Hågvar and Abrahamsen (1990).

Hazardous Concentration	mg/kg	Effect level (# of species)	mg/kg	95% C.I.
HC_5	64	EC5	219	(83 -580)
HC_{50}	488	EC50	14500	(5460 - 38500)

- <u>The Wageningen-copper case</u>. The importance of bioavailability is further supported by a field study on nematodes exposed to copper at different soil pH values (Korthals *et al.* 1996a). The composition of the nematode fauna was studied at a site near Wageningen (The Netherlands) artificially contaminated with a range of concentrations of copper sulphate. The pH(KCl) was set at various levels between 4 and 6. Ten years after establishment of the plots, samples were taken from each treatment, and copper concentrations, pH and nematode numbers were determined. The observations are compared to Hazardous Concentrations 5 and 50% for 'species', as calculated from recently collected copper toxicity data for a broad range of species (Crommentuijn *et al.* , 1997). The HC_5 and HC_{50} values were 24 and 304 mg added Cu/kg standard soil, respectively. Recalculated to the local soil type (4% clay and 1.9% organic matter, and a background copper concentration of 11 mg Cu/kg soil, Korthals *et al.*, 1996b), the local HC_5 and HC_{50} are (added + background: 12+11=) 23 and (157+11)=168 mg Cu/kg soil. The effects of copper on the nematode numbers appeared to depend on total copper concentrations and pH (Fig. 9.3, left panel). At high pH, exceedance of the HC_{50} for copper is *not* associated with decreased nematode numbers, while at low pH severe effects are observed at the same total copper concentration. This shows that the ecological relevance of Hazardous Concentrations for which no bioavailability correction has been made differs for different situations. Nonetheless, since acidification of soils may occur on the long-term (e.g., when the function of agricultural fields change to nature areas), the generic risk limits should be sufficiently protective to allow for this change. Figure 9.3 (left panel) suggests that the Hazardous Concentrations would be too strict for soils with a high pH, but that they offer the required level of protection at low pH. Toxic effects in the field plots were also expressed on the basis of $CaCl_2$-exchangeable copper concentrations (right panel), assuming that this copper fraction is a better estimate of nematode exposure than total concentrations. This yielded overlapping effect curves, except for the low exposure concentrations. In this concentration range, low acidity directly affects nematode numbers. These curves may in principal be used to evaluate bioavailability-corrected Hazardous Concentrations, similar to the comparisons for zinc in the Validation project. However, no single-species laboratory toxicity data based on $CaCl_2$ exchangeable fractions are available for copper. Nonetheless, the copper data demonstrate that pH correction is *vital* to improve field relevance of Hazardous Concentrations.

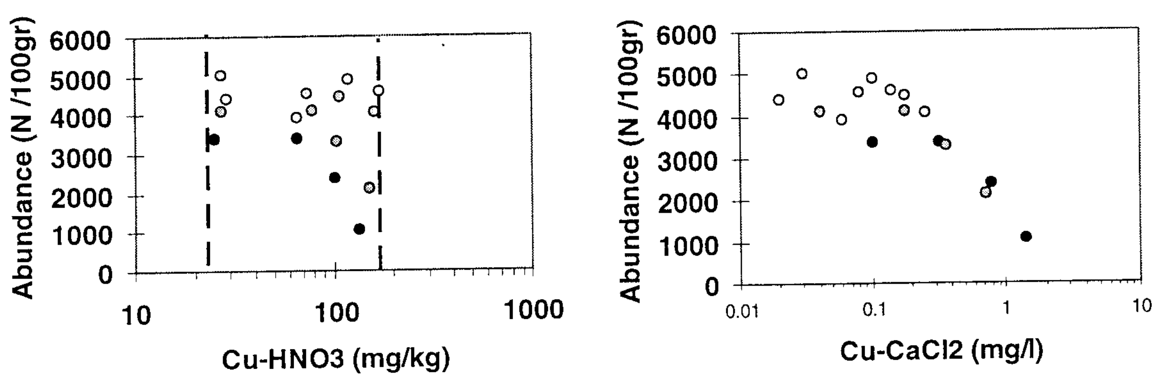

Figure 9.3. Abundance of nematodes living in close association with the soil pore water (all species summed) after 10 years of exposure to copper in an outdoor agricultural field plot with different copper concentrations at 4 pH levels (the degree of circle coloration depicts soil acidity: white pH=6.1, light grey: pH=5.4, dark grey: pH=4.7, black: pH=4.0. Left: based on total copper concentrations, right: based on $CaCl_2$-exchangeable copper concentrations. Dotted verticals represent HC_5 and HC_{50} of copper after soil type correction; these cannot be calculated for $CaCl_2$-exchangeable copper concentrations (data from Korthals *et al.* 1996a).

<u>The Gusum-zinc and copper case</u>. A third study illustrates the possibilities for evaluating the field relevance of risk levels when site contamination consists of a mixture of toxicants. The impact of metals on forest floor ecosystems has been studied intensively near the brassworks at Gusum (SE Sweden), Data from various groups of organisms have been compiled (Tyler 1984). These brassworks are the only industrial sites in that forested area and almost exclusively zinc and copper (98 %) are emitted. The concentrations of lead are low. Because there is no primary smelting, the emission of acidic compounds is insignificant.

Several studies on the occurrence of plant and animal species, and on soil processes have been performed along the contamination gradient (western direction). Because metals accumulated mainly in the organic layer of the top soil (mor horizon), the studies focused on that part of the soil system.

The ecotoxicological risk limits were addressed by comparing the predicted effects (assuming concentration additivity at the level of 'potential risks') with observed effects for a mixture of metals, the effects of different metals could not be separated. To this end, soil metal concentrations were expressed in Contamination Units. This approach follows the principles of the Toxic Unit approach for studying mixture effects on the species level (see Chapter 2). This was done by first subtracting the background concentrations (measured at approx. 8 km of the emission source) from the measured concentrations at each site. The resulting values were assumed to be the *added* concentrations, and by definition the reference site is characterized by CU_{added}=0. Subsequently, the added concentrations were expressed as dimensionless fractions of the appropriate Hazardous Concentrations. The latter were calculated from a recent inventory of toxicity data by Crommentuijn *et al.* (1997), HC_5 and HC_{50} values were calculated separately for 'species' and 'microbial processes'.

The data are summarized in Table 9.7. For lead, the low local soil concentrations and the relatively high Hazardous Concentration imply that the contribution of lead to the summed Contamination Units was low. Therefore, the contribution of lead was considered negligible. For the soil type correction it was assumed that the forest top soil at Gusum contains 2 % clay and 30 % organic matter (alternative realistic assumptions do not affect the conclusions reached within the bandwidth of a factor of ten).

The analyses show that almost all functional and structural parameters have a reduced performance concomitant with the increase of the Cu+Zn concentrations in the soil (Figure 9.4); microfungi and vascular plants showed the weakest responses.

Focusing on the HC_5 value for 'microbial processes' (upper left panel), the figure shows that the reference site had a mixture-$HC_{5,\ processes}$ of 1 CU. This means, that the reference site is exposed to copper and zinc at the level of the regulatory chosen Dutch Maximum Permissible Concentration. Exceedance of this HC_5, by e.g. 10 times or more, is associated with reduction of all process-parameters. For 'species' (lower left panel), the effects were less severe. The reference site at 8 km downwind was exposed at a level of one-tenth of the Dutch MPC. Exceedance of the $HC_{5,\ species}$ is associated with a reduction of species number. Following similar reasoning for the right panels, bearing on exceedance of the mixture HC_{50} for 'microbial processes' and 'species', shows that both types of parameters show severe reduction beyond 1 CU, i.e. beyond the HC_{50} level calculated for the mixture.

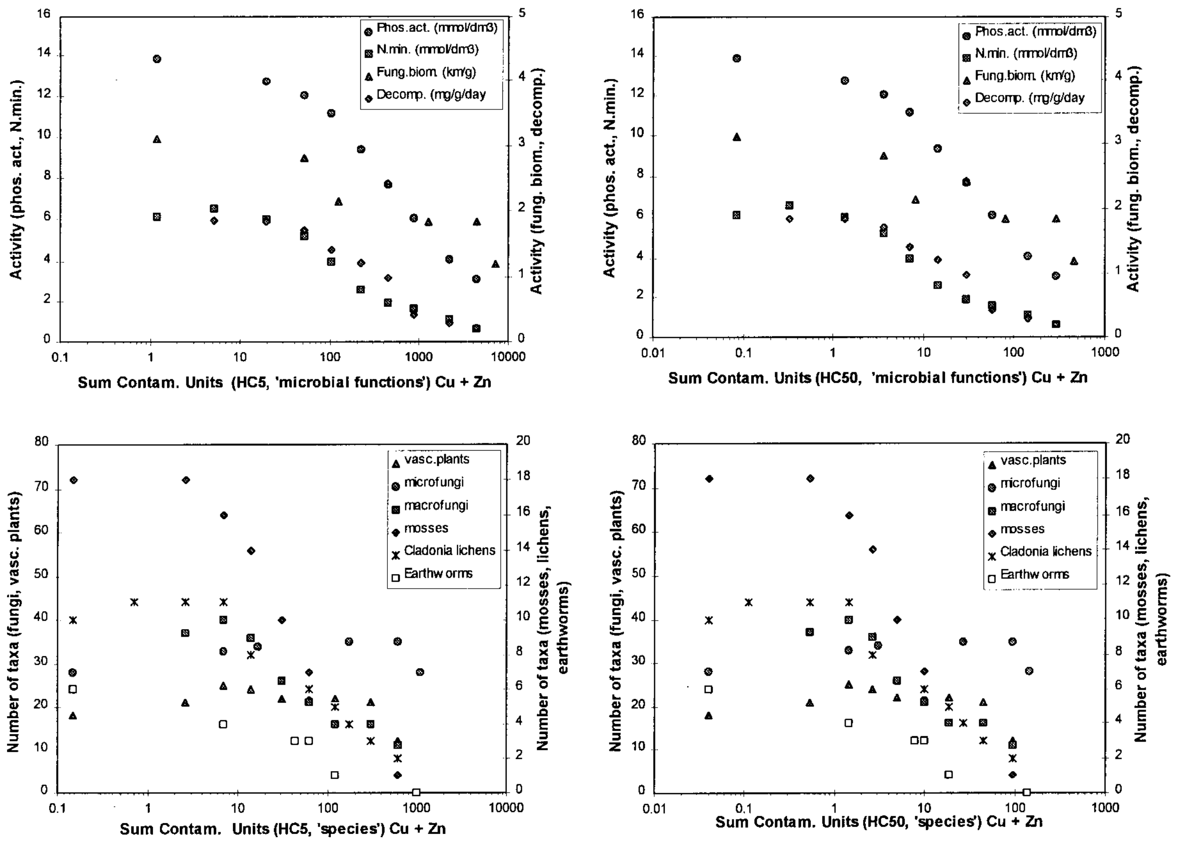

Figure 9.4. Impact of copper and zinc on the ecological performance of various functional (upper panels) and structural characteristics (lower panels) of soil biota inhabiting the organic layer (litter and humus) of forest soils in the neighbourhood of Gusum (SE Sweden). Contamination is due to the emission by brassworks. Endpoints are plotted against the summed Contamination Units of copper and zinc as explained in the text. These units are calculated by dividing soil concentrations (minus background) by the appropriate ('species' or 'microbial processes', for standard soil) HC_5 (left panels) and HC_{50} (right panels), respectively. Data from Tyler (1984).

Table 9.7. Hazardous Concentrations for added metals in standard soil derived from Crommentuijn *et al.* (1997), and in Gusum soil (assuming 2% clay and 30% organic matter). Data were used for the calculation of Contamination Units for the Gusum-case.

HC-level	Soil	Cu (mg added / kg)		Zn (mg added / kg)	
		'microbial processes'	'species'	'microbial processes'	'species'
5	Standard	3.5	24	16	132
50	Standard	59.8	304	207	385
5	Gusum	3	23	12	95
50	Gusum	57	289	149	278

In the Gusum-case, the mixture-HC_{50} values are associated with severe effects for a range of organism types, not just micro-organisms and nematodes as in the previous examples. The analysis of the Gusum case also demonstrates that for mixtures of contaminants Hazardous Concentrations can in principle be judged in the same way as for single contaminants. The applied principle boils down to using concentration additivity as the null-hypothesis for combined action. This might be slightly precautionous when considering joint effects of metals on separate species in controlled experiments (Chapter 4, 5 and 6).

9.2.2.2 Effects on function parameters

The occurrence of PICT in microbial communities exposed to metals has been established in some studies. Doelman and Haanstra (1979) studied the occurrence of PICT for lead, comparing the community tolerance in samples from reference sites and sites contaminated with lead. For each sampling site, colony-forming units were counted in two media with different levels of added lead. This yielded two observations on increased microbial tolerance (PICT), based on two different sub-samples from the local communities. The EC10 in the artificial media was chosen to quantify community tolerance, and was calculated following Notenboom and Posthuma (1995). This study showed that the EC10 of the community samples from five reference sites varied between approx. 0.01 and 1 mg Pb/kg in the media, whereas the samples from the contaminated sites showed EC10 values of approx. 1 mg Pb/kg in the medium for a smelter site, up to ranges between 10 and 100 mg Pb/kg in the medium for samples from other smelter areas and samples collected near ore deposits. PICT for lead occurred at total lead concentrations of 200 mg Pb/kg, and higher. The HC_5 and HC_{50} for 'microbial processes' using data of Crommentuijn *et al.* (1997) are 55 andd 523 mg added Pb/kg. Evidently, exceedance of the HC_{50} for lead is associated with responses in the microbial community. The data do not allow for conclusions at the HC_5 level.

Following an approach that is grossly similar to the PICT-measurement in our experimental field plot, Diaz-Raviña *et al.* (1994) studied the occurrence of PICT after adding different concentrations of various metals to a non-polluted field-collected soil. Nine months after the additions, PICT was established in samples from the microbial communities. Instead of measuring substrate decomposition (as in Chapter 8), these authors applied thymidine incorporation rates to estimate bacterial population growth. Again, PICT was demonstrated at the appropriate HC_{50} level derived from Crommentuijn *et al.* (1997). Since all exposure concentrations added at the beginning of the experiment were much higher than the appropriate HC_5 values, the HC_5 could not be evaluated as accurate predictor of absence of PICT-development.

9.2.2.3 Data from multi-species experiments and other observations

Many researchers have compared single- and multi-species toxicity experiments. Approximately 2000 literature data have been reviewed by Okkerman et al. (1993), bearing on organic contaminants in aquatic organisms. According to this review single-species toxicity data provide a good starting point for the derivation of ecotoxicological risk limits. The extrapolated Hazardous Concentrations were generally slightly lower than the NOEC of multi-species experiments, i.e., they apparently offer sufficient protection in a simplified multi-species setting. This has been confirmed recently in more complex studies in experimental ditches. Effects of application of the insecticide chloropyrifos on the invertebrate community and its recovery were quantified using univariate statistics and multivariate ordination techniques (e.g., Van den Brink et al. 1996). The ordination technique was successful in deriving a concentration-effect curve for the total invertebrate community while still providing information at the species level. Comparisons between short-term responses of indigenous species in the experimental ditches (Van Wijngaarden et al. 1996) and long-term effects showed that NOECs for short-term exposed sensitive indigenous species and the long-term exposed invertebrate community were similar.

ECETOC (1997) recently evaluated laboratory-to-field extrapolation in a two-step approach, first from laboratory to multi-species experiment, second from multi-species experiment to the field. For the first step, the ratio between the lowest species NOEC and the NOEC of the most sensitive multi-species endpoint was (on average) 1.45, with a 90 percentile of 8.1. This implies that a safety factor of 8 could be applied to the lowest species-NOEC to protect most community endpoints. This value is lose to the value of 10 which is often applied as standard safety factor in risk assessment procedures. The second sub-extrapolation did not show the need for an extra safety factor, given well-designed multi-species studies.

Finally, Van Wijngaarden et al. (in press) and Lahr et al. (in press) reviewed the ecological relevance of the Dutch MTR for insecticides and herbicides, as were recently derived by Crommentuijn et al. (1997b). This was done by comparison of this risk limit with effects observed in aquatic micro- and mesocosm studies. It appeared that the Dutch MTR offered sufficient protection to freshwater ecosystems, both with respect to structure and function, even when the aquatic communities were exposed to (sub-)chronic concentrations of pesticides. The Dutch MTR was generally "overprotective" in case of a single application of non-persistent pesticides.

