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No. 64, *Guidance Document on Overview of Residue Chemistry Studies (2006, revised 2009)*

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No. 85, *Report of the Validation Peer Review for the Hershberger Bioassay, and Agreement of the Working Group of the National Coordinators of the Test Guidelines Programme on the Follow-up of this Report (2007)*

No. 86, *Report of the OECD Validation of the Rodent Hershberger Bioassay: Phase 2: Testing of Androgen Agonists, Androgen Antagonists and a 5 α-Reductase Inhibitor in Dose Response Studies by Multiple Laboratories (2008)*

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No.94 *Report of the Validation Peer Review for the 21-Day Fish Endocrine Screening Assay and Agreement of the Working Group of the National Coordinators of the Test Guidelines Programme on the Follow-up of this Report (2008)*

No.95 *Detailed Review Paper on Fish Life-Cycle Tests (2008)*

No.96 *Guidance Document on Magnitude of Pesticide Residues in Processed Commodities (2008)*

No.97 *Detailed Review Paper on the use of Metabolising Systems for In Vitro Testing of Endocrine Disruptors (2008)*

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No.106 Guidance Document for Histologic Evaluation of Endocrine and Reproductive Test in Rodents (2009)

No.107 *Preservative treated wood to the environment for wood held in storage after treatment and for wooden commodities that are not cover and are not in contact with ground. (2009)*

No.108 *Report of the validation of the Hershberger Bioassay (weanling model) (2009)*

No. 109 *Literature review on the 21-Day Fish Assay and the Fish Short-Term Reproduction Assay (2009)*

No. 110 *Report of the validation peer review for the weanling Hershberger Bioassay and agreement of the working of national coordinators of the test guidelines programme on the follow-up of this report (2009)*

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No. 119 *Classification and Labelling of chemicals according to the UN Globally Harmonized System: Outcome of the Analysis of Classification of Selected Chemicals listed in Annex III of the Rotterdam Convention (2010)*

No. 120 *Explanatory Background Document to the OECD Draft Test Guideline on in vitro Skin Irritation Testing (2010)*

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No. 128 *Validation Report of the 21-day Androgenised Female Stickleback Screening Assay (2010)*

No. 129 *Guidance Document on using Cytotoxicity Tests to Estimate Starting Doses for Acute Oral Systemic Toxicity Tests*

No.130 *Draft Guidance Document On Using Cytotoxicity Tests To Estimate Starting Doses For Acute Oral Systemic Toxicity Tests (2010)*

No.131 *Report of the Test Method Validation of Avian Acute Oral Toxicity Test (OECD test guideline 223) (2010)*

No.132 *Report of the Multi-Laboratory Validation of the H295R Steroidogenesis Assay to Identify Modulators (2010)*

No.133 *Peer Review Report for the H295R Cell-Based Assay for Steroidogenesis (2010)*

No.134 *Report of the Validation of a Soil Bioaccumulation Test with Terrestrial Oligochaetes by an International ring test (2010)*

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The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 31 industrialised countries in North and South America, Europe and the Asia and Pacific region, as well as the European Commission, meet to co-ordinate and harmonise policies, discuss issues of mutual concern, and work together to respond to international problems. Most of the OECD's work is carried out by more than 200 specialised committees and working groups composed of member country delegates. Observers from several countries with special status at the OECD, and from interested international organisations, attend many of the OECD's workshops and other meetings. Committees and working groups are served by the OECD Secretariat, located in Paris, France, which is organised into directorates and divisions.

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The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNEP, UNIDO, UNITAR, WHO and OECD. The World Bank and UNDP are observers. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

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FOREWORD

This document presents the report of validation of a soil bioaccumulation test with terrestrial oligochaetes by an international ring test.

Within the *OECD Guidelines for the Testing of Chemicals* relating to environmental fate, the Bioconcentration Flow-through Fish Test (TG 305) and the Bioaccumulation in Sediment-dwelling Benthic Oligochaetes (TG 315) were published in 1996 and 2008 respectively. Because of the difficulty to extrapolate aquatic bioaccumulation data to terrestrial organisms like earthworms, it turned out to be necessary to develop a soil compartment-specific bioaccumulation test. Soil-ingesting invertebrates are exposed to soil bound substances. Among these animals, terrestrial oligochaetes play an important role in the structure and function of soil.