For soils, multi-species experiments relevant for a validation of risk limits are scarce. For this compartment, it is important to assure that the true exposure under laboratory and mesocosm or field conditions is similar. In principle, the change of the nematode community in the experimental field plot during the years can be analysed with the ordination technique applied in the experimental ditches. In addition, only Bembridge et al. (in press) have collected data of similar kind, describing the changes in earthworm communities after pesticide treatments. Again, concentration-effect curves for the earthworm community could be established. The technique allows for the derivation of the time needed for recovery, which could be used as a relevant endpoint for community-level effects.

9.2.2.4 Conclusions on the field relevance of Hazardous Concentrations

-Zinc. The results obtained within the Validation project have allowed for a comparison of predicted and observed toxic effects on two community endpoints, viz. nematode diversity and microbial PICT. Although it is not known whether the chosen community endpoints are relatively sensitive compared to a 'generic' community, it can be stated that the Hazardous Concentrations correctly discriminated, within the bandwidth of a factor of ten, between negligible and severe effects at the 5 and 50% level, respectively. This held both for the experimental field plot and the Budel field gradient. Correction for bioavailability differences is

scientifically justified, and altered the observed patterns of prediction versus observations for the experimental field plot. However, the change did not exceed the bandwidth of 10.
The question arises whether the findings of the Budel field gradient and the experimental field plot are valid for other zinc-contaminated soils as well. Various authors have advocated that the range of variation in soil quality among sites, and of the possible toxicity endpoints, makes the task of a *generic* validation of Hazardous Concentrations extremely difficult if not impossible (e.g., Chapman *et al.* 1998). These authors emphasize that location-specific aspects should always be incorporated in a scientifically-based application of risk limits. It was the mission of the Validation project, however, to consider *generic* risk limits. The investigated cases (experimental field plot and Budel gradient) have a very different contamination background, but yielded similar conclusions. Therefore, the conclusion is considered robust, also for other soil types.
- Other data. In the studies taken from the literature, no corrections could be applied for the uncertainty factors mentioned in Table 1.1. Nonetheless, the gross overview of the collected studies suggests that the extrapolation method does *not* result in non-sense Hazardous Concentrations within a bandwidth of ten: the comparisons indicate that toxic effects on community endpoints in the field indeed occur at concentrations within one order of magnitude of the extrapolated values. In most cases, the bandwidth was even considerably less than a factor of 10. In relation to the zinc data, it has already been mentioned that it is not clear whether the agreement between predicted and observed effects is coincidental, or that it is caused by an intrinsic correctness of the extrapolation method. The observation that generic Hazardous Concentrations can not be categorized as non-sense is now repeated with other data. This supports the argument that the extrapolation methods yield *generic* protection levels (5% level) and *generic* levels of concern (50% level). Exceedance of Hazardous Concentrations implies *that* adverse effects will become likely (beyond HC_5) or will be severe (beyond HC_{50}), but does not specify *which* effects are to be expected.
On the basis of this conclusion it can be stated that there is no motivation in the scientific data for changing the chosen protection levels at 5 and 50% to higher or lower values. At the 50% level, adverse effects were obvious, whereas at the 5% level uncertainty about the reference situation and statistical uncertainties hamper a scientifically-based proposal for alternative cut-off points of the sensitivity distribution.

It should be noted that the investigated data concerned Hazardous Concentrations for metals. For these compounds, it may be expected that the sensitivity distribution is unimodal, and this has been confirmed by the data of Crommentuijn *et al.* (1997). For compounds with a specific mode of action, for example for pesticides, a bimodal distribution of species sensitivities may be found, or other important compound- or intoxication aspects may differ. However, as argued in Section 9.1, the order of toxicity of chemicals has more often been found to be grossly similar in laboratory and field studies. Often, literature data do not contain the data required for validation purposes. This holds in particular for pesticides, for which field assays often only bear on one or two exposure levels. Eco-epidemiological reasoning (requiring further circumstantial evidence) may improve plausibility of conclusions for specific problems (Bro-Rasmussen and Løkke 1984, Fox 1991). Exceptions to the rule that *generic* risk levels identify the levels of protection and concern may, of course, exist, but it is beyond the scope of this report to specify these cases. Only repeated and extreme disparity between predicted and observed effects should be considered as a trigger for the reconsideration of *generic* risk limits. This may hold for cases where the cause of the disparity is obvious, so that it can be solved; otherwise, safety factors can be applied. In addition, it should be kept in mind that *generic* Hazardous Concentrations bear on a first-tier evaluation. False positives, i.e. the prediction of effects when true effects are absent, will appear in the second-tier, the location-specific evaluation that is currently developed further.

9.3 Environmental Quality Criteria (EQC) for soil: present status and future developments

Although in the Validation project attention has mainly been focused on experimental work with zinc as contaminant, and some selected organisms in specific soils and situations as response variables, a more general assessment of the approaches and results reveals the following four elements:

1. The design of laboratory toxicity experiments
2. The choice of relevant community endpoints
3. The derivation of Hazardous Concentrations and their use in soil protection policy
4. The evaluation of risks of local pollution within the framework of soil remediation.

These aspects are related to validity of toxicity data (1) and validity of risk limits (2, 3 and 4), and will be briefly discussed, assessing the relevance of the issue and the perspectives for actions to be taken.

9.3.1 Design of toxicity experiments

<u>9.3.1.1 Improvement of laboratory-to-field extrapolation.</u>

To improve the field relevance of laboratory-to-field extrapolation the following issues should be tackled:

- Understanding bioavailability is of prime importance for understanding the ecotoxicity of chemicals in soils (Allen 1997, Chapman *et al.* 1998), both for laboratory toxicity data and for the evaluation of contamination risks at contaminated field sites. This conclusion is not only based on the quantitative importance of this issue, but also by its basic position in the cause-effect chain: e.g., one cannot address joint toxicity properly without addressing bioavailability (Van Gestel and Hensbergen 1997, Posthuma *et al.* 1997). In laboratory toxicity experiments, measurements should be made on the true exposure of the test organism under test conditions. As advocated by, e.g., Van Wensem *et al.* 1994 and Posthuma *et al.* in prep.), the measurement of body residues may be the crucial factor through which exposure differences in different situations can be understood. It is emphasized again that this approach should be handled with care for essential elements.

- Within toxicity experiments, there is a need for an ecologically underpinned selection of toxicity endpoints. Optimally, the endpoint should discriminate the toxicity level that truly affects the population viability, and it should integrate all relevant sublethal effects. At present, this criterion is not necessarily valid for current sublethal endpoints used in standardized test systems (e.g., cocoon number in *Eisenia andrei*). In the chain of cause to effects, this aspects is basic for understanding toxic effects per species. Just as for bioavailability in the abiotic domain, population fitness should be understood before one can succesfully address aspects like species interactions. Population viability analyses are frequently made in the fields of wildlife toxicology and nature preservation. Approaches to address this question for soil invertebrates have been suggested amongst others by Van Straalen *et al.* (1989) and Kammenga *et al.* (1997).

9.3.1.2 - Perspectives for implementation.

Adopting novel experimental designs in these ways will improve the field relevance of single-species toxicity data, and thereby the scientific underpinning of the derived Hazardous Concentrations. Adaptations of experimental schemes are not applicable, however, when retrospective literature searches are the only source of toxicity data. For many chemicals, only few data exist, and safety factors upon the lowest NOEC are often applied instead of statistical extrapolation. Considering literature data, a stricter confinement to data that fit the new criteria can be reached by expert judgement, although this should preferably not lead to the use of assessment factors (that have no ecological basis). It should be noted that the implementation of improved experimental designs will yield better data for laboratory-to-field extrapolation only on the medium term (years to decades). Control of this factor is thus rather restricted, i.e. relating to the definition of selection criteria by which existing studies can be evaluated rather than relating to the development of new standardized assays.

9.3.2 The choice of community endpoints

9.3.2.1 Addressing structure and function.

Once the extrapolation method has been used to derive Hazardous Concentrations, the question arises 'what is (not) protected'? Initially, when the extrapolation method mainly addressed toxicity data for soil invertebrates, there was the notion that the Hazardous Concentrations only concerned the protection of community structure, and that only indirectly community function was protected. In the Validation project structural and functional toxicity endpoints were however studied simultaneously. This is in agreement with the fact that the extrapolation method is presently used separately for 'species' and 'microbial functions' so that separate HC_5 and HC_{50} values are derived for these two entries (see, e.g. the data for zinc collected in Table 1.2).

In the Validation project, the chosen community endpoints were related to toxic effects for cryptobiota, which is a choice that may be disputed in view of certain policy aims (e.g., no relationship with a Target Species approach in Nature Conservation policy). However, any chosen endpoint should be biologically plausible (linkage of cause and effect, and an *ecological* relevance of response), and it should appeal to regulatory and public interest. This means that

exceedance of Hazardous Concentrations in the field should lead to truly adverse effects on community endpoints that are generally accepted as 'valuable'.
Some remarks can be made on the relevance of cryptobiota for the validation of generic risk limits. First, the linkage of cause and effect often trades off with the issues of ecological relevance and regulatory and public interest of the chosen endpoint. Amongst others, statistical considerations render vertebrate diversity, as an alternative option for 'species', unsuitable for the purpose of evaluating Hazardous Concentrations. Such considerations yielded the endpoints in the cryptobiota, for which regulatory and public interest is not inherently present. From the point of view of validation of *generic* risk limits for soils, however, it is defensible to address the *primary* responses in the exposed ecosystem, using organisms that live in close contact with the soil (pore water), that have a low response time and an inherently high (species or functional) variability within the sub-system.
Also from other viewpoints the choice of cryptobiota gains growing attention. Firstly, Dutch environmental policy increasingly focuses on function next to structure, in particular for areas outside the national Nature Target Types. For those areas, the issue of Life Support Functions has been brought forward, to focus on those functions that are as yet hidden in the soil, but that are vital to maintain the biogeochemical cycles on which life depends.
Secondly, there are ecological clues to consider structure and function simultaneously, instead of the notion of 'indirect functional protection' once structure is protected. This is clearly illustrated in various literature data on Pollution Indiced Community Tolerance (PICT). PICT studies show that changed function is associated with changed community structure. Millward and Grant (1995) showed that the increased community tolerance of an exposed estuarine nematode community was associated with a structural change: the most sensitive species were absent or occurred at lower density at the most polluted site. Blanck and co-workers also mentioned such relationships for communities of exposed algae, with sometimes structure and sometimes function being the (slightly) more sensitive endpoint (e.g., Blanck *et al.* 1988 and Molander and Blanck 1992).
Further, there exists evidence that PICT is associated with a reduced ecological amplitude (Figure 8.7). The frequency distribution of the exposed microbial community was narrowed compared to the reference community. Moreover, there may be an associated loss of metabolic functions. Doelman *et al.* (1994) showed that tolerance evolution in soil microbial communities evolves at the expense of the degradation capability for organic substrates. On the one hand, the potential socio-economic importance of this is the change of biogeochemical cycles basic to ecosystem functioning, while on the other hand the efficacy of such organisms in remediation of organically-contaminated soils is affected. Both aspects, however, need further support from field studies.

9.3.2.2 Perspectives for implementation.

Critical evaluation of generic risk limits may for certain chemicals be needed in the future, due to lack of laboratory data of sufficient quality, or for specific ecological or economical concerns (i.e., risk limits for a certain chemical are seemingly under- or overprotective). In such cases, the results of (semi-) field studies should be interpreted, and study endpoints and study design should be carefully chosen. The endpoints studied in the Validation project are good candidates for such studies, for the characteristics mentioned above (fast, variable and direct responses). The study design of PICT-studies, with an internal control per sample site, should preferably be considered, to avoid uninterpretable results from complex field studies in which many factors vary, as was observed along the Budel gradient in the field.

9.3.3 Derivation of Hazardous Concentrations and their use in soil protection policy

9.3.3.1 Improvement of generic Hazardous Concentrations.

In general, reconsideration of the available laboratory toxicity data will not yield better scientifically underpinned Hazardous Concentrations in the near future, simply due to lack of data (9.3.1.). The accuracy of Hazardous Concentrations can, however, be improved in the Dutch system by transforming the method of soil-type correction into a scientifically underpinned approach for the correction of bioavailability differences between soils. The Validation project and other recent investigations (Van Gestel *et al.* 1995, Allen 1997, De Rooij and Smits 1997, Peijnenburg *et al.* 1997, Janssen *et al.* 1997ab) have yielded strong evidence to incorporate soil acidity, in addition to clay and organic matter contents, to the correction system. The notion that bioavailability correction is crucial for a proper estimation of risks has already been adopted in the calculation of the Potentially Affected Fraction (PAF) of species, which is based on the same extrapolation principles as used to derive Hazardous Concentrations (Klepper and Van De Meent 1997).

It is intrinsically impossible to derive Hazardous Concentrations that are equally applicable to all circumstances: Hazardous Concentrations cannot yield the same level of (non-) protection under different field conditions for different endpoints (Chapman *et al.* 1998). After proper bioavailability correction, for example, differences still remain among ecosystems in which contaminants are released. In fact, generic Hazardous Concentrations will remain to be a first-tier yardstick approach, against which potential effects of chemicals in ecosystems can be judged. Second-tier evaluation systems, to evaluate risks at contaminated sites more specifically, are currently receiving increasing regulatory and scientific attention.

9.3.3.2 Perspectives for improvements.

Currently, lack of toxicity data of sufficient quality may be the limiting factor for improving the accuracy of generic Hazardous Concentrations for many compounds. However, the collected data have not shown a general reason to adopt a different first-tier methodology. It predicts, given sufficient toxicity data and no obvious deviation from the hypothesized sensitvity distribution, *that* some adverse effect is likely to occur when the Hazardous Concentration is exceeded, although it does not specify *which* effect.

9.3.4 Location-specific judgement

9.3.4.1 Bioassays in the second-tier assessment.

The results of the Validation project can be used in the further development of the second-tier system for the evaluation of risks at contaminated sites. This is related to the frequent application of bioassays within the project. At present, decisions on soil remediation and on the need for further research are based (in the Dutch system) on local exceedance of the IV or the criterion for further investigations (exceedance of [0.5*(IV+TV)]), respectively (VROM, 1994b). Location-specific risk assessment asks for a further critical evaluation, on the *meaning* of the exceedance of Hazardous Concentrations. In line with the TRIAD-approach followed in the evaluation of sediment quality (e.g. Chapman, 1986; Van De Guchte, 1992), the critical evaluation should consider environmental chemistry (concentrations, availability and accumulation), bioassay results and results of field inventories. The second-tier is likely to consist of various levels of refinement (Rutgers and Notenboom, pers. comm.). For the bioassays, this can take the form of fast screening tests and more elaborative diagnostic tests. The results of the bioassays of the Validation project suggest that bioassays should be interpreted in concert with proper data on the reference situation, with (environmental) chemical data, with data on potentially disturbing factors, etcetera. One aspect needs specific attention when looking at bioassay studies with soil animals for second-tier assessments of soil quality. This concerns the pH-preference of most organisms used in the standardized laboratory assays. For most of the investigated species, the soil from the Budel gradient was too acid, and required pH control. Apparently, the selection of potentially suitable bioassay species is quite limited, and not suitable for many Dutch soil types.

9.3.4.2 Perspectives for improvement.

Screening- and diagnostic bioassay tests for soil require further development, and should concern a broader array of test species than currently available in the 'pool' that consists of the species used for standardized toxicity testing. The further development and width is needed for bioassays to be implemented succesfully in a second-tier evaluation system for evaluating risks at contaminated sites that can be used throughout the Netherlands. Current experience with soil bioassays is limited.

9.4 Alternative endpoints for alternative policy aims

The current system of ecotoxicologically based EQOs has been operationalized (for a number of chemicals) on the basis of toxicity data for soil invertebrates and microbial functions. This approach is basic to Dutch legislation for the regulation of chemicals. The Validation project implicitly followed this notion, by using soil-invertebrate and microbial toxicity data, extrapolating the data into Hazardous Concentrations, and evaluating those concentrations again with observations on the occurrence of soil organisms at contaminated sites. Although intrinsically a coherent approach, risk limits or Hazardous Concentrations should not only protect soil organisms. Validation could also have focused on other endpoints, like the population viability of certain Target Species. This would imply that population rather than community endpoints should be taken into account when validity of risk limits is concerned. In the Netherlands, the protection of valuable wild-life species (Target Species) has been operationalized by defined Nature Target Types ("Natuurdoeltypen"), with the particular wild-life species as key species (usually vertebrates, plants) (Jansen *et al.* 1995). This approach, which is associated with nature conservation endpoints, requires (a) food chain models to estimate exposure of wild-life species, (b) appropriate population models, and (c) data on the intrinsic sensitivity of the species for chemicals, in order to predict toxic effects at the population level. Next to the protection of structural parameters, attention increasingly focuses on ecosystem functions (e.g., Breure 1996). Risk assessment systems may eventually develop into a tiered approach, in which toxic effects of chemicals on both cryptobiota and wild-life species, and on functions, can be evaluated at a generic or specific manner. The present project addressed the validity of toxicity data and risk limits for cryptobiota that are directly exposed by chemicals in soils. The development of a coherent system that can be accomodated to be used for the different endpoint types (structure - function, cryptobiota - vertebrates/plants, etc.) will be a major challenge for the future.

10. CONCLUSIONS AND RECOMMENDATIONS

Field relevance of laboratory toxicity data

1. Bioavailability is a very important factor affecting the extrapolation of laboratory toxicity data for metals to the field. This conclusion was confirmed for plants, collembolans, earthworms and enchytraeids, but not for micro-organisms.
2. Soil acidity and contaminant ageing are important factors influencing bioavailability, and consequently toxicity of metals in laboratory tests, semi-field tests and bioassays with contaminated field soils. Soil acidity has a major influence on metal solubility in soils and on bioavailability of metals to soil organisms. Ageing often reduces metal solubility in field soils. Reduced solubility in field soils due to ageing can be counteracted by low pH of some field soil types, or by breakdown of organic matter. It should be noted that the soil pH of most standardized test systems is approximately neutral, and higher than in many field soils.
3. The soil type correction system currently used in Dutch soil protection policy does not adequately correct for differences in bioavailability of metals among soils that are caused by other factors than organic matter and clay content.
4. It must be realised that the soil type correction has a physico-chemical basis that does not take into account differences in contaminant uptake between exposed species. Soil factors influence both physico-chemical 'supply' and biological 'demand', as shown for pH. At low pH uptake in earthworms in soil increases, whereas it decreases in micro-organisms in water. Research on metal bioavailibility, aimed at understanding physico-chemical processes and species-specific aspects to improve the soil type correction system, is currently executed in a related Dutch research programme, called "Methodology for determination of heavy metal standards for soil".
5. Expression of toxicity data (e.g., EC_{50}) on the basis of (1) 0.01 M $CaCl_2$ exchangeable or (2) water soluble concentrations reduces differences in metal toxicity levels between soils in bioassays with soil invertebrates and plants in many cases. It can be applied as a practical approximation of metal bioavailability in location-specific assessment of soil quality.
6. Expression of toxicity data on the basis of internal concentrations (body residues) has been suggested as a useful instrument to reduce differences in metal toxicity between bioassays with different soils. However, for a regulated metal like zinc, internal concentrations in the test organisms may have only limited value to predict toxic effects for sublethal endpoints. Effects of zinc on reproduction of collembolans and earthworms already occur before internal concentrations start to increase. Hence, homeostatic body concentration ranges do not represent a no-risk area.
7. Based on mixture toxicity experiments with collembolans, earthworms and enchytraeids, it can be concluded that effects of metal mixtures differ between biological species and, within a single species, between toxicity endpoints. Mixture effects are influenced by sorption interactions in soil. Joint effects of metals were mostly similar to a concentration-additive response or slightly less than concentration additive. The potential risks of exposure to

multiple substances can be evaluated on the basis of concentration additivity, unless strong evidence exists for another type of response (e.g., synergistic, antagonistic, response additive).

8. The influence of uncontrolled environmental (climatic) conditions on species sensitivity is fairly small and negligible compared to the effect of bioavailability, as long as climatic conditions are in the range that the species normally encounters in the field.
9. Heterogeneity in laboratory tests is usually minimized. Outdoors, conditions may differ over small spatial scales, as shown in the experimental field plot experiments with plants and springtails. The variation of environmental conditions apparently causes variation in species performance, that is superimposed on the variation normally observed in biological tests. In most (semi-)field studies, heterogeneity is insufficiently accounted for in the sample density, so that toxic effect levels are difficult to establish due to statistical noise in the data.
10. Due to heterogeneity and variation in additional stress factors, the absence of a species at a contaminated field site does not necessarily indicate the presence of adverse effects of contaminants, and the presence of species does not indicate the absence of such adverse effects.
11. For a versatile bioassay system, useful for characterization of risks in a wide range of soil types, the natural soil factors should fit within the ecological range for the species. This asks for the use of a range of species with different ecological amplitudes (e.g., different pH preferences), or of species with a broad ecological amplitude. In this respect, it is important to know autecological features of species used in bioassays. Artificial optimization of the soil prior to a bioassay will influence bioavailability of the contaminant, which reduces the power of the bioassay results to elucidate local risks of contamination.
12. Current standardized laboratory toxicity tests for invertebrates and plants usually focus on lethal or sublethal toxicity endpoints under (considerably) less than life-time exposure. For the organisms used in standardized test systems there are limited data on the relationship between the studied (sub)lethal toxicity endpoints and long-term performance of the population. Hence, limited conclusions about long-term effects at the population level are possible.
13. Toxic effect levels obtained for laboratory-bred species cannot directly be extrapolated to other species. However, inter-species extrapolation of toxicity data is deemed unnecessary for generic risk assessment when toxicity data from a broad spectrum of species can be used in the statistical extrapolation procedure as applied to obtain Hazardous Concentrations.
14. Many literature reviews on the comparison of toxicity data obtained in laboratory and field for metals and pesticides yield the conclusion of a similar ranking of relative toxicities in laboratory and field tests. The interpretation of field effects on the basis of laboratory toxicity data is severely hampered by the lack of knowledge on actual exposure concentrations and the low number of concentrations or replications. Consistent characterization of exposure concentrations is needed.

Ecological relevance of Hazardous Concentrations

15. Correction for bioavailability before using zinc toxicity data in the extrapolation procedure affected the value of the Hazardous Concentrations for zinc at maximum with a factor of four.
16. The influence of correction of toxicity data for bioavailability (or other factors that influence the sensitivity of species) on the outcome of the statistical extrapolation procedure may be numerically negligible when the variation in species sensitivity is high. Depending on the type of substance under consideration, this may be a practical reason not to put too strong emphasis on scientifically justified, but numerically irrelevant correction factors for laboratory-to-field extrapolation.
17. Consistent documentation of test conditions is needed to be able to choose the most reliable input data from literature and to decide whether an observed sensitivity distribution reflects differences in sensitivity or is biased by peculiar test conditions.
18. The observations in the experimental field plot after ageing show that the HC5 and HC50 for added zinc, as determined from a recent compilation of toxicity data (in which no deviations from the log-logistic sensitivity distribution were found)[1] discriminate between respectively the absence and presence of toxic effects on two community toxicity endpoints, viz. (1) structural effects on the nematode community (loss of nematode diversity) and (2) functional effects on the microbial community (increase of microbial community tolerance for zinc). Within a bandwidth (a factor of 10 but usually less), the HC5 is related to no or minor effects on the studied endpoints, whereas the HC50 is related to measurable effects.
19. In the Budel field gradient it is not possible to disentangle effects of zinc (and the other metals copper, cadmium and lead) on enchytraeid and nematode community parameters from the effects of the covarying soil pH. Furthermore, there was a high heterogeneity of other soil factors, such as organic matter and clay content, and biological variation was largely not related to measured soil factors.
20. Heterogeneity of soil factors and covariation of metal concentrations with soil pH were experimentally eliminated in the data on zinc tolerance of the indigenous microbial communities along the Budel field gradient. Even at the lowest concentration levels in the field range, below the HC5 for microbial processes, an increase of zinc tolerance can be measured. At the HC50 level for microbial functions, a large increase of community tolerance is again present.
21. Effects on individual nematode species are detected at concentrations below the effect levels for community endpoints (e.g. species diversity indices). The abundance of opportunistic species may increase at increasing exposure concentration due to reduced competition or changed ecological interactions, before it declines due to direct effects of the contaminant.

[1] See Crommentuijn et al. (1997); Methods applied therein to derive Hazardous Concentrations follow the 'added risk approach', and derive HC5 and HC50 values separately for 'species' and 'microbial processes'. Further conclusions and recommendations also refer to this report.

22. Hazardous Concentrations for metals from the recent compilation of toxicity data (see footnote 1) have been compared to various literature data on toxic effects on community endpoints in the field. Again, the HC5 is related to no or minor effects in pertinent endpoints, whereas the HC50 is related to severe effects.
23. In the Validation project and literature studies, the measured community endpoints concern fast responding, intrinsically diverse and ubiquitous groups of organisms (soil invertebrates and micro-organisms), which are highly exposed due to their living in the top soil layers. Community endpoints for such groups of organisms are especially suited for validation of generic Hazardous Concentrations. This is concurrent with the policy viewpoint that generic risk limits should be sufficiently protective in all possible situations in the Netherlands.
24. The studies in the Validation project support that currently used method for the derivation of generic ecotoxicological risk limits (including ascertaining of a log-logistic distribution of sensitivities) yields plausible results. Exceedance of the HC5 in the field predicts *that* effects are likely, although it does not specify *what* the nature of the effect will be. Exceedance of the HC50 predicts severe effects.
25. Procedures for the derivation of Hazardous Concentrations are a plausible tool for a preventive, first-tier evaluation of ecotoxicological risks of contamination. From a scientific viewpoint, a generic system cannot accurately predict effects for all possible soil conditions and exposed ecosystem types. In this respect, a second-tier risk assessment system is needed for a further quantification of contaminant risks at specific sites.

Recommendations

1. The incorporation of soil pH as an additional parameter in the soil type correction method should be considered.
2. Extraction methods can be applied for a practical approximation of metal availability in location-specific assessment of soil quality.
3. Exposure concentrations in laboratory toxicity tests and bioassays should be characterized consistently. This can be done by application of extraction methods or by using total concentrations including the measurement of soil characteristics modulating availability. To relate external concentrations to toxicity, insight into uptake kinetics and internal distribution processes in the organisms' tissues is required.
4. Since the frequency distribution of species sensitivities is a crucial aspect in the derivation of Hazardous Concentrations, and due to the numerical importance of factors such as bioavailability in modulating the outcome of toxicity tests, it is important to consistently document all (potentially) relevant test conditions. This documentation can be used to implement the newest scientific insights on species sensitivity in regular updating of Hazardous Concentrations. It is also useful for the development of bioassay systems, and for the interpretation of their outcomes.

5. The potential risks of mixtures should be evaluated on the basis of concentration additivity, unless strong evidence exists for synergistic or antagonistic effects of the chemicals under consideration.
6. Modification of the soil in bioassays, in order to reduce adverse effects of soil characteristics on the test species, should be avoided. Hence, bioassay systems for versatile use should comprise a 0range of species with different ecological preferences, or species with broad ecological amplitudes.
7. Methods should be further finalized and implemented to assess the long-term impact of contaminants on population performance. Efforts should be put into the implementation of test parameters and population dynamic models that allow for a estimation of long-term effects of contaminants on population sustainability.
8. Validation of generic risk limits should preferably be based on (community) endpoints related to fast responding, ubiquitous and intrinsically diverse groups of organisms that are likely to be highly exposed.
9. In addition to the first-tier risk assessment system, using Hazardous Concentrations derived according to the current Dutch methodology, the development of a second-tier system should be considered for location-specific risk assessment. In second tier assessments, a sequential approach with step-wise upgrading of sensitivity may be considered.

11. REPORT ON THE DISCUSSIONS AT THE WORKSHOP FEBRUARY 5, 1998, AT RIVM, BILTHOVEN

11.1 Organization

A workshop was held at RIVM, Bilthoven, on February 5, 1998. The purpose of this workshop was a critical discussion and evaluation of the results and conclusions of the Validation project in the light of the policy basis for environmental quality objectives for soils. In the morning session, members of the project team presented an overview of the project in relation to the questions raised at the start of the project (see Section 11.5). The afternoon session comprised a critical evaluation of the project by external referees, who were asked to formulate critical opinions on the project on the basis of a draft version of the final report (Chapter 1 - 9). This was followed by a discussion chaired by Prof. Dr. N.M. Van Straalen using the points raised by the referees and other participants during the morning session.
This workshop report summarises the major remarks made by the referees (par. 1.2) and the audience (par. 1.3), the (possible) answers raised, and the agreement reached on various aspects. Since the introductory lectures were inevitably less detailed than the final report, the project team felt free to annotate the workshop report with some clarifying remarks, indicated by footnotes. Where applicable, reference is made to particular issues addressed in the report. This report was made on the basis of notes made by Hans Vonk, Kees van Gestel, Els Smit and Leo Posthuma during the discussions, with a tape record as back up.

11.2 Introductions by the referees

Dr. Th.C.M. Brock, The Winand Staring Centre for Integrated Land, Soil and Water Research (SC-DLO), Wageningen.

The combination of laboratory toxicity experiments, semi-field experiments and field monitoring was considered by Brock as an ideal combination to underpin soil protection policy. He recognised that the Validation project followed a quality criteria-directed approach, aimed at predicting field effects on the basis of laboratory toxicity data (bottom-up). He would have welcomed, however, more emphasis on a system-directed, ecological approach (top-down), in which it is attempted to understand effects observed in the field on the basis of controlled toxicity experiments. In this respect, some critical questions were raised by Brock. He questioned the similarity of the soil used in the experimental field plots with the Budel gradient soils and the issue of using laboratory test animals in the Budel soil[1]. Another point brought forward was the use of zinc chloride in the laboratory and experimental field plot experiments. In the Budel situation, emissions of zinc have been in the oxide form, and although presently the

[1] As noted in Chapter 2, the experimental field plot soil was chosen to mimic the Budel gradient soil with respect to most soil characteristics. Major differences were, fresh versus aged contamination, zinc versus a mixture of metals, chloride versus oxide 'immission' and pH. The experimental field plot soil had a neutral indigenous pH, whereas the Budel gradient soils were mostly acidic. The low acidity of the Budel gradient soils introduced problems with rearing the chosen test species, since these all have an optimum near neutral pH.

speciation of zinc has considerably changed in the soil, the presence of chloride might have affected the results, especially at the higher concentrations[2].

A more ecologically oriented approach in future research would require the use of species occurring at the relevant site (e.g., Budel). Therefore, more toxicity data should be collected on wild species, because the laboratory species cannot be considered as representative for the field[3].

For future work on validation of toxicity data for soil, Brock advised to develop laboratory tests with populations of nematodes, because of their field relevance (omnipresence, high intrinsic variability within the species group). Also, he wondered whether the promising microbial PICT approach could be applied to organisms with a more complex life cycle, like nematodes and oligochaetes, or even other organisms[4].

For the interpretation of laboratory toxicity data, it is obvious that for terrestrial situations semi-field (mesocosm) experiments are a good intermediate between laboratory and real field, similar to the situation in aquatic ecotoxicology. For the interpretation of results of semi-field tests, Brock favoured the use of multivariate techniques like Principal Response Curves, supplementary to log-logistic dose-effect relationships.

With respect to current risk assessment, Brock said that uncertainty about bioavailability in the field requires that the derivation of quality objectives should be stringent. If potential risks are indicated, a location-specific ecological risk assessment should follow. For such an adequate risk assessment, it is important to consider the exposure route and the true bioavailable concentration, and to use ecologically sensitive endpoints. In this respect both structure *and* function of the ecosystem should be taken into account. Also, long-term direct and indirect effects on key species and functions should be considered.

Some general remarks on validation studies made by Brock were further:

- it is important to maintain sufficient expertise in the field of generic and location-specific risk assessment; it should be possible to make use of a combination of adequate instruments like: the availability of various test systems, microcosm facilities, PICT, statistical techniques, and simulation models
- interdisciplinary approaches are necessary, and in this respect
- more co-operation between aquatic and terrestrial experts would be useful.

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[2] The possible adverse effects of excess chloride were confirmed by studies on *F. candida*, in which it was shown that percolation of spiked soil before testing reduced the toxicity. Chloride is supposed not to have influenced the results of the experimental field plot experiments, since excess chloride will have been leached with the first rainfall after the construction of the test field plot.

[3] The latter aspect was not tackled by the project, because it was beyond its aims. Literature data do not suggest consistent differences in sensitivity between laboratory and field species (see Chapter 9). This suggests that this aspect might be of minor importance for the derivation of generic quality objectives.