The Test Guideline on Bioaccumulation in Terrestrial Oligochaetes, which was proposed by Germany, describes a method that consists of two phases: the uptake (exposure) phase and the elimination (post-exposure) phase. During the uptake phase, worms are exposed to soil which has been spiked with the test substance; for the elimination phase, the worms are transferred to a soil free of the test substance. The concentration of the test substance in/on the worms is monitored throughout both phases.

Ring testing was carried out in 2006-2007 with two chemicals using Lumbricid and Enchytraeid oligochaete species; fourteen laboratories from seven member and non-member countries participated. The ring test results and literature references are presented in chapters 1 to 11. Chapter 12 contains two Annexes: the draft test method on bioaccumulation in terrestrial oligochaetes (Annex 1) and the report of a workshop on ring testing of the terrestrial oligochaetes bioaccumulation test method that was held in May 2007 in Coimbra, Portugal (Annex 2).

The validation report was endorsed at the 22nd WNT Meeting on 23-25 March 2010, and the Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides, and Biotechnology agreed to its declassification on 19 July 2010.

Research and Development Project of the German Federal Environmental Agency

FKZ: 204 67 458

Technical Report:

Validation of a Soil Bioaccumulation Test with Terrestrial Oligochaetes by an International Ring Test

(Validierung einer Methode zur Messung der Bioakkumulation in terrestrischen Organismen mittels eines internationalen Ringtests)

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ON BEHALF
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Berichts - Kennblatt

1. Berichtsnummer UBA-FB 204 67 458	2.	3.
4. Titel des Berichts Validation of a Soil Bioaccumulation Test with Terrestrial Oligochaetes by an International Ring Test (Validierung einer Methode zur standardisierten Messung der Bioakkumulation mit terrestrischen Oligochaeten)		
5. Autor(en) (Name, Vorname(n)) Egeler, Philipp; Gilberg, Daniel; Scheffczyk, Adam; Moser, Thomas; Römbke, Jörg		6. Abschlussdatum 15. November 2007
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15. Zusätzliche Angaben		
16. Zusammenfassung/Kurzfassung Aufgabe des Vorhabens war die Organisation und Durchführung eines internationalen Ringtests mit dem vor einigen Jahren im Auftrag des Umweltbundesamtes entwickelten Bioakkumulationstest mit terrestrischen Oligochaeten. Dabei sollten bestehende Kenntnisse, insbesondere die UBA-Berichte F + E 206 03 909 (1997) und 298 64 416 (2001) berücksichtigt werden. Auf Basis der Ergebnisse eines Startworkshops wurde eine Prüfvorschrift erstellt, anhand welcher der Test bei den einzelnen Ringtestteilnehmern durchgeführt wurde. Insgesamt nahmen 14 Institutionen (Behörden, Universitäten, Auftragslabors und Industrie) aus 7 Ländern am Ringtest teil. Lumbriciden (*Eisenia fetida*, *E. andrei*) und Enchyträen (*Enchytraeus albidus*, *E. crypticus*) wurden als Testorganismen verwendet. Als Testsubstanzen wurden ^{14}C-markiertes Hexachlorobenzol (HCB) und Cadmiumchlorid ausgewählt. Die Einzeltests wurden je einmal pro Teilnehmer durchgeführt und bestanden aus einer Aufnahme- und einer Eliminationsphase. Insgesamt wurden 33 Tests durchgeführt und vollständig ausgewertet. Während des Ringtests wurden mehrere Workshops sowie ein praktisches Training der Teilnehmer organisiert. Die Aufnahme- und Eliminationskinetik der einzelnen Tests wurde mittels nichtlinearer Regressionsanalyse ausgewertet. Im Anschluss wurden in einer Gesamtschau die Vergleichbarkeit und Variabilität der Testergebnisse sowie zentrale Aspekte der Testmethodik ausgewertet und im Rahmen eines Workshops von den Ringtestteilnehmern diskutiert. Anhand dieser Auswertung wurden die am besten geeigneten Endpunkte und die Randbedingungen für ein adäquates Testdesign festgelegt sowie die vorläufigen Validitätskriterien überprüft. Nach Auswertung des Ringtests wurde ein Richtlinienentwurf entsprechend OECD-Format formuliert, der im Anschluss an das Vorhaben bei der OECD eingereicht werden kann.		
17. Schlagwörter Bioakkumulation, Boden, Oligochaeten, Testrichtlinie, Ringtest		
18. Preis	19.	20.