[4] An example of the use of PICT in field studies has shown that the method can also be applied with multi-cellular organisms (nematodes), by means of studying survival time at a single artificial exposure concentration (see Chapter 9).

Drs. C. Van De Guchte (Institute for Inland Water Management and Waste Water Treatment (RIZA), Lelystad).

Van de Guchte highlighted few aspects that require further attention when using the data collected in the Validation project. First, as far as ageing of the contaminant is concerned, a distinction should be made between diffuse historically deposited metal pollutions for which remediation is considered, and recent and ongoing pollution from emitting sources, for which an emission reducing policy is necessary (e.g. leaching from contaminant-containing structures). For the latter form of pollution, ageing is not so relevant, and should not be taken into account for the derivation of generic risk limits. From this it follows that biological availability of pollutants should be taken into account as an assessment aspect as far as remediation is concerned. For the setting of priorities in the prevention of emissions, bioavailability plays a minor role in the derivation of environmental quality criteria. In view of this, Van De Guchte emphasized the distinction between generic soil standards which can be used to set priorities for different substances and sources, as is done in the present soil protection policy, and risk limits which are used as a starting point for *in situ*, site-specific risk assessments. For the latter purpose (and thus for validation), indicators of effects in the field should be sensitive, have sufficient contact with the pollutant, be "appealing" and should differentiate between relevant and irrelevant parameters[5].

Secondly, Van De Guchte noted that the exposure situations chosen in the project did not sufficiently take into account the vertical distribution and concentration gradients of the pollution in the Budel field situation. In the field the highest concentration will be present in the litter layer, with its particular abiotic and biotic conditions. More attention therefore should have been focused on the specific exposure of litter-dwelling organisms[6].

Thirdly, Van De Guchte would have welcomed some reference to background concentrations in relation to risk values, in particular in view of the choice of zinc as model contaminant for the project.

Van De Guchte ended with the well-known casus of high zinc concentrations in sediment deposits in the alluvial plains of the Rhine river delta as an illustration of the importance of having accurate soil quality criteria and methods for site-specific risk assessment. In the example, approx. 100×10^6 m^3 soil is involved. The zinc concentrations in the alluvial plains exceed the local target value, which impedes execution of planned nature development projects. The problem would be solved if environmental quality criteria for zinc were a factor 3 higher.

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[5] In relation to this first point raised by Van De Guchte, it should be noted that, in the course of the project, developments in the regulatory field have been fast, i.e., in contrast to the situation in 1992 emphasis is now on the evaluation of location-specific risks. This development in the project setting has been recognized by the project team (e.g., in the Introductory lectures by Denneman and Posthuma, and in Chapter 9), by expressing opinions on the usefulness of the project results for the latter evaluations. Thereby, the results of standardized laboratory toxicity tests already obtained remained the basic motivation for the execution of the project.

[6] This point was raised again later in the open discussion. In view of this it should be mentioned that in the field study on enchytraeids in Budel the vertical distribution of animals over different soil layers was taken into account.

11.3 Discussion

11.3.1 Way of addition of the test substance (fresh addition versus ageing)

The way of addition was not considered to be very important, *but only* as long as the speciation or the actual exposure is properly measured. This implies that a good insight into the environmental chemistry of the toxicant should be available, which is, however, often missing for existing toxicity data. In addition, it was also commented that bioavailability might differ between different organisms, so that a chemical characterisation of bioavailability alone would not be sufficient.

Eijsackers stressed the importance of long-term effects of low concentrations near the background (e.g., 14 km versus 20 km from the source in Budel) for which it was shown that microbial PICT differed between these sites in (aged) Budel soil. This means that biological effects can occur even when chemical analysis does not indicate serious pollution. In general, an observation like this focuses on the question of the (im)possibility to predict long-term effects in aged soils on the basis of laboratory toxicity experiments with freshly contaminated soil, and reinforces the remark made on this by Brock.

11.3.2 Representativity of test species

Are the species used in the Validation project sufficiently representative and sensitive to extrapolate results to the field? This question can only be answered if one knows *for what* they should be representative. Van Gestel pointed out that the species used in the Validation project were chosen simply because they are widely used in internationally standardised tests. Due to this, and merely for practical reasons, these species are widely considered as 'representative' for the derivation of quality objectives. In cases where these species can not be considered representative, e.g. in site-specific evaluations, the relevance of test results obtained with the species have to may be questioned, and might ask for observations on locally occurring species. It was remarked, however, that the number of test species should be of restricted size for practical reasons. Eijsackers supported the opinion that in this respect, again, a distinction should be made between generic quality criteria and location specific assessments. For the derivation of generic criteria the presently chosen species fulfil their purpose, for site-specific assessment other species can be used.

Some discussion was focused on the suitability of plants to be used in soil protection policy. It was felt that plants were principally as important as model organisms as the other test organisms. The experiences with application of field bioassays with plants in the experimental field plot showed that a careful design is important to avoid unwanted climatic influences..

The aim of the project was to compare effects on test organisms of *standard tests* in the laboratory with effects on the same organisms in the field. How related field organisms (e.g. other plants, other worms) react is another step, which can only in part be solved by literature

study. According to some participants it may be predicted that the variation in sensitivities will be rather large, and it would require a major effort to establish useful sensitivity patterns[7].
It was agreed that more attention and research is needed to find out to what extent the presently used standard species are representative (e.g. sensitivity) for local species. With respect to the predictive value of standardised toxicity tests in general, the participants felt that these tests can predict *that* some effect could occur. standard tests however cannot predict the kind and size of effect.

11.3.3 Representativity of project results for other contaminants than zinc

Apart from the fact that zinc is an important contaminant, there was a general feeling that the conclusions drawn for zinc were also valid for other metals. There was some discussion about the role of the essentiality of zinc for its representativity. A remark was made that the Aldenberg-Slob extrapolation method for HC_5 derivation was not valid for essential elements. It was however also felt that deficiency did not play a role in the situations studied in the Validation project. Moreover, data from the literature on *in situ*-effects of copper and lead also show that effects at the community level in the field are found between HC_5 and HC_{50}, which confirms the zinc story[8].

11.3.4 Prediction of effects in situ

It was questioned whether the underpinning of generic criteria by the Validation project was still valuable in view of the recent attention for a local evaluation of soil pollution (so called BEVER-approach, a Dutch acronym for "BEleids VERnieuwing" (new policy perspectives) in relation to soil protection and remediation). It was felt that the results were still valuable, because bioavailability also plays an important role in the local situation, especially for the assessment of uncertainties in interlocalities comparisons. In relation to this, again, the importance of the environmental chemistry of pollution was stressed.
For the implications in the field of nature development the attention has also to be directed on specific receptors, i.e. the organisms which should play a role in that nature development project. In this respect attention should be paid to the lower end of the dose-effect relationship or the contamination gradient, to find out what is minimal possible and maximal acceptable for nature development. Also, attention should be given to prediction of long-term changes in the abiotic factors affecting bioavailability (pH, redox potential), e.g. the release of metals from sludge by decomposition of the organic matter.

[7] This has, for example, been shown with the studies on enchytraeids, in which all three experimental levels were performed, i.e.: standardized laboratory assays were compared with enchytraeid performance in the experimental field plot, and in the Budel gradient soils (Chapter 6). That comparison demonstrated that confounding factors may impede a full cause-effect interpretation of observational data along a field gradient, even in a case where toxicity was expected on the basis of laboratory data.

[8] Most literature data concern the validity of single-species laboratory toxicity data, and only few studies are available to judge the validity of risk limits. For the former comparisons, data are available on various classes of contaminants (see Section 9.1).

11.3.5 Vertical gradients and micro-exposure

Some criticism was encountered that the heterogeneity of the Budel gradient soils was not taken into account in all investigations. In Budel, a vertical gradient of pollution is present, as appears from difference in tolerance development by plants between deep rooting and surface rooting species. Notenboom pointed out that sampling of enchytraeids was carried out in several layers, thus taking into account the possibility of vertical gradients in contamination and response (see Chapter 6).

It is important to know the sensitivity of organisms present in the layer where the toxicant actually is encountered, i.e., zinc in relation to the litter and humus-dwelling organisms. Therefore, the representativity of the test organisms for those species actually present has to be established to improve site-specific assessment. It should be realised that this may bring along many technical difficulties in transferring these organisms to the laboratory, culturing them and carrying out tests. Perhaps focus should be on unravelling the field situation and/or testing fractions (e.g., water soluble extracts) of the contaminated field soils.

11.3.6 General discussion points

Soil extraction and analysis

It was not recommended to focus on one type of bioavailability-imitating soil extraction method "imitating" bioavailability, because none of the (partial) extraction methods (water, $CaCl_2$) was (or can be) valid for all biological species that come into contact with a contaminant. Extraction methods for determining total metal concentrations are now well standardised. Denneman is of the opinion that, therefore, in the first generic step of risk assessment total concentrations are satisfactory. For further steps other methods can be applied for a differentiation[9].

For some participants it was clear that a bioavailability-imitating extraction and investigation of speciation was essential, (e.g. in the case of lead shot pollution total concentrations would yield gross over-estimation of risk). It was noted that schemes for such investigations are available and further into development in Germany (DECHEMA).

Available bioassays for soils

Work is in progress to develop soil bioassays for the evaluation of situations with a potential contaminant risk. The Validation project certainly has a spin-off in that respect, and considerable experience has been gained, especially for executing bioassays focusing on sublethal parameters. One of the problems encountered in bioassays is how to find the correct reference situation, and to distinguish between common soil factors with an adverse effect on the test species (e.g., pH) and contaminant effects. PICT might be a promising tool for this type of approach.

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[9] It should be noted that, although methods for the determination of total metal concentrations are standardised, the large number of different methods (HNO_3; HNO_3/HCl in different proportions; with or without HF, etc.) still can lead to different results. Compared to this, 0.01 M $CaCl_2$ might be more unequivocal.

The relation with field-monitoring networks and scenario calculations

Present field-monitoring networks (e.g., the Dutch 'Landelijk Meetnet Bodem') have yielded some data on the relationship between soil factors and the occurrence of nematodes[10]. The monitoring network can possibly be extended to include other organisms. This would allow for investigations on the abundance of the studied species in relation to toxicants and abiotic soil factors, in order to predict the abundance on specified sites at which contaminant risks might occur (scenario calculations). This requires a large database of ecologically base line information in addition to the database already collected in the networks on abiotic factors in background reference situations.

Toxicity of mixtures

The Budel gradient soils contain a mixture of metals. The Validation project gives only restricted insight in, and solutions for, problems with the toxicity of mixtures. Although this is an important and complex problem, soil protection policy needs rules of thumb to work with.
A safety factor of 100 between the Maximum Permissible Concentration and the Negligible Risk Concentration (see Chapter 1) takes into account, among others, the uncertainties with regard to effects of mixtures. At present, the issue of mixtures is under further consideration as part of a thorough evaluation of the applicability of Intervention Values in daily practice.

11.4 Overall conclusions

Scientific and regulatory merits

The Validation project showed a lot of progress in the validation of toxicity data and risk limits in soil in general, which is helpful for both the scientific support of a quality criteria-oriented (first-tier) approach in soil protection, as well as for the development of location-specific evaluation methods. It added also to the general knowledge on the toxicity of zinc for soil organisms.
Some aspects requiring further attention were: the importance of metal speciation, the use of multivariate statistics and modelling, and the effects on populations and communities of groups other than nematodes and micro-organisms.

General validity

Although it was questioned whether the conclusions drawn on the basis of zinc can be extrapolated to other metals and pollutants, many participants felt that some conclusions (e.g. effects of ageing) were generally valid. However, one should be careful when a different combination of soils, organisms and contaminants is evaluated. One may be guided by the project results obtained here for zinc to focus primarily on the factors that modulate toxicity most, for example bioavailability.

[10] The nematode study in Chapter 7.

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REFERENCES

Adema, D.M.M. and Henzen, L. (1986) De invloed van tien milieugevaarlijke stoffen op de groei van drie soorten hogere planten. Report no. R 86/181a, TNO, Delft.

Aldenberg, T., and Slob, W. (1993) Confidence limits for Hazardous Concentrations based on logistically distributed NOEC toxicity data. *Ecotoxicol. Environ. Saf.* 25: 48-63.

Alexander, M. (1995) How toxic are toxic chemicals in soil? *Environ. Sci. Technol.* **29**, 2713-2717.

Alkemade, J.R.M. and M.L.P. van Esbroek (1994) Naar een effecten-voorspellingsmodel voor de bodemfauna: BOEF. MOVE-BOdEmFauna versie 1. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 712901001

Alkemade, J.R.M., Schouten, A.J., Kersten, P. and Van Esbroek, M.L.P. (1996) Vergelijking van effectniveaus voor bodemorganismen met het maximaal toelaatbaar risico. Een veldstudie naar de invloed van zware metalen in een gradiënt te Budel. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 607505001

Allen, H.E (1997) Standards for metals should not be based on total concentrations. *SETAC-Europe News* **8**, 7-9

Alloway, B.J. (1990) Soil processes and the behaviour of metals. In *Heavy metals in soils* (B.J. Alloway, Ed.), Blackie, Glasgow/London, pp. 7-28.

Bååth, E. (1992) Measurement of heavy metal tolerance of soil bacteria using thymidine incorporation into bacteria after homogenization centrifugation. *Soil Biol. Biochem.*, **24**, 1167-72.

Bååth, E., Frostegård, Å. Díaz-Raviña, M. and Tunlid, A. (1998) Microbial community-based measurements to estimate heavy metal effects in soil: the use of Phospholipid Fatty Acid Patterns and Bacterial Community Tolerance. *Ambio* 27: 58-61.

Baes, C.F. and Sharp, R.D. (1983) A proposal for estimation of soil leaching and leaching constants for use in assessment models. *J. Environ. Qual.*, **12**, 17-28.

Bagy, M.M.K., El-Sharouny, H.M.M. and El-Shanawany, A.A. (1991) Effect of pH and organic matter on the toxicity of heavy metals to growth of some fungi. *Folia Microbiol.*, **36**, 367-374.

Bakker, J., Posthuma, L. and Baerselman, R. (1997) Toxische effecten van zink op de worm *Eisenia andrei*: resultaten van proefveldexperimenten Amsterdam 1995 en 1996. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 60750001.

Bakker, M.I. and Notenboom, J. (1994) Literatuurstudie naar de invloed en structuur van organisch materiaal op sorptie van hydrofobe organische verbindingen. National Institute of Public Health and the Environment, Bilthoven, The Netherlands. Report nr. 672760001.

Balsberg Påhlsson, A-M. (1989) Toxicity of heavy metals (Zn, Cu, Cd, Pb) to vascular plants. *Water Air Soil Pollut.*, **47**,287-319.

Barrow, N.J. (1986a) Testing a mechanistic model. IV. Describing the effects of pH on zinc retention by soils. *J. Soil Sci.*, **37**, 295-302.

Barrow, N.J. (1986b) Testing a mechanistic model. II. The effects of time and temperature on the reaction of zinc with a soil. *J. Soil Sci.*, **37**, 277-286.

BBA (1994) Richtlinien für die Zulassung von Pflanzenschutzmitteln im Zulassungsverfahren; Teil VI, 2-2; Auswirkungen von Pflanzenschutzmitteln auf die Reproduktion und das Wachstum von *Eisenia fetida/Eisenia andrei*. Ribbesbüttel, Germany, Saphir-Verlag.

Belfroid, A.C., Seinen, W., Van Gestel, C.A.M., Hermens, J.L.M. and Van Leeuwen, K.J. (1995) Modelling the accumulation of hydrophobic organic chemicals in earthworms. *Environ. Sci. Pollut. Res.*, **2**, 5-15.

Belfroid, A.C., Sijm, D.T.H.M. and Van Gestel, C.A.M. (1996) Bioavailability and toxicokinetics of hydrophobic aromatic compounds in benthic and terrestrial invertebrates. *Environ. Rev.*, **4**, 276-299.

Bembridge, J.D., Kedwards, T.J., Edwards, P.J. (in press) Variation in earthworm populations and methods for assessing responses to perturbations. In: Sheppard S, Bembridge JD, Holmstrup M, Posthuma L, (Eds.) *Progress in earthworm ecotoxicology*. SETAC Publishers,

Bengtsson, G. and Rundgren, S. (1982). Population density and species number of enchytraeids in coniferous forest soils polluted by a bras mill. *Pedobiologia,* **24**, 211-218.

Bengtsson, G. and Rundgren, S. (1992) Seasonal variation of lead uptake in the earthworm *Lumbricus terrestris* and the influence of soil liming and acidification. *Arch. Environ. Contam. Toxicol.*, **23**, 198-205.

Bengtsson, G., Ohlsson, L. and Rundgren, S. (1985) Influence of fungi on growth and survival of *Onychiurus armatus* (Collembola) in a metal polluted soil. *Oecologia,* **68**, 63-68.

Bettger, W.J. and O'Dell, B.L. (1981) A critical physiological role of zinc in the structure and function of biomembranes. *Life Sci.,* **28**, 1425-1438.

Blanck, H., Wängberg, S-Å., Molander, S. (1988). Pollution-induced community tolerance - A new ecotoxicological tool. In: Cairns J.Jr, Pratt JR, Eds. *Functional testing of aquatic biota for estimating hazards of chemicals.* ASTM STP 988. American Society for Testing and Materials; 1988; Philadelphia. p 219-30.

Bogacz, W. (1993) Influence of soil properties on the distribution of zinc in polish soils. *Geomicrobiology J.* **11**, 299-308.

Bogomolov, D.M., Chen, S-K., Parmelee, R.W., Subler, S. and Edwards, C.A. (1996) An ecosystem approach to soil toxicity testing: a study of copper contamination in laboratory soil microcosms. *Appl. Soil Ecology,* **4**, 95-105.

Bongers, T., (1990) The Maturity Index: an ecological measure of environmental disturbance based on nematode species composition. *Oecologia,* **83**, 14-19

Borovik, A.S. (1990) Characterization of metals ions in biological systems. In *Heavy metal tolerance in plants: evolutionary aspects,* edited by A. J. Shaw. Boca Raton, Florida: CRC Press.

Breure, A.M. (ed.) (1996) Wat en hoe met biodiversiteit. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. No report number.

Bro-Rasmussen, F. and Løkke, H. (1984) Eco-epidemiology. A casuistic discipline describing ecological disturbances and damages in relation to their specific causes; examplified by chlorinated phenols and chlorophenoxy acids. *Reg. Toxicol. Pharmacol.* **4,** 391-399

Brümmer, G., Tiller, K.G., Herms, U. and Clayton, P.M. (1983) Adsorption-desorption and/or precipitation-dissolution processes of zinc in soils. *Geoderma,* **31**, 337-354.

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.

Buchter, B., Davidoff, B., Amacher, M.C., Hinz, C., Iskandar, I.K. and Selim, H.M. (1989) Correlation of Freundlich Kd and n retention parameters with soils and elements. *Soil Sci.,* **148**, 370-379.

Cairns, J.J., Heath, A.G. and Parker, B.C. (1975) The effects of temperature upon the toxicity of chemicals to aquatic organisms. *Hydrobiologia,* **47**, 135-171.

Calamari, D. and Alabaster, J.S. (1980) An approach to theoretical models in evaluating the effects of mixtures of toxicants in the aquatic environment. *Chemosphere,* **9**, 533-538.

Calamari, D. and Vighi, M. (1992) A proposal to define quality objectives for aquatic life for mixtures of chemical substances. *Chemosphere,* **25**, 531-542.

Calow, P. , Sibly, R.M, and Forbes, V. (1997) Risk assessment on the basis of simplified life-history scenarios. *Environ. Toxicol. Chem.* **16**,1983-1989.

Chandini, T. (1988) Changes in food [*Chlorella*] levels and the acute toxicity of cadmium to *Daphnia carinata* (Daphnidae) and *Echinisca triserialis* (Macrothricidae) [Crustacea: Cladocera]. *Bull. Environ. Contam. Toxicol.,* **41**, 398-403.

Chandini, T. (1989) Survival, growth and reproduction of *Daphnia carinata* (Crustacea: Cladocera) exposed to chronic cadmium stress at different food (Chlorella) levels. *Environ. Pollut.,* **60**, 29-45.

Chaney, R.L., Bruins, R.J.F., Baker, D.E., Korcak, R.F., Smith, J.E. and Cole, D. (1987) Transfer of sludge-applied trace elements to the food chain. In *Land application of sludge - Food chain implication,* edited by A. L. Page, T. J. Logan and J. A. Ryan. Chelsea: Lewis Publishers Inc.

Chapman, P.M., Fairbrother, A.F., Brown, D. (1998) A critical evaluation of safety (uncertainty) factors for ecological risk assessment. *Environ. Toxicol. Chem.* **17**,:99-108

Chapman, P.M. (1986) Sediment quality criteria from the sediment quality triad data: an example. *Environ. Toxicol. Chem.* 3: 957-964.

Christensen, T.H. (1987) Cadmium soil sorption at low concentrations: VI. A model for zinc competition. *Water Air Soil Pollut.,* **34**, 305-314.

Clarkson, D.T. and Hanson, J.B. (1980) The mineral nutrition of higher plants. *Ann. Rev. Plant Physiol.,* **31**, 547-552.

Clarkson, D.T. and Lüttge, U. (1989) III Mineral nutrition: divalent cations, transport and compartmentation. *Prog. Bot.,* **51**, 93-112.

Cleven, R.F.M.J., Janus, J.A., Annema, J.A., and Slooff, W. (1993) Integrated Criteria Document Zinc. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 710401028.

Corp, N. and Morgan, A.J. (1991) Accumulation of heavy metals from polluted soils by the earthworm, *Lumbricus rubellus*: Can laboratory exposure of control worms reduce biomonitoring problems? *Environ. Pollut.*, **74**, 39-52 .

Cowgill, U.M. (1987) Critical analysis of factors affecting the sensitivity of zooplankton and the reproducibility of toxicity test results. *Wat. Res.*, **21**, 1453-1462.

Crommentuijn, G.H., Van De Plassche E.J., and J.H. Canton (1994) Guidance document on the derivation of ecotoxicological criteria for serious soil contamination in view of the Intervention Value for soil clean-up. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 950011003.

Crommentuijn, T., Doodeman, C.J.A.M., Doornekamp, A., and Van Gestel, C.A.M. (1997) Life-table study with the springtail *Folsomia candida* (Willem) exposed to cadmium, chlorpyrifos and triphenyltin hydroxide. In N.M. van Straalen and H. Løkke (Eds.). *Ecological risk assessment of contaminants in soil,* London: Chapman & Hall, pp 275-291.

Crommentuijn, T., Doodeman, C.J.A.M., Doornekamp, A., Van der Pol, J.J.C., Bedaux, J.J.M. and Van Gestel, C.A.M. (1994) Lethal body concentrations and accumulation patterns determine time-dependent toxicity of cadmium in soil arthropods. *Environ. Toxicol. Chem.*, **13**, 1781-1789.

Crommentuijn, T., Doodeman, C.J.A.M., Van der Pol, J.J.C., Doornekamp, A., Rademaker, M.C.J. and Van Gestel, C.A.M. (1995b) Sublethal sensitivity index as an ecotoxicity parameter measuring energy allocation under toxicant stress: application to cadmium in soil arthropods. *Ecotoxicol. Environ. Saf.*, **31**, 192-200.

Crommentuijn, T., Doornekamp, A. and Van Gestel, C.A.M. (1997) Bioavailability and ecological effects of cadmium on *Folsomia candida* (Willem) in an artificial soil substrate as influenced by pH and organic matter. *Appl. Soil Ecol.* **5**, 261-271.

Crommentuijn, T., Polder, M.D. and Van de Plassche, E.J. (1997) Maximum Permissible Concentrations for metals, taking background concentrations into account. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 601501001.

Crommentuijn, T., Kalf, D.F., Polder, M.D., Posthumus, R., and Van de Plassche, E.J. (1997a) Maximum permissible concentrations and negligible concentrations for pesticides. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 601501002.

Crommentuijn, T., Stäb, J.A., Doornekamp, A., Estoppey, O. and Van Gestel, C.A.M. (1995a) Comparative ecotoxicity of cadmium, chlopyrifos and triphenyltin hydroxide for four clones of the parthenogenetic collembolan *Folsomia candida* in an artificial soil. *Funct. Ecol.*, **9**, 734-742.

Crossland, N.O. (1994) Extrapolating from mesocosms to the real world. *Toxicol. Ecotoxicol. News,* **1**, 15-22.

De Bruijn, J.H.M. and Denneman, C.A.J. (1992) Achtergrondgehalten van negen sporen-metalen in oppervlaktewater, grondwater en grond van Nederland. VROM-publicatiereeks Bodembescherming. nr. 1992/1 (In Dutch).

De March, B.G.E. (1987) Simple similar action and independent action - two similar models for the joint effects of toxicants applied as mixtures. *Aquatic Toxicol.*, **1987**, 291-304.

De Rooij, N.M. and Smits, J.G.C. (1997) Methodology for determination of heavy metal standards for soil. Phase I: Definition study. Report nr. T2004T01.01, Delft Hydraulics, Delft.

De Zwart, D. (1997) Ordination of the integrated monitoring data gathered under auspices of ICP-IM (UN-ECE Convention on Long-Range Transboundary Air Pollution): 1989-1994. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 259101006.

Demon, A. and Eijsackers, H. (1985) The effects of lindane and azinphosmethyl on survival time of soil animals, under extreme or fluctuating temperature and moisture conditions. *Zeitschr. ang. Entomol.*, **100**, 540-510.

Denneman, C.A.J. (1998) Aanpassing van bodemnormen? Biobeschikbaarheid als onderdeel van een norm of als flexibel element in een risicobeoordeling? *Bodem* Feb. 98, pp. 23 -25.

Denneman, C.A.J. and Van Gestel, C.A.M. (1990). Bodemverontreiniging en bodemecosystemen: voorstel voor C-(toetsings)waarden op basis van ecotoxicologische risico's. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 725201001.

Díaz-Raviña, M., Bååth, E. and Frostegård, Å. (1994) Multiple heavy metal tolerance of soil bacterial communities and its measurement by a thymidine incorporation technique. *Appl. Environ. Microbiol.*, **60**, 2238-2247.

Didden, W. A. M. (1993) Ecology of Terrestrial Enchytraeidae - *Review. Pedobiologia,* **37**, 2-29.

Dijkshoorn, W., Van Broekhoven, L.W., and Lampe, J.E.M. (1979) Phytotoxicity of zinc, nickel, cadmium, lead, copper and chromium in three pasture plant species supplied with graduated amounts from the soil. *Nether. J. Agri. Sci.*, **27**, 241-253.

Dirven-van Breemen, L., Baerselman, R. and Notenboom, J. (1994) Onderzoek naar de geschiktheid van de potwormsoorten *Enchytraeus albidus* en *E. crypticus* (Oligochaeta, Annelida) in bodemecotoxicologisch onderzoek. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 719110025.

Doelman, P. and Haanstra, L. (1979) Effects of lead on the soil microflora. *Soil Biol. Biochem.*, **11**, 487-491.

Doelman, P., Jansen, E., Michels, M. and Van Til, M. (1994) Effects of heavy metals on microbial diversity and activity as shown by the sensitivity-resistance index, an ecologically relevant parameter. *Biol. Fertil. Soil*, **17**, 177-184.

Donker, M.H. (1992) Energy reserves and distribution of metals in populations of the isopod *Porcellio scaber* from metal-contaminated sites. *Funct. Ecology*, **6**, 445-454.

Donker, M.H., Van Capelleveen, H.E. and Van Straalen, N.H. (1992) Metal contamination affects size-structure and life history dynamics in isopod field populations. In *Ecotoxicology of metals in invertebrates* (R. Dallinger and P.S. Rainbow, eds.), Lewis Publishers, Boca Raton, pp. 384-399.

Driscoll, M., Dean, E., Reilly, E., Bergholz, E. and Chalfie, M. (1989) Genetic and molecular analysis of a *Caenorhabditis elegans* beta-tubulin that conveys benzimidazole sensitivity. *J. Cell Biol.*, **109**, 1993-3004.

ECETOC (1997) The value of aquatic model ecosystem studies in ecotoxicology. Technical report No. 73. European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), Brussels, Belgium.

Edelman, T. (1984) Achtergrondgehalten van een aantal anorganische en organische stoffen in de bodem van Nederland. Staatsuitgeverij. Bodembescherming 34.

Edelman, Th., Ma, W.C and Kuypers, J.J.M. (1985) Verontreiniging van de bodem met de zware metalen cadmium, lood en zink in de omgeving van een zinksmelterij in de Brabantse Kempen. *Land en Water Nu, bijlage Milieutechnik*, **25**, 12-14

EEC (1985) Annex V. Part C. Methods for the determination of ecotoxicity. Level I. C (L1)4: Toxicity for earthworms. Artificial soil test. DG XI/128/82. Final.

Elliott, H.A., Liberati, M.R. and Huang, C.P. (1986) Competitive adsorption of heavy metals by soils. *J. Environ. Qual.*, **15**, 214-219.

Elrashidi, M. and O'Connor, G.A. (1982) Influence of solution composition on sorption of zinc by soils. *Soil Sci. Soc. Am. J.*, **46**, 1153-1158.

Enserink, E.L., Kerkhofs, M.J.J., Baltus, C.A.M. and Koeman, J.H. (1995) Influence of food quantity and lead exposure on maturation in *Daphnia magna*; evidence for a trade-off mechanism. *Funct. Ecol.*, **9**, 175-185.

Faber, J.H. and Verhoef, H.A. (1991) Functional differences between closely related soil arthropods with respect to decomposition processes in the presence or absence of pine-tree roots. *Soil Biol. Biochem.*, **23**, 15-23.

Folkerts, A.-J. and Zweers, G.P.C.(1996). Het effect van zink op de reproduktie van *Enchytraeus crypticus* in semi veld- en laboratorium-experimenten. ECO-notitie 96-07 (RIVM - Laboratorium voor Ecotoxicologie).

Forge, T.A., Berrow, M.L., Darbyshire, J.F. and Warren, A. (1993) Protozoan bioassays of soil amended with sewage sludge and heavy metals, using the common soil ciliate *Colpoda steinii*. *Biol. Fertil. Soils*, **16**, 282-286.

Fox, G.A. (1991) Practical causal inference for ecoepidemiologists. *J. Toxicol. Environ. Health* **33**, 359-373

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

Hågvar, S. and Abrahamsen, G. (1990) Microarthropoda and Enchytraeidae (Oligochaeta) in naturally Lead-contaminated soil: A gradient study. *Environ. Entomol.* **19**, 1263-1277

Haight, M., Mudry, T. and. Pasternak, J (1982) Toxicity of seven metals on *Pnagrellus silusiae*: the efficacy of the free-living nematode as an *in vivo* toxicological bioassay. *Nematologica*, **28**, 1-11

Hamers, T., Notenboom, J. and Eijsackers, H.J.P. (1996). Validation of laboratory toxicity data on pesticides for the field situation. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 719102046.

Hames, C.A.C. and Hopkin, S.P. (1991) Assimilation and loss of ^{109}Cd and ^{65}Zn by the terrestrial isopods *Oniscus asellus* and *Porcellio scaber*. *Bull. Environ. Contam. Toxicol.*, **47**, 440-447.

Harmens, H. , Gusmão, N.G.C.P.B., Den Hartog, P.R., Verkleij, J.A.C. and W.H.O. Ernst. (1993). Uptake and transport of zinc in zinc-sensitive and zinc-tolerant *Silene vulgaris*. *J. Plant Physiol.*, **141**, 309-315.

Hartenstein, R., Neuhauser, E.F., and Narahara, A. (1981) Effects of heavy metal and other elemental additives to activated sludge on growth of *Eisenia foetida*. *J. Environ. Qual.*, **10**, 372-276.

Hatch, D.L., Jones, L.H.P. and Burau, R.G. (1988) The effect of pH on the uptake of cadmium by four plant species grown in flowing solution culture. *Plant and Soil*, **105**, 121-126.

Hatzinger, P.B. and Alexander, M. (1995). Effect of aging of chemicals in soil on their biodegradability and extractability. *Environ. Sci. Technol.* **29**, 537-545.

Heimbach, F. (1992) Correlation between data from laboratory and field tests for investigating the toxicity of pesticides to earthworms. *Soil Biol. Biochem.*, **24**, 1749-1753.

Henry, C.L. and R.B.C Harrison. (1992). Fate of trace metals in sewage sludge compost. In *Biogeochemistry of Trace Metals*, edited by D. C. Adriano: Lewis Publishers.