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12. Funding Agency (Name, Address) Umweltbundesamt, Wörlitzer Platz 1, 06844 Dessau		13. No. of Tables and Diagrams 31 14. No. of Figures 6
15. Supplementary Notes		
16. Abstract The aim of the project was the validation of a soil bioaccumulation test with endobenthic oligochaetes by an international ring test. The test method was developed taking into account existing methods (UBA-reports 206 03 909 (1997) and 298 64 416 (2001)). A test protocol was prepared and used to run the test. A total of 14 institutions (governmental, universities, contract laboratories and industry) from 7 different countries participated in the ring test. Lumbricids (*Eisenia fetida*, *E. andrei*) and enchytraeids (*Enchytraeus albidus*, *E. crypticus*) were used as test species. The test compounds were ^{14}C-labelled hexachlorobenzene (HCB) and cadmium chloride. The ring test runs were performed once by each laboratory, and consisted of an uptake and an elimination phase. Analysis of total radioactive residues and Cd-concentrations in soil, and tissue samples was performed. In total, 33 tests were performed, and evaluated. Several courses and workshops were organised to inform and train the participants. In a first step following practical ring-testing, the kinetic results of the single test runs were evaluated by nonlinear regression analysis. Additionally, comparability and variability of the results, as well as advantages and difficulties of the method were assessed and discussed by the ring test participants at a workshop. The outcome of the workshop was used to refine evaluation of the results and the test methodology, to define the most appropriate endpoints, the prerequisites for establishing a suitable test design and the validity criteria of this test. As a major outcome of the ring test, the draft test guideline, which closely follows formal requirements of the OECD test guidelines, was updated and prepared for submission to OECD`s Test Guideline Programme.		
17. Keywords Bioaccumulation, Soil, Oligochaetes, Test Guideline, Ring Test		
18. Price	19.	20.

Report Cover Sheet 06/2000

Contents Page

List of Tables Page

List of Figures Page

Definitions

Bioaccumulation is the increase in concentration of the test substance in or on an organism relative to the concentration of the test substance in the surrounding medium. Bioaccumulation results from both bioconcentration and biomagnification processes (see below).

Bioconcentration is the increase in concentration of the test substance in or on an organism, resulting exclusively from uptake via the body surface, relative to the concentration of the test substance in the surrounding medium.

Biomagnification is the increase in concentration of the test substance in or on an organism, resulting mainly from uptake from contaminated food or prey, relative to the concentration of the test substance in the food or prey. Biomagnification can lead to a transfer or accumulation of the test substance within food webs.

The elimination of a test substance is the loss of this substance from the test organism tissue by active or passive processes, that occurs independently of presence or absence of the test substance in the surrounding medium.

The bioaccumulation factor (BAF) at any time during the uptake phase of this bioaccumulation test is the concentration of test substance in/on the test organism (C_a in g kg^{-1} wet or dry weight) divided by the concentration of the substance in the surrounding medium (C_s as g kg^{-1} of wet or dry weight of soil). In order to refer to the units of C_a and C_s, the BAF has the units of g soil kg^{-1} worm.

The steady state bioaccumulation factor (BAF_{ss}) is the BAF at steady state and does not change significantly over a prolonged period of time, the concentration of the test substance in the surrounding medium (C_s as g kg^{-1} of wet or dry weight of soil) being constant during this period of time.

The biota-soil accumulation factor (BSAF) is the lipid-normalised steady state concentration of test substance in/on the test organism divided by the organic carbon-normalised concentration of the substance in the soil at steady state. C_a is then expressed as g kg^{-1} lipid content of the organism, and C_s as g kg^{-1} organic content of the soil.

A plateau or steady state is defined as the equilibrium between the uptake and elimination processes that occur simultaneously during the exposure phase. The steady state is reached in the plot of the BAF at each sampling period against time when the curve becomes parallel to the time axis and three successive analyses of BAF made on samples taken at intervals of at least two days are within 20% of each other, and there are no statistically significant differences among the three sampling periods.