Hensbergen, P.J. and Van Gestel, C.A.M. (1995) Combinatie-toxiciteit in het terrestrische milieu. Report nr. R04, Technische Commissie Bodembescherming, Den Haag, The Netherlands.

Hesterberg, D., Bril, J. and Del Castilho, P. (1993) Thermodynamic modeling of zinc, cadmium, and copper solubilities in a manured, acidic loamy-sand topsoil. *J. Environ. Qual.*, **22**, 681-688.

Hopkin, S.P. (1993) Ecological implications of '95% protection levels' for metals in soil. *Oikos* 66: 137-141

Houba, V.J.G., Lexmond, Th. M., Novozamsky, I. andVan der Lee, J.J. (1996). State of the art and future developments in soil analysis for bioavailibility assessment. *Sci. Tot. Environ.*, **178**, 21-28.

Howel, R. (1984) Acute toxicity of heavy metals to two species of marine nematodes. *Mar. Environ. Res.*, **11**, 13-161

ISO (1993) Soil quality. Effects of pollutants on earthworms (*Eisenia fetida*). Part 2. Determination of effects on reproduction.: ISO 11268-2.

ISO (1994) ISO/DIS 11267. Soil quality-Effects of soil pollutants on Collembola (*Folsomia candida*): method for the determination of effects on reproduction.

Jagers op Akkerhuis, G.A.J.M. and Hamers, T.H.M. (1992) Substrate-dependent bioavailability of deltamethrin for the epigeal spider *Oedothorax apicatus* (Blackwell) (Aranea, Erigonidae). *Pestic. Sci.*, **36**, 59-68.

Jansen, S.R.J, Bal, D, Beije, H.M., Hoogeveen, Y.R., Van Der Reest, P.J. (1995) Handboek natuurdoeltypen in Nederland. Wageningen, The Netherlands., IKC-N. Report IKC Natuurbeheer 11

Janssen, M.P.M. and Bergema, W.F. (1991) The effect of temperature on cadmium kinetics and oxygen consumption in soil arthropods. *Environ. Toxicol. Chem.*, **10**, 1493-1501.

Janssen, M.P.M., Bruins, A., De Vries, T.H. and 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., Peijnenburg, W.J.G.M., Posthuma, L., and Van Den Hoop, M.A.G.T. (1997a) Equilibrium partitioning of heavy metals in Dutch field soils. I. Relationships between metal partition coefficients and soil characteristics. *Environ. Toxicol. Chem.* **16** 2470 - 2478.

Janssen, R.P.T., Posthuma, L., Baerselman, R., Den Hollander, H.A., Van Veen, R.P.M. and Peijnenburg, W.J.G.M. (1997b) Equilibrium partitioning of heavy metals in Dutch field soils. II. Prediction of metal accumulation in earthworm. *Environ. Toxicol. Chem.* **16**, 2479-2488.

Janus, J.A. Integrated Criteria Document Zinc: Ecotoxicity. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 710401019.

Johnson, D.L. and Wellington, W.G. (1980) Post-embryonic growth of the collembolans *Folsomia candida* and *Xenylla grisea* at three temperatures. *Can. Ent.*, **112**, 687-695.

Jones, R. and. Burgess, M.S.E. (1984) Zinc and cadmium in lettuce and radish grown in soils collected near electrical transmission (hydro)powers. *Water Soil Pollut.*, **19**, 389-395.

Juste, C. and Mench, M.. (1992) Long-term application of sewage sludge and its effects on metal uptake by crops. In *Biogeochemistry of trace metals*, edited by D. C. Adriano. Boca Raton: Lewis Publishers.

Kammenga, J.E., Van Koert, P.H.G., Koeman, J.H,. Bakker, J. (1997) Fitness consequences of toxic stress evaluated within the context of phenotypic plasticity. *Ecol. Applic.* 7, 726-734

Kammenga, J. (1995). Phenotypic plasticity and fitness consequences in nematodes exposed to toxicants. PhD thesis, Agricultural University, Wageningen.

Kammenga, J.E,. Van Gestel, C.A.M and Bakker, J. (1994) Patterns of sensitivity to cadmium and pentachlorophenol among nematode species from different taxonomic and ecological groups. *Arch. Environ. Contam. Toxicol.*, **27**, 88-94

Kammenga, J.E. and Riksen, J.A.G. (1996) Comparing differences in species sensitivity to toxicants: phenotypic plasticity versus concentration-response relationships. *Environ. Toxicol. Chem.*, **15**, 1649-1653.

Kammenga, J.E., Van Koert, P.H.G., Riksen, J.A.G., Korthals, G.W. and Bakker, J. (1996) A toxicity test in artificial soil based on the life-history strategy of the nematode *Plectus acuminatus*. *Environ. Toxicol. Chem.*, **15**, 722-727

Kelsey, J.W. and Alexander, M. (1997) Declining bioavailability and inappropriate estimation of risk of persistent compounds. *Environ. Toxicol. Chem.* **16**, 582-585.

Kessels, B.G.F., Belde, P.J.M. and Borst-Pauwels, G.W.F.H. (1985) Protection of *Saccharomyces cerevisae* against Cd^{2+} toxicity by Ca^{2+}. *J. General Microbiol.* **131**, 2533

Khalil, M.A., Abdel-Lateif, H.M., Bayoumi, B.M., Van Straalen, N.M. and Van Gestel, C.A.M. (1996a) Effects of metals and metal mixtures on survival and cocoon production of the earthworm *Aporrectodea caliginosa. Pedobiologia,* **40,** 548-556.

Khalil, M.A.A.-L.H.M., Bayoumi, B.M. and Van Straalen, N.M (1996b) Analysis of separate and combined effects of heavy metals on the growth of *Aporrectodea caliginosa* (Oligochaeta: Annelida), using the toxic unit approach. *Appl. Soil Ecol.*, **4**, 213-219.

Kiekens, L. (1990) Zinc. In *Heavy metals in soils* (B.J. Alloway, Ed.), Blackie, Glasgow/London, pp. 261-277.

Kiewiet, A.T. and Ma, W.C. (1991) Effect of pH and calcium on lead and cadmium uptake by earthworms in water. *Ecotoxicol. Environ. Saf.*, **21**, 32-37.

Kirk, L. and Krogh, P.H. (1995) Predator-prey interactions. Poster presented at the 5th SETAC Europe Congress, 25-28 June, Copenhagen.

Klepper, O. and Van De Meent, D. (1997) Mapping the Potentially Affected Fraction (PAF) of species as an indicator of toxic stress. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 607504001.

Klepper, O., Alkemade, J.R.M. and Van Grinsven, J.J.M. (1997) Oorzaken en gevolgen van de aanwezigheid van zware metalen in het landelijk gebied. *Bodem* ,(7) no 3, 112-116

Klironomos, J.N., Widden, P. and Deslandes, I. (1992) Feeding preferences of the collembolan *Folsomia candida* in relation to microfungal successions on decaying litter. *Soil Biol. Biochem.*, **24**, 685-692.

Klok, C. and De Roos, A.M. (1996) Population level consequences of toxicological influences on individual growth and reproduction in *Lumbricus rubellus* (Lumbricidae, Oligochaeta). *Ecotoxicol. Environ. Saf.*, **33**, 118-227.

Korthals, G.W., Alexiev, A.D., Lexmond, Th.M., Kammenga, J.E., Bongers, T. (1996a) Long-term effects of copper and pH on the terrestrial nematode community in an agroecosystem. *Environ. Toxicol. Chem.* **15**, 979-985

Korthals, G.W. (1997) Pollution induced changes in terrestrial nematode communities. Ph. D. Thesis Agricultural University Wageningen.

Korthals, G.W., Van de Ende, A., Van Megen, H., Lexmond, Th. M., Kammenga, J. E. and Bongers, T. (1996) Short-term effects of cadmium, copper, nickel and zinc on soil nematodes from different feeding and life-history strategy groups. *Appl. Soil Ecol.*, **4**, 107-117

Krogh, P.H. (1995) Does a heterogenous distribution of food or pesticide affect the outcome of toxicity tests with Collembola ? *Ecotoxicol. Environ. Saf.,* **30**, 158-163.

Kula, H. (1995) Comparison of laboratory and field testing for the assessment of pesticide side effects on earthworms. *Acta Zool. Fenn.,* **196**, 338-341.

Låg, J., Hvatum, O.O., Bolviken, B. (1970) An occurence of naturally lead-poisoned soil at Kastad near Gjovik, *Norway. Nor. Geol. Unders.* **266,** 141-157.

Lahr, J. and Brock, T.C.M. (in press) Ecologische risico's van bestrijdingsmiddelen in zoetwaterecosystemen. SC-DLO report.

Lebourg, A., Sterckeman, T., Ciesielski, H. and Proix, N. (1996) Intérêt de différents réactifs d'extraction chimique pour l'évaluation de la biodisponibilité des métaux en traces du sol. *Agronomie,* **16**, 201-215.

Lee, Q. and Widden, P. (1996) *Folsomia candida*, a "fungivorous" collembolan, feeds preferentially on nematodes rather than soil fungi. *Soil Biol. Biochem.*, **28**, 689-690.

Lin, Z-Q., Barthakur, N.N., Schuepp, P.H. and Kennedy, G.G. (1996) Uptake and translocation in balsam fir seedlings with ^{54}Mn and ^{65}Zn radioisotopes applied to soil surfaces. *J. Environ. Qual.*, **25**, 92-96.

Lo, K.S.L., Yang, W.F. and Lin, Y.C. (1992) Effects of organic matter on the specific adsorption of heavy metals by soil. *Toxicol. Environ. Chem.*, **34**, 139-153.

Ma, W. and Bodt, J. (1993) Differences in toxicity of the insecticide chlorpyrifos to six species of earthworms (Oligochaeta, Lumbricidae) in standardized soil tests. *Bull. Environ. Contam. Toxicol.*, **50**, 864-870.

Mackay, D., Puig, H. and McCarty, L.S. (1992) An equation describing the time course and variability in uptake and toxicity of narcotic chemicals to fish. *Environ. Toxicol. Chem.*, **11**, 941-951.

Macnicol, R.D., and Beckett, P.H.J. (1989) Critical tissue concentrations of potentially toxic elements. *Plant Soil,* **85**, 107-129.

Malecki, M.R, Neuhauser, E.F. and Loehr, R.C. (1982) The effect of metals on the growth and reproduction of *Eisenia fetida* (Oligochaeta, Lumbricidae). *Pedobiologia,* **24**, 129-137.

Mann, S.S. and Ritchie, G.S.P. (1994) Changes in the forms of cadmium with time in some western Australian soils. *Aust. J. Soil Res.,* **32**, 241-250.

Marinussen, M.P.J.C. (1997) Heavy metal accumulation in earthworms exposed to spatially variable soil contamination. Ph.D. Thesis, Agricultural University Wageningen.

Marinussen, M.P.J.C. and Van der Zee, S.E.A.T.M. (1996) Conceptual approach to estimating the effect of home-range size on the exposure of organisms to spatially variable soil contamination. *Ecological Modelling,* **87**, 83-89.

Marschner, H. (1986) *Mineral nutrition in higher plants*. London: Academic Press.

McCarty, L.S., Mackay, D., Smith, A.D., Ozburn, G.W. and Dixon, D.G. (1992) Residue-based interpretation of toxicity and bioconcentration QSARs from aquatic bioassays: neutral narcotic organics. *Environ. Toxicol. Chem.*, **11**, 917-930

McGrath, S.P., Brookes, P.C. and Giller, T. (1988) Effects of potentially toxic metals in soil derived from past applications of seqage sludge on nitrogen fixation by *Trifolium repens*. *Soil Biol. Biochem.*, **21**, 841-848

Migula, P., Kafel, A., Kedziorski, A. and Nakonieczny, M. (1989a) Combined and separate effects of cadmium, lead and zinc on growth and feeding in the house cricket (*Acheta domesticus*). *Biologia,* **44**, 911-921.

Migula, P., Kedziorski, M., Makonieczny, M. and Kafel, A. (1989b) Combined and separate effects of heavy metals on energy budget and metal balances in *Acheta domesticus*. Uttar *Pradesh J. Zool.*, **9**, 140-149.

Millward, R.M. and Grant, A. (1995) Assessing the impact of copper on nematode communities form a chronically metal-enriched estuary using pollution-induced community tolerance. *Mar. Pollut. Bull.*, **30**, 701-706.

Molander, S. and Blanck, H. (1992) Detection of Pollution-Induced Community Tolerance (PICT) in marine periphyton communities established under diuron exposure. *Aquat. Toxicol.*, **22**, 129-144.

Morgan, J.E. and Morgan, A.J. (1991) Differences in the accumulated metal concentrations in two epigeic earthworm species (*Lumbricus rubellus and Dendrodrilus rubidus*) living in contaminated soils. *Bull. Environ. Contam. Toxicol.*, **47**, 296-301.

Moriarty, F., Hanson, H.M. and Freestone, P. (1984) Limitations of body burden as an index of environmental contamination: heavy metals in fish *Cottus gobio* L. from the river Ecclesbourne, Derbyshire. *Environ. Pollut. Ser. A*, **34**, 297-320

Mudry, T., Height, M. and Pasternak, J. (1982) The effects of some heavy metals on the kinetics of pharyngeal pumping in *Panagrellus silusiae. Nematologica,* **28**, 12-20

Mullins, G.L., and Sommers, L.E. (1986) Cadmium and zinc influx characteristics by intact corn (*Zea mays* L.) seedlings. *Plant Soil,* **96**, 153-164.

Nederlof, M.M., Van Riemsdijk, W.H. and De Haan, F.A.M. (1993) Effect of pH on the bioavailability of metals in soils. In *Integrated soil and sediment research: a basis for proper protection* (H.J.P. Eijsackers and T. Hamers, Eds.), Kluwer Academic Publishers, Dordrecht, pp. 215-219.

Notenboom, J. and Posthuma, L. (1994) Validatie toxiciteitsgegevens en risicogrenzen bodem: Voortgangsrapportage 1993. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 719102029.

Notenboom, J. and Posthuma, L. (1995) Validatie toxiciteitsgegevens en risicogrenzen bodem: Voortgangsrapportage 1994. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 719102045.

Notenboom, J. and Van Beelen, P. (1992) Validatie van risicogrenzen voor de bodem. Verslag van een workshop gericht op de opzet van een onderzoekprogramma. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 719102020.

Novozamsky, I., Lexmond, Th. M, and Houba, V.J.G.. (1993) A single extraction procedure of soil for evaluation of uptake of some heavy metals by plants. *Intern. J. Environ. Anal. Chem.,* **51**, 47-58.

O'Conner, F.B. (1971) The Enchytraeidae. In: Philipson, J. (ed.). *Methods of study in quantitative soil ecology*. Blackwell, Oxford and Edinburgh. p. 83-106.

OECD (1984a) Guideline for testing of chemicals no. 207. Earthworm acute toxicity tests. Paris, 1984.

OECD (1984b) Guideline for testing of chemicals no. 208. Terrestrial plants, growth test. Paris, 1984

Okkerman, P.C., Van de Plassche, E.J., Emans ,H.J.B., Canton, J.H. (1993) Validation of some extrapolation methods with toxicity data derived from multiple species experiments. *Ecotoxicol.Environ. Saf.* **25,** 341-359.

Parmelee, R.W., Wentsel, R.S., Phillips, C.T., Simini, M. and Checkai, R.T. (1993) Soil microcosm for testing the effects of chemical pollutants on soil fauna communities and trophic structure. *Environ. Toxicol. Chem.*, **12**, 1477-1486.

Peijnenburg, W.J.G.M., Posthuma, L., Eijsackers H.J.P., Allen, H.E. (1997) A conceptual framework for implementation of bioavailability of metals for environmental management purposes. *Ecotoxicol. Environ. Saf.* **37**, 163-172

Pellegrini, M., Laugier, A., Sergent, M., Phan-Tan-Luu, R., Valls, R. and Pellegrini, L. (1993) Interactions between the toxicity of the heavy metals cadmium, copper, zinc in combinations and the detoxifying role of calcium in the brown alga *Cystoseira barbata. J. Appl. Phycol.* **5**, 31-351-361.

Pierzynski, G.M. and Schwab, A.P. (1993) Bioavailability of zinc, cadmium and lead in a metal-contaminated alluvial soil. *J. Environ. Qual.,* **22**, 247-254.

Plette, A.C.C. (1997) Cadmium and zinc interactions with a Gram-positive soil bacterium-from variable charging behavior of the cell wall to bioavailability of heavy metals in soils. Ph.D. Thesis Agricultural University Wageningen.

Posthuma, L. (1997) Effects of toxicants on population and community parameters in field conditions, and their potential use in the validation of risk assessment methods. In: N.M van Straalen N.M. and H Løkke. (Eds.), *Ecological risk assessment of contaminants in soil.* Chapman & Hall, London, pp. 85-117.

Posthuma, L. and Notenboom, J. (1996) Toxic effects of heavy metals in three worm species (*Eisenia andrei* , *Enchytraeus crypticus* and *Enchytraeus albidus*: Oligochaeta) exposed in artifcially polluted soil substrates and contaminated field soils. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 719102048.

Posthuma, L. and Van Straalen, N.M. (1993) Heavy metal adaptation in terrestrial invertebrates: a review of occurrence, genetics, physiology and ecological concequences. *Comp. Biochem. Physiol.*, **106C**, 11-38.

Posthuma, L., Baerselman, R., Van Veen, R.P.M. and Dirven-Van Breemen, E.M. (1997) Single and joint toxic effects of copper and zinc on reproduction of *Enchytraeus crypticus* in relation to sorption of metals in soils. *Ecotoxicol. Environ. Saf.*, **38**, 108-121.

Posthuma, L., Boonman, H., Mogo, F.C. and 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. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 719102033.

Posthuma, L., Notenboom, J., De Groot, A. C. and Peijnenburg, W.J.G.M. (in press) Soil acidity as a major determinant of zinc partitioning and zinc uptake in two oligochaete worms (*Eisenia andrei and Enchytraeus crypticus*) exposed in contaminated field soils. In: S.C. Sheppard, J.J.B. Bembridge, M Holmstrup and L. Posthuma (Eds.) *Progress in earthworm ecotoxicology*, SETAC Publishers.

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

Postma, J.F. and Davids, C. (1995) Tolerance induction and life-cycle changes in cadmium exposed *Chironomus riparius* (Diptera) during consecutive generations. *Ecotoxicol. Environ. Saf.* **30**, 195-202.

Postma, J.F., Buckert-De Jong, M.C., Staats, N. and Davids, C. (1994) Chronic toxicity of cadmium to *Chironomus riparius* (Diptera) at different food levels. *Arch. Environ. Contam. Toxicol.* **26**, 143-148.

Rademaker M., Van Esbroek, M.L.P. and Schouten, A.J. (1994) Methodiekontwikkeling voor een bioassay met nematoden in natuurlijke grond. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 719102030

Reinecke, A.J. and Reinecke, S.A. (1996) The influence of heavy metals on the growth and production of the compost worm *Eisenia fetida* (Oligochaeta). *Pedobiologia,* **40**, 439-448.

Ristola, T. (1995) Effect of feeding regime on the results of sediment bioassays and toxicity tests with chironomids. *Ann. Zool. Fenn.*, **32**, 257-264.

Römbke, J. (1989) *Enchytraeus albidus* (Enchytraeidae, Oligochaeta) as a test organism in terrestrial laboratory systems. *Arch. Toxicol.*, **13**, 402-405.

Römbke, J. (1995) Enchytraeen (Oligochaeta) als Bioindikator. *UWSF - Z. Umweltchem. Ökotox.* **7**, 246-249.

Römbke, J. (1997a) The enchytraeid reproduction test (ERT): current status of the international ringtest. Second international workshop on earthworm ecotoxicology, Amsterdam, April 3-5, 1997.

Römbke, J. (1997b) Experiment 1996. Field sampling of Enchytraeidae at Budel (The Netherlands). Report of RPR GbR for National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, The Netherlands.

Römbke, J. and Moltmann, J.F. (1995) *Applied ecotoxicology.* Lewis Publishers, Boca Raton.

Rutgers, M., Van 't Verlaat, I., Wind, B., Posthuma, L. and Breure, A.M. (in press) Rapid method to assess pollution-induced community tolerance in contaminated soil. *Environ. Toxicol. Chem.*

s' Jacob, J.J. and Van Bezooijen, (1984) Manual for practical work in nematology. Agricultural University Wageningen

Salomons, W. (1993) Non-linear and delayed responses of toxic chemicals in the environment. In *Contaminated Soil '93* In F. Arendt, G.J. Annokkée, R. Bosman and W.J. van den Brink, (Eds.), Kluwer Academic Publishers, Dordrecht, pp. 225-238.

Salomons, W. and U. Förstner. (1984). *Metals in the hydrocycle.* Berlin: Springer Verlag.

Samoiloff, M.R., and Bogaert, T. (1984). The use of nematodes in marine ecotoxicology. In: G. Persoone, E. Jaspers and C. Claus (eds) *Ecological testing for the marine environment.* State Univ. Ghent and Inst. Mar. Scient. Res., Bredene, Belgium, vol. 1, 798 p.

Samoiloff, M.R., Schulz, S., Jordan, Y., Denich, K. and Arnott, E. (1980) A rapid simple longterm toxicity assay for aquatic contaminants using the nematode *Panagrellus redivivus. Can. J. Fish. aquat. Sci.*, **37**, 1167-1174

Sánchez-Martin, M.J. and Sánchez-Camazano, M. (1993) Adsorption and mobility of cadmium in natural, uncultivated soils. *J. Environ. Qual.*, **22**, 737-742.

Schouten, A.J., Van Esbroek, M.L.P. and Alkemade, J.R.M. (in prep.) Validatie toxiciteitsgegevens en risicogrenzen bodem: Ecologische effecten van zink op bodemorganismen (nematoden) bij een chronische belasting en semi-veldomstandigheden. National Institute for Public Health and the Environment, Bilthoven, The Netherlands.

Sheppard, S.C. and Evenden, W.G. (1992) Bioavailability indices for uranium: effect of concentration in eleven soils. *Arch. Environ. Contam. Toxicol.*, **23**, 117-124.

Slooff, W., Van Oers, J.A.M., and De Zwart, D. (1986) Margins of uncertainty in ecotoxicological hazard assessment. *Environ. Toxicol. Chem.* **5**, 841-852.

Smit, C.E. (1997) Field relevance of the *Folsomia candida* soil toxicity test. Ph.D. Thesis Vrije Universiteit Amsterdam.

Smit, C.E. and Van Gestel, C.A.M. (1995) Influence of temperature and humidity fluctuations on the sensitivity of *Folsomia candida* for zinc. In *Contaminated Soil '95* In W.J. Van den Brink, R. Bosman and F. Arendt, (Eds.), Kluwer Academic Publishers, Dordrecht, pp. 645-646.

Smit, C.E. and Van Gestel, C.A.M. (1996) Comparison of the toxicity of zinc for the springtail *Folsomia candida* in artificially contaminated and polluted field soils. *Appl. Soil Ecol.*, **3**, 127-136. erratum *Appl. Soil Ecol.*, **4**, 93-94.

Smit, C.E. and Van Gestel, C.A.M. (1997) Influence of temperature on the regulation and toxicity of zinc in *Folsomia candida* (Collembola). *Ecotoxicol. Environ. Saf.*, **37**, 213-222.

Smit, C.E. and Van Gestel, C.A.M. (1998a) Effects of soil type, pre-percolation and ageing on bioaccumulation and toxicity of zinc for the springtail *Folsomia candida. Environ. Toxicol. Chem.* **17**: 1132-1141

Smit, C.E., Van Beelen, P. and Van Gestel, C.A.M. (1997) Development of zinc bioavailability and toxicity for the springtail *Folsomia candida* in an experimentally contaminated field plot. *Environ. Pollut.* **98**, 73-80

Smit, C.E., Van Overbeek, I. and Van Gestel, C.A.M. (1998b) The influence of food supply on the toxicity of zinc for *Folsomia candida. Pedobiologia* **42**, 154-164.

Snider, R.M. and Butcher, J.W. (1973) The life history of *Folsomia candida* (Willem) (Collembola: Isotomidae) relative to temperature. *The Great Lakes Entomologist*, **6**, 97-106.

Sokal, R.R. and Rohlf, F.J. (1995) *Biometry.* W.H. Freeman, New York.

Sprague, J.B. (1970) Measurement of pollution toxicity to fish. II. Utilizing and applying bioassay results. *Water Res.*, **1970**, 3-32.

Spurgeon, D.J. and 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. and Hopkin, S.P. (1996a) Effects of metal-contaminated soils on the growth, sexual development, and early cocoon production of the earthworm *Eisenia fetida*, with particular reference to zinc. *Ecotoxicol. Environ. Saf.*, **35**, 86-95.

Spurgeon, D.J. and Hopkin, S.P. (1996b) Effects of variations of the organic matter content and pH of soils on the availability and toxicity of zinc to the earthworm *Eisenia fetida*. *Pedobiologia*, **40**, 80-96.

Spurgeon, D.J. and Hopkin, S.P. (1996c) The effects of metal contamination on earthworm populations around a smelting works: quantifying species effects. *Appl. Soil. Ecol.* **4**, 147-160

Spurgeon, D.J., Hopkin, S.P. and 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.

Stam, E.M., Van de Leemkule, M.A. and Ernsting, G. (1996) Trade-offs in the life history and energy budget of the parthenogenetic collembolan *Folsomia candida*. *Oecologia*, **107**, 283-292.

Stephenson, R.R. and Watts, S.A. (1984) Chronic toxicity tests with *Daphnia magna*: the effects of different food and temperature regimes on survival, reproduction and growth. *Environ. Pollut.*, **36**, 95-107.

Struijs, J., Van De Meent, D., Peijnenburg, W.J.G.M., Van Den Hoop, M.A.G.T., and Crommentuijn, T. (1997) Added risk approach to derive maximum permissible concentrations for heavy metals: how to take into account the natural background levels? *Ecotoxicol. Environ. Saf* 37:112-118.

Suter II, G.W. (1996) Abuse of hypothesis testing statistics in ecological risk assessment. *Hum. Ecol. Risk Assess.* **2**, 331-347

Suter, G.W. (1983) Ecological risk assessment. Lewis Publishers, Boca Raton, Florida, USA.

Taiz, L., and Zeiger, E. (1991) *Plant Physiology*. Redwood city: The Benjamin/Cumming Publish. Comp.

Temminghoff, E.J.M., Van der Zee, S.E.A.T.M. and De Haan, F.A.M. (1995) Speciation and calcium competition effects on cadmium sorption by sandy soil at various pHs. *J. Soil Sci.* **46**, 649-655.

Tyler, G. (1984) The impact of heavy metal pollution on forests: a case study of Gusum, Sweden. *Ambio* **13**, 18-24

Ure, A.M. and Davidson, C.M. (1995) *Chemical speciation in the environment*, Blackie Academic and Professional, Glasgow.

Usher, M.B. and Stoneman, C.F. (1977) *Folsomia candida*- an ideal organism for population studies in the laboratory. *J. Biol. Educ.*, **11**, 83-90.

Vaal, M.A. Van Der Wal, J.T., Hermens, J., Hoekstra, J. (1997a) Pattern analysis of te variation in sensitivity of aquatic species to toxicants. *Chemosphere,* **35**, 1291-1309

Vaal, M.A. Van Der Wal, J.T., Hoekstra, J. Hermens, J., (1997b) Variation in the sensitivity of aquatic species in relation to the classification of environmental pollutants. *Chemosphere,* **35**: 1311-1327

Van Beelen, P. and Doelman, P. (1997) Significance and application of microbial toxicity tests in assessing ecotoxicological risks of contaminants in soil and sediment. *Chemosphere,* **34**, 455-499.

Van Beelen, P. and Fleuren-Kemilä, A.K. (1997) Influence of pH on the toxic effects of Zinc, Cadmium, and pentachlorophenol on pure cultures of soil microorganisms. *Environ. Toxicol. Chem.*, **16**, 146-155.

Van Beelen, P. and Notenboom, J. (1996) Validatie toxiciteitsgegevens en risicogrenzen bodem: Voortgangsrapportage 1995. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 607505002.

Van Beelen, P., Fleuren-Kemilä, A.K., Huys, M.P.A., Van Montfort, A.C.P. and Van Vlaardingen, P.L.A. (1991) The toxic effects of pollutants on the mineralization of acetate in subsoil microcosms. *Environ. Toxicol. Chem.*, **10**, 775-790.

Van Capelleveen, H.E. (1987) The ecotoxicology of metals for terrestrial isopods. Ph.D. Thesis, Vrije Universiteit, Amsterdam.

Van De Guchte, C. (1992) The sediment quality triad: an integrated approach to assess contaminated sediments. In: P.J. Newman, M.A. Piavaux & R.A. Sweeting (Eds.) *River Water Quality. Ecological Assessment and Control.* ECSC-EEC-EAEC, Brussels, Luxembourg, pp. 425-431.

Van De Meent, D., Aldenberg, T., Canton, J.H., Van Gestel, C.A.M. and Slooff, W. (1990) Desire for levels. Background study to the policy document "Setting environmental quality standards for water and soil". National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 670101002.

Van Den Brink, P.J., Van Wijngaarden, R.P.A., Lucassen, W.G.H.B., Brock, T.C.M., Leewangh, P. (1996) Effects of the insecticide Dursban 4E (active ingredient chlorpyrifos) in outdoor experimental ditches: II Invertebrate community responses and recovery. *Environ. Toxicol. Chem.* **15**, 1143-1153.

Van der Hoeven, N. and Henzen, L. (1994a) Combinatietoxicologie van de metalen zink en cadmium en de plantensoort *Trifolium pratense* op kunstrmatige grond samengesteld volgens OECD guideline 207 (wormengrond). TNO report MW-R94/297.

Van der Hoeven, N. and Henzen, L. (1994b) Groei van de plantensoort *Trifolium pratense* op elf gronden uit Budel gemonsterd in een gradient. TNO report IMW-R94/041.

Van Ewijk, P.A. and Hoekstra, J.A. (1993) Calculation of the EC_{50} and its confidence interval when subtoxic stimulus is present. *Ecotoxicol. Environ. Saf.,* **25**, 25-32.

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

Van Gestel, C.A.M. and Hensbergen, P.J. (1997) Interaction of Cd and Zn toxicity for *Folsomia candida* Willem (Collembola Isotomidae) in relation to bioavailability in soil. *Environ. Toxicol. Chem.*, **16**, 1177-1186.

Van Gestel, C.A.M. and Ma, Wei-chun (1990) An approach to quantitative structure-activity relationships in terrestrial ecotoxicology. *Chemosphere,* **21**, 1023-1033.

Van Gestel, C.A.M. and Van Diepen, A.F.M. (1997) The influence of soil moisture content on the bioavailability and toxicity of cadmium for *Folsomia candida* Willem (Collembola: Isotomidae). *Ecotoxicol. Environ. Saf.*, **36**, 123-132.

Van Gestel, C.A.M. and Van Straalen, N.M. (1994) Ecotoxicological test systems for terrestrial invertebrates. In *Ecotoxicology of soil organisms* (M.H. Donker, H. Eijsackers and F. Heimbach, Eds.), Lewis publishers, Boca Raton, pp. 205-227.

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

Van Gestel, C.A.M., Dirven-van Breemen, E.M. and Kamerman, J.W. (1992b) Beoordeling van gereinigde grond. IV.Toepassing van bioassays met planten en regenwormen op referentiegronden. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 216402004.

Van Gestel, C.A.M., Rademaker, M.C.J. and Van Straalen, N.M. (1995) Capacity controlling parameters and their impact on metal toxicity in soil invertebrates. In *Biogeodynamics of pollutants in soil and sediments-risk assessment of delayed and non-linear responses* (W. Salomons and W.M. Stigliani, Eds.), Springer-Verlag, Berlin, pp. 171-192.

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

Van Gestel, C.A.M., Van Dis, W.A., Van Breemen, E.M. and 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. *Ecotoxicol. Environ. Saf.,* **18**, 305-312

Van Rhee, J.A. (1970) De regenwormen (Lumbricidae) van Nederland. KNNV-Mededelingen no. 84

Van Riemsdijk, W.H. and Hiemstra, T. (1993) Adsorption to heterogeneous surfaces. In *Metals in groundwater* (H.E. Allen, E.M. Perdue and D.S. Brown, Eds.), Lewis Publishers, Chelsea, pp. 1-36.

Van Straalen, N.M. (1993) Soil and sediment quality criteria derived from invertebrate toxicity data. In: Dallinger R, Rainbow PS, (Eds.*) Ecotoxicology of metals in Invertebrates.* Lewis publishers, Boca Raton, FL, U.S.A. p 427-441

Van Straalen, N.M. (1993) An ecotoxicologist in politics. *Oikos* 66: 142-143.