Bioaccumulation factors calculated directly from the ratio of the soil uptake rate constant divided by the elimination constant kinetic rate constants (k_s and k_e, respectively - see below) are termed kinetic bioaccumulation factor (BAF_K).

The uptake or exposure phase is the time period during which the test organisms are exposed to the test substance.

The soil uptake rate constant (k_s) is the numerical value defining the rate of increase in the concentration of the test substance in/on the test organism resulting from uptake from the soil phase. k_s is expressed in g soil kg^{-1} of worm d^{-1}.

The elimination phase is the time period, following the transfer of the test organisms from a contaminated medium to a medium free of the test substance, during which the elimination (or the net loss) of the substance from the test organisms is studied.

The elimination rate constant (k_e) is the numerical value defining the rate of reduction in the concentration of the test substance in/on the test organism, following the transfer of the test organisms from a medium containing the test substance to a substance-free medium; k_e is expressed in d^{-1}.

The organic carbon-water partitioning coefficient (K_{oc}) is the ratio of the concentration of a substance in/on the organic carbon fraction of a soil and the concentration of the substance in water at equilibrium.

The octanol-water partitioning coefficient (K_{ow}; also sometimes expressed as P_{ow}) is the ratio of the solubility of a substance in n-octanol and water at equilibrium and represents the lipophilicity of a substance. The K_{ow} or its logarithm of K_{ow} (log K_{ow}) is used as an indication of the potential of a substance for bioaccumulation by aquatic organisms.

The conditioning period is used to stabilise the microbial component of the soil and to remove e.g. ammonia originating from soil components; it takes place prior to spiking of the soil with the test substance.

The equilibration period is used to allow for distribution of the test substance between the solid phase, and the porewater; it takes place after spiking of the soil with the test substance and prior to addition of the test organisms.

Artificial soil, or formulated, reconstituted or synthetic soil, is a mixture of materials used to mimic the physical components of a natural soil.

Spiked soil is soil to which test substance has been added.

Porewater or interstitial water is the water occupying space between sediment or soil particles.

1 INTRODUCTION

Within the EU, adopted bioaccumulation standardised guidelines exist only for fish up to now (OECD 1996). However, the extrapolation of fish bioaccumulation data to terrestrial organisms like earthworms is difficult, if possible at all. Therefore, model calculations based on a compound's lipophilicity (e.g., Connell & Markwell 1990) are currently used for the assessment of bioaccumulation of chemicals in the soil ecosystem, e.g. in the EU Technical Guidance Document (EC 2003). The need for a compartment-specific test method was already addressed more than a decade ago (e.g., Phillips 1993).

Several national guidelines address the issue of bioaccumulation in organisms other than fish, (e. g., ASTM International 2000, and U.S. EPA 2000). More importantly, a national test guideline describing the measurement of bioaccumulation from contaminated soils in earthworms (*Eisenia fetida*, Savigny) and potworms has recently been adopted (ASTM International 2004). The development, standardisation and implementation of an internationally accepted, standardised method for the experimental determination of bioaccumulation in spiked soil can therefore considerably improve the risk assessment for chemicals in terrestrial ecosystems (e.g., EPPO 2003, Füll et al. 2003).

In order to fill this gap, a standardisable test method to measure bioaccumulation of organic chemicals from spiked soil was developed on behalf of the German Federal Environmental Agency (Umweltbundesamt, UBA) Berlin, Germany (Bruns et al. 2001a). In addition, several studies were published recently dealing with the issue of bioaccumulation in terrestrial oligochaetes (e.g. Römbke et al. 1998, Sample et al. 1999, Jager et al. 2000, Bruns et al. 2001b, Conder & Lanno 2003, Didden 2003). Based on the method above, and taking into account literature and existing national guidelines (e.g. ASTM 2000 (draft; recently adopted: ASTM International 2004)), a draft guideline according to OECD format was formulated (Bruns et al. 2001c).

In order to validate this test method, an international ring test was performed in agreement with the main recommendations given in the "Guidance Document for the Development of OECD Guidelines for Testing of Chemicals, Environment Monographs No. 76" (OECD 2006) and the "Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment, Series on Testing and Assessment No. 34" (OECD 2005). Fourteen institutions (governmental, universities, contract laboratories and industry) from seven countries participated in the ring test.

The study was carried out based on the Draft Test Guideline: "Bioaccumulation: Soil Test Using Terrestrial Oligochaetes" (Egeler et al. 2005). The performance and documentation was done in general agreement with GLP-rules, but no formal certificate was required for the participating laboratories.

This report describes methods and results of the ring test. Based on the outcome of this ring test project, the draft guideline was revised and updated for submission to the OECD testing programme.

Discussion of results and data from the ring test (Workshop Minutes) as well as the revised draft guideline are included in the Annexes to this report.

2 ORGANISATION OF THE RING TEST

In the ring test presented in this report, the main recommendations of the OECD (OECD 2005, OECD 2006) were fulfilled.

2.1 Ring Test Participants

A total of 28 institutions were contacted, of which 15 agreed to participate to the ring test. One institution declined during the ring test, thus 14 institutions performed 33 tests in total.

Contact persons		Institution, Country
Monica	Amorim	Universidade de Aveiro, PT
Jitka	Bezchlebova	RECETOX, CZ
Gladys	Stephenson	Stantec Ltd., CDN
Sabine Pierre Yves	Dodard Robidoux	NRC, CDN
Kerstin Markus	Hund-Rinke Simon	Fraunhofer-IME, DE
Roman Michael	Kuperman Simini	U.S. Army, USA
Thorsten Annette	Leicher Hauger	BayerCropScience, DE
Tiago	Natal da Luz	Universidade de Coimbra, PT
Claudia	Norr	BBA, DE
Irina	Panchenko	Russian Acad. of Sciences, RUS
Juliska	Princz	Environment Canada, CDN
Kees	van Gestel	Vrije Universiteit Amsterdam, NL
Roman	Lanno	Ohio State University, USA
Philipp Thomas	Egeler Moser	ECT Oekotoxikologie GmbH, DE

2.2 Co-ordinating Institute

ECT Oekotoxikologie GmbH
Boettgerstr. 2 - 14
D-65439 Floersheim am Main
Germany

2.3 Monitoring

Dr. Gabriele Studinger
Umweltbundesamt
Fachgebiet IV 2.3
Wörlitzer Platz 1
06844 Dessau
Germany

2.4 Distribution of Materials

The test compounds including Certificate of Analysis and Safety Data Sheet, as well as peat (air-dried and ground) were used of one batch and were distributed to the ring test participants. Due to import restrictions, the participants located in North America worked with separate batches of peat powder.

The radiolabelled test compound was obtained and distributed from Institute of Isotopes Co., Ltd., 1121 Budapest, Hungary. Cadmium chloride hemipentahydrate was obtained and distributed by Sigma-Aldrich, Taufkirchen, Germany.

Those laboratories requiring starter cultures of *E. fetida* or *E. andrei* and/or *E. albidus* were provided with animals from ECT's culture, and with culture instructions. All other institutes worked with oligochaetes from their own in-house cultures.

The ring test protocol including addenda, excel files for calculating application rates as well as form sheets and excel files for the recording of raw data were also sent to all participants prior to start of the definitive tests.

3 ACKNOWLEDGEMENTS

The authors thank the German Federal Environmental Agency (UBA) for funding and supporting this ring test. Additionally, the authors wish to thank all participants of the ring test for their dedicated work during ring testing. Sonia Chelinho, Tjago Natal da Luz and Paulo Sousa of the Instituto do Ambentie e Vida, Dep. Zoologia da Universidade de Coimbra, (Coimbra Portugal) are thanked for hosting the final workshop. Acknowledgements are also due to Gabriele Studinger (UBA), for encouraging monitoring throughout the project.

4 PRACTICAL PERFORMANCE OF THE RINGTEST - TEST METHODS

The study was carried out based on the Draft Test Guideline: "Bioaccumulation: Soil Test Using Terrestrial Oligochaetes" (Egeler et al. 2005).

To determine the bioaccumulation of the test chemical, adult oligochaetes were exposed to soil spiked with the test chemical. Artificial and field soils were used as substrate. Test vessels without the addition of the test chemical served as controls. The soil was equilibrated for 4 days after application of ^{14}C-labelled hexachlorobenzene (3 weeks equilibration for cadmium). The test animals were exposed to the spiked, equilibrated soil for a period of up to 21 days to assess the bioaccumulation kinetics of the test chemical in the test organisms (uptake phase). After the uptake phase, the remaining test animals were exposed to control soil to eliminate accumulated test chemical for a period of up to 21 days (elimination phase). With respect to the low nutrient content of the soil, the worms were fed during the test with the same type of food as in the cultures (e.g. with finely ground dung (earthworms) or ground oat flakes (enchytraeids)). The food was mixed into the soil immediately before adding the test organisms.

Endpoints to be determined were 1. the bioaccumulation factor (BAF) as the ratio of the concentration of the test chemical in the test organisms and the concentration of the test chemical in the soil at the end of the uptake phase; 2. the soil uptake rate constant k_s and the elimination rate constant k_e; 3. the Non-Eliminated Residues (NER), which is the concentration of the test chemical in the test organisms at the end of the

elimination phase compared to the concentration of the test chemical in the test organisms at the end of the uptake phase.

Hexachlorobenzene was used in radiolabelled form (^{14}C) in order to facilitate the quantification of the test chemical (i.e. parent compound and its major metabolites) in soil and worm samples. Total radioactive residues were measured by liquid scintillation counting (LSC) following extraction, combustion or tissue solubilisation. Therefore, the bioaccumulation factor (BAF) is based on the parent compound including any retained metabolites. Additionally, and in a separate run under identical conditions, the percentage of radioactivity associated with the parent compound was quantified in aged OECD soil and earthworms at the end of the uptake phase.

Cadmium was used as cadmium chloride. The concentrations were measured as mg metal per kg dry soil, e.g. by atomic absorption spectroscopy.

The ring test protocol as it was distributed to the ring test participants and used during the ring test contained methods to be applied during practical performance in the ring test. It was optional to the ring test participants to apply other methods, e.g. for lipid and TOC determination, than the ones outlined in the protocol, as long as these alternative methods were summarised and referenced. In the following sections, the test method and procedures are summarised.

4.1 Test Organisms

The following test species were used in this ring test:

Earthworms (Lumbricidae, Oligochaeta):

Eisenia fetida Savigny 1826
Eisenia andrei Bouché 1972

Enchytraeids (Enchytraeidae, Oligochaeta):

Enchytraeus albidus Henle 1837
Enchytraeus crypticus Westheide & Graefe 1992

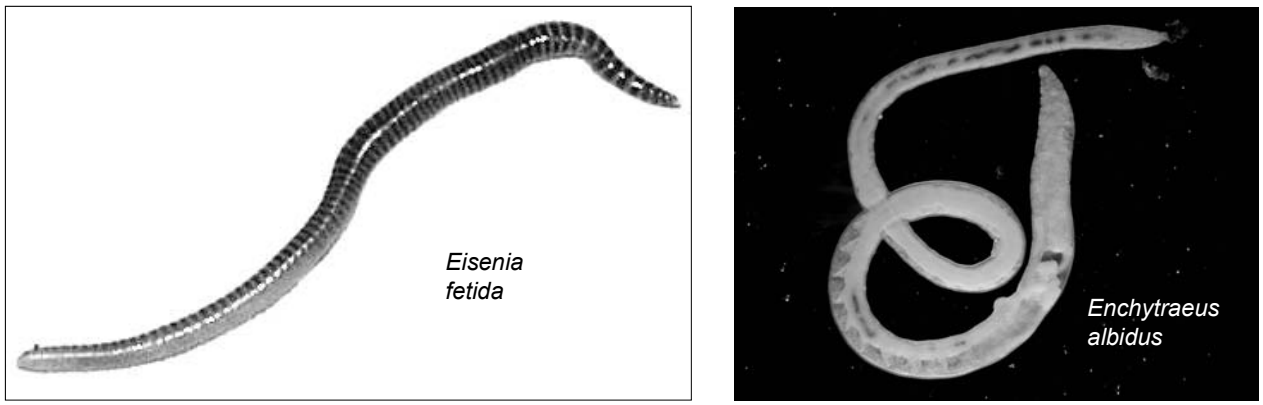


Figure 1: *Eisenia fetida* (left) and *Enchytraeus albidus* (right).

4.2 Culture Conditions

The participating laboratories used their regular culture conditions. Additionally, some recommendations for culturing of the oligochaete species used in the ring test are given in the draft guideline.

4.3 Soil

4.3.1 Artificial Soil

For the ring test, artificial soil was used in order to provide comparable test conditions between the laboratories. The composition of this artificial soil is is based on OECD Guideline No. 207 (OECD 1984); the percentages refer to the soil dry weight as shown in Table 1.

Table 1: Composition of the artificial soil.

Constituent	Characteristics	% of soil dry weight
Peat	Sphagnum peat without additives, no visible plant remains, air dried and finely ground (particle size ≤ 5 mm), degree of decomposition: "medium"	10
Quartz sand	Grain size: > 50% of the particles should be in the range of 50-200 µm	70
Kaolinite clay	Kaolinite content ≥ 30%	20
Calcium carbonate	$CaCO_3$, pulverised, analytical grade, in addition to dry soil	ca. 1
Food	in addition to dry soil: e.g. dung powder (earthworms): e.g. ground oat flakes (enchytraeids):	 2 0.4 - 0.5

The peat was air dried and ground to a fine powder. Then the peat was mixed with the kaolinite clay and the quartz sand. One batch of artificial soil was prepared, sufficient for control and treated soil, for uptake and elimination phase.

For spiking via quartz sand (see chapter 0), 10% of the total amount of quartz sand was to be retained for spiking. In this case, the control soil and the soil for the elimination phase was to be mixed with a corresponding amount of quartz sand before use.

The pH in soil was measured in a mixed sample in a 1 M solution of potassium chloride (KCl) or a 0.01 M solution of calcium chloride ($CaCl_2$) according to ISO-Norm 10390 (1994). If necessary, the pH of the final mixture was adjusted to 6 ± 0.5 with calcium carbonate ($CaCO_3$).

Thereafter the maximum water holding capacity (WHC) was measured (see chapter 0).

At least two days before spiking, the soil was moistened with deionised water (40 - 60% of WHC) for conditioning.

For spiking via water (see chapter 0), the soil was moistened with 40 - 60% of WHC minus the volume of application solution needed (e.g. moistened to 10 or 20% of WHC). After spiking the test item, the final soil moisture was adjusted.

The artificial soil to be used in the test was conditioned for four days (^{14}C-HCB), and three weeks (Cd) prior to spiking with the test chemical.

4.3.2 Field Soils

The performance of test with field soils was an option in this ring test. In the following, field (reference) soils used by individual labs are described. Characteristics are reported as provided by the respective laboratories.

LUFA standard soil

LUFA standard soil 2.2 was used in this ring test (Labs O2, O5). The properties of this reference soil are shown in Table 2.

Table 2: Typical characteristics of LUFA standard soil.

Standard Soil Type	**2.1**	**2.2**	**2.3**
Batch-No. (Sp=stored; F=field fresh)		SP223903	
Sampling Date		26.09.03	
Org. C in %	1,23 ± 0,30	2,26 ± 0,12	1,02 ± 0,17
Particles <0.02 mm in %	7,8 ± 1,4	15,7 ± 2,6	20,8 ± 2,6
pH-Value (0,01 M $CaCl_2$)	6,2 ± 0,7	5,8 ± 0,3	6,3 ± 0,4
Cation Exchange Capacity (mval/100 g)	8 ± 1	11± 2	10 ± 2
Particle Sizes According to German DIN (%)			
< 0,002 mm	3,6 ± 1,2	8,0 ± 1,1	9,1 ± 1,7
0,002 - 0,006 mm	1,5 ± 0,9	1,8 ± 1,3	2,5 ± 1,3
0,006 - 0,02 mm	2,5 ± 0,7	5,9 ± 1,4	9,3 ± 1,1
0,02 - 0,063 mm	6,5 ± 1,6	8,7 ± 2,7	20,1 ± 2,7
0,063 - 0,2 mm	27,4 ± 2,7	34,5 ± 2,4	30,9 ± 3,84
0,2 - 