Van Straalen, N.M. (1996) Critical body concentrations: their use in bioindication. In *Bioindicator systems for soil pollution* (N.M. Van Straalen and D.A. Krivolutsky, Eds.), Kluwer Academic Publishers, Dordrecht, pp. 5-16.

Van Straalen, N.M. and De Goede, R.G.M., (1987) Productivity as a population performance index in life-cycle toxicity tests. *Water Sci. Technol,* **19**, 13-20.

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

Van Straalen, N.M. and Verkleij, J.A.C. (1993) *Leerboek Oecotoxicologie* VU-Uitgeverij, Amsterdam.

Van Straalen, N.M., Schobben, J.H.M. and De Goede, R.G.M. (1989) Population consequences of cadmium toxicity in soil microarthropods. *Ecotoxicol. Environ. Saf.,* **17**, 190-204.

Van Tilborg, W.J.M. and Van Assche, F. (1996) Risk assessment of essential elements: proposal for a fundamentally new approach. *SETAC News*, **Sept. 1996**, 28-29.

Van Wensem, J., Vegter, J.J. and Van Straalen, N.M. (1994) Soil quality criteria derived from critical body concentrations of metals in soil invertebrates. *Appl. Soil Ecol.*, **1**, 185-191.

Van Wensum, J. (1997) Actief bodembeheer voor de zinkerfenis in de Kempen. *Bodem*, **3**, 119-121.

Van Wijngaarden, R.P.A., Van Den Brink, P.J., Crum, S.J.H., Oude Voshaar, J.H., Brock, T.C.M., Leewangh, P. (1996) Effects of the insecticide Dursban 4E (active ingredient chlorpyrifos) in outdoor experimental ditches: I. Comparison of short-term toxicity between laboratory and the field. *Environ. Toxicol. Chem.* **15**, 1133-1142.

Vegter, J.J. (1995) Soil protection in the Netherlands. In: Heavy metals - Problems and solutions (W. Salomons, U. Förstner and P. Mader, Eds.), Springer Verlag, Berlin-Heidelberg, pp. 79-100.

Verkleij, J.A.C. and Schat, H. (1990). Mechanisms of metal tolerance in higher plants. In *Heavy metal tolerance in plants: evolutionary aspects*, edited by A. J. Shaw. Boca Raton, Florida: CRC Press.

Verma, A. and Pillai, M.K.K. (1991) Bioavailability of soil-bound residues of DDT and HCH to earthworms. *Current Sci.* **61**, 840-843.

Vogel, W.R. (1988) Zur Aufnahme und Auswirkung der Schwermetalle Zink und Cadmium beim Mehlkäfer *Tenebrio molitor* L. (Col., Tenebrionidae) unter Berücksichtigung möglicher Wechselwirkungen. *Zool. Anz.*, **220**, 25-32.

Vonk, J.W and Matla, Y.A. (1993) A test for effects of chemicals on glutamate mineralisation in soil. A draft guideline. Report IMW-R 93/097. TNO-Institute for Environmental Sciences, Delft, The Netherlands.

Vonk, J.W., Matla, Y.A., Van Gestel, C.A.M., Koolhaas - van Hekezen, J., Gerritsen, A.A.M. and Henzen, L. (1996) The influence of soil characteristics on the toxicity of cadmium for *Folsomia candida, Eisenia fetida* and glutamate mineralisation. Report MEP-R 96/144. TNO-Environmental Science, Energy Research and Process Innovation, Delft, The Netherlands.

Vranken, G., Tiré, C. and Heip, C. (1988) The toxicity of paired metal mixtures on the nematode *Monhystera disjuncta* (Bastian, 1865). *Mar. Environ. Res.*, **26**, 161-179

Vranken, G., Vanderhaegen, R. and Heip, C. (1985) Toxicity of cadmium to freeliving marine and brackish water nematodes (*Monhystera microphthalma, Monhystera disjuncta and Pelliodítis marina*). *Dis. Aquat. Org.*, **1**, 49-58

Vranken, G., Vanderhaegen, R. and Heip, C. (1986) Effect of pollutants on life history parameters of the marine nematode *Monhystera disjuncta. J. Mar. Sci.*, **48**, 325-334

Vranken, G., Vanderhaegen, R. and Heip, C. (1991) Effects of pollutants on life history parameters of the marine nematode *Monhystera disjuncta. ICES J. mar. Sci.*, **48**, 325-334

VROM (1989a) National Environmental Policy Plan. To choose or to lose. Second Chamber, session 1988-1989, 21137, no 1-2.

VROM (1989b) Notitie Milieukwaliteitsdoelstellingen Bodem en Water (MILBOWA). Tweede Kamer, vergaderjaar 1990-1991, 21990, no 1 (In Dutch).

VROM (1994) Circulaire interventiewaarden bodemsanering. Bijlage: Interventie- en streefwaarden voor de bodem.

VROM (1994a) Environmental Quality Objectives in the Netherlands. A review of environmental quality objectives and their policy framework in the Netherlands. Ministry of Housing, Spatial Planning and The Environment, The Netherlands.

VROM (1994b) Circulaire Inwerkingtreding Saneringsregeling Wet Bodembescherming (Tweede Fase). Ministerie van Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer, The Netherlands.

VROM (1997) Integrale Normstelling Stoffen. Milieukwaliteitsnormen bodem, water, lucht. Ministry of Housing, Spatial Planning and The Environment, Publication 97759/h/12-97.

Wallace, A. (1989) Plant responses to some hardly known trace elements and trace element composition and distribution in plants. *Soil Sci.*, **147**, 461-464.

Wang, W. (1991) Literature review on higher plants for toxicity testing. *Water, Air, Soil Pollut.*, **59**, 381-400.

Warne, M.S.J. and Hawker, D.W. (1995) The number of components in a mixture determines whether synergistic and antagonistic or additive toxicity predominate: the funnel hypothesis. *Ecotoxicol. Environ. Saf.*, **31**, 23-28.

Weltje, L., Posthuma, L., Mogo, F.C., Dirven-Van Breemen, E.M. and Van Veen, R.P.M. (1995) Toxische effecten van mengsels van cadmium, zink en koper op terrestrische oligochaeten in relatie tot bodem-chemische interacties. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 719102043.

Wheeler, D.M., and. Power, I.L. (1995) Comparison of plant uptake and plant toxicity of various ions in wheat. *Plant Soil,* **172**, 167-173.

White, J.C. Kelsey, J.W., Hatzinger, P.B. and Alexander, M. (1997) Factors affecting sequestration and bioavailability of phenanthrene in soils. *Environ. Toxicol. Chem.* **16**, 2040-2045.

Winner, R.W., Keeling, T., Yeager, R. and Farrell, M.P. (1977) Effect of food type on the acute and chronic toxicity of copper to *Daphnia magna. Freshwater Biol.,* 7, 343-349.

Wolt, J.D. (1994) *Soil solution chemistry: applications to environmental science and agriculture.* John Wiley and Sons, New York.

Woolhouse, H.W. (1983) Toxicity and tolerance in the response of plants of metals. In *Encyclopaedia of Plant Physiology*, edited by A. Pirson and M. H. Zimmermann. Berlin: Springer-Verslag.

Xian, X. (1989) Effect of chemical forms of cadmium, zinc, and lead in polluted soils on their uptake by cabbage plants. *Plant Soil,* **113**, 257-264.

Zelibor, J.L., Doughten, M.W., Grimes, D.J. and Colwell, R.R. (1987) Testing for bacterial resistance to arsenic in monitoring well water by the direct viable counting method. *Appl. Environ. Microbiol.*, **53**, 2929-34.

PROJECT BIBLIOGRAPHY

Within the framework of the Validation project, the following papers and reports have been published (or are of direct relevance):

RIVM, Laboratory for Ecotoxicology, Bilthoven

Alkemade, J.R.M.,. Schouten, A.J., Kersten, P. and Van Esbroek, M.L.P. (1996) Vergelijking van effectniveaus voor bodemorganismen met het maximaal toelaatbaar risico. Een veldstudie naar de invloed van zware metalen in een gradiënt te Budel. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 607505001

Bakker, J., Posthuma, L. and Baerselman, R. (1997) Toxische effecten van zink op de worm *Eisenia andrei*; resultaten proefveldexperimenten Amsterdam 1995 en 1996. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 607505003.

Klepper, O., Alkemade, J.R.M. and Van Grinsven, J.J.M (1997) Oorzaken en gevolgen van de aanwezigheid van zware metalen in het landelijk gebied. Bodem (7) no. 3, 112-116

Notenboom, J. and Posthuma, L. (Eds.) (1994) Validatie toxiciteitsgegevens en risicogrenzen bodem: voortgangsrapportage 1993. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 719102029.

Notenboom, J. and Posthuma, L. (Eds.) (1995) Validatie toxiciteitsgegevens en risicogrenzen bodem: voortgangsrapportage 1994. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 719102045

Notenboom, J. and Van Beelen, P. (Eds.) (1992) Validatie van risicogrenzen voor de bodem. Verslag van een workshop gericht op de opzet van een onderzoeksprogramma. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 719102020.

Posthuma, L. (1997) Effects of toxicants on population and community parameters in field conditions, and their potential use in the validation of risk assessment methods. In: Van Straalen N.M., Løkke H. (Eds.), *Ecological risk assessment of contaminants in soil*. Chapman and Hall, p 85-123.

Posthuma, L., Baerselman, R., Van Veen, R.P.M. and Dirven-Van Breemen, E.M. (1997) Single and joint toxic effects of copper and zinc on reproduction of *Enchytraeus crypticus* in relation to sorption of metals in soils. *Ecotoxicol. Environ. Saf.* **38**, 108-121.

Posthuma, L., Boonman, H., Mogo, F.C. and Baerselman, R. (in press) Antagonism and bioavailability determine low toxicity of heavy metals for *Eisenia andrei* in smelter soil in comparison to OECD-test results. *Soil Biol. Biochem.*

Posthuma, L., Boonman, H., Mogo, F.C. and 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. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 719102033.

Posthuma, L. and Notenboom, J. (1996) Toxic effects of heavy metals in three worm species (*Eisenia andrei, Enchytraeus crypticus* and *Enchytraeus albidus*: Oligochaeta) exposed in artificially polluted soil substrates and contaminated field soils. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 719102048

Posthuma, L., Notenboom, J., De Groot, A.C. and Peijnenburg, W.J.G.M. (in press) Soil acidity as major determinant of zinc partitioning and zinc uptake in two oligochaete worms (*Eisenia andrei* and *Enchytraeus crypticus*) exposed in contaminated field soils. In: Sheppard S.C., Bembridge J.J.B., Holmstrup M., Posthuma L., (Eds.) *Progress in earthworm ecotoxicology*. SETAC Publishers.

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

Rademaker M., Van Esbroek, M.L.P. and Schouten, A.J. (1994) Methodiekontwikkeling voor een bioassay met nematoden in natuurlijke grond. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 719102030

Römbke, J. (1996) Field sampling of Enchytraeidae at Budel (The Netherlands). Pilot Experiment. Report of RPR GbR, Mörfelden-Walldorf, Germany.

Römbke, J. (1997) Field sampling of Enchytraeidae at Budel (The Netherlands). Experiment 1996. Report of RPR GbR, Mörfelden-Walldorf, Germany.

Rutgers, M., Van 't Verlaat, I.M., Wind, B., Posthuma, L. and Breure, A.M. (in press) Rapid method to assess pollution-induced community tolerance in contaminated soil. *Environ. Toxicol. Chem.*

Schouten, A.J., Van Esbroek, M.L.P. and Alkemade, J.R.M. (in prep.) Validatie toxiciteitsgegevens en risicogrenzen bodem: Ecologische effecten van zink op bodemorganismen (nematoden) bij een chronische belasting en semi-veldomstandigheden. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report In preparation.

Van Beelen, P.and Doelman P. (1997) Significance and application of microbial toxicity tests in assessing ecotoxicological risks of contaminated soils and sediments. *Chemosphere*, **34**, 55-499

Van Beelen, P. and Notenboom, J. (Eds.) (1996) Validatie toxiciteitsgegevens en risicogrenzen bodem: voortgangsrapportage 1995. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Report no. 607505002

Van Beelen, P. and Fleuren-Kemilä, A. (1997) Influence of pH on the toxic effects of zinc, cadmium and pentachlorophenol on pure cultures of soil microorganisms. *Environ. Toxicol. Chem.*, **16**, 146-153

TNO, Institute of Environmental Sciences, Energy Research and Process Innovation and Nutrition and Food Research Institute, Den Helder and Zeist

Van der Hoeven, N. and Henzen, L. (1994) Groei van de plantensoorten Lolium perenne, *Vicia sativa* en *Trifolium pratense* op grond uit Budel en effecten van zink en cadmium op die groei. TNO rapport MW-R94/004.

Van der Hoeven, N. and Henzen, L.(1994) Groei van de plantensoort *Sinapis alba* op elf gronden uit Budel gemonsterd in een gradiënt.TNO rapport MW-R94/045.

Van der Hoeven, N. and Henzen, L.(1994) Combinatietoxicologie van de metalen zink en cadmium en de plantensoort *Trifolium pratense* op relatief schone grond uit Budel. TNO rapport MW-R94/130.

Van der Hoeven, N. and Henzen, L. (1994) Combinatietoxicologie van de metalen zink en cadmium en de plantensoort *Trifolium pratense* op kunstmatige grond samengesteld volgens OECD Guideline no. 207 (wormengrond). TNO rapport MW-R94/297.

Van der Hoeven, N. and Henzen, L. (1994). De effecten van zink op de groei van de plantensoort *Trifolium pratense* (onder laboratorium- en veldomstandigheden) op grond uit Panheel. TNO rapport MW-R94/304.

Van der Hoeven, N. and Henzen, L. (1994) Groei van de plantensoort *Trifolium pratense* op elf gronden uit Budel gemonsterd in een gradiënt. TNO rapport MW-R94/041.

Kaag, N.H.B.M. and van der Veen, D.P.C. (1998) De invloed van zink op de groei van rode klaver, *Trifolium pratense*, in een proefveldexperiment. TNO-MEP rapport R98/097

Vrije Universiteit, Department of Ecology and Ecotoxicology, Amsterdam

Smit, C.E. and Van Gestel, C.A.M. (1995) Influence of temperature and humidity fluctuations on the sensitivity of *Folsomia candida* (Collembola) for zinc. In *Contaminated Soil '95* (W.J. Van Den Brink, R. Bosman and F. Arendt, eds.) Kluwer Academic Publishers, Dordrecht, pp. 645-646.

Smit, C.E. and Van Gestel, C.A.M. (1996) Comparison of the toxicity of zinc for the springtail *Folsomia candida* in artificially contaminated and polluted field soils. *Appl. Soil Ecol.*, **3**, 127-143.

Smit, C.E. and Van Gestel, C.A.M. (1997) Influence of temperature on the regulation and toxicity of zinc in *Folsomia candida* (Collembola). *Ecotoxicol. Environ. Saf.* **37**, 213-222

Smit, C.E. (1997) Field relevance of the *Folsomia candida* soil toxicity test. Ph.D. Thesis, Vrije Universiteit Amsterdam.

Smit, C.E., Van Beelen, P. and Van Gestel, C.A.M. (1997) Development of zinc bioavailability and toxicity for the springtail *Folsomia candida* in an experimentally contaminated field plot. *Environ. Pollut.* **98**, 73-80.

Smit, C.E.and Van Gestel, C.A.M. (1998a) Effects of soil type, pre-percolation and ageing on bioaccumulation and toxicity of zinc for the *springtail Folsomia candida. Environ. Toxicol. Chem.* **17**, 1143-1141

Smit, C.E., Van Overbeek, I. and Van Gestel, C.A.M. (1998b) The influence of food supply on the toxicity of zinc for *Folsomia candida* (Collembola). *Pedobiologia* **42**, 154-164.

Van Gestel, C.A.M. and Hensbergen, P.J. (1997). Interaction of Cd and Zn toxicity for *Folsomia candida* Willem (Collembola: Isotomidae) in relation to bioavailability in soil. *Environ. Toxicol. Chem.*, **16**, 1177-1186.

Van Gestel, C.A.M. and Van Diepen, A.M.F. (1997) The influence of soil moisture content on the bioavailability and toxicity of cadmium for *Folsomia candida* Willem (Collembola: Isotomidae). *Ecotoxicol. Environ. Saf.*, **36**, 123-132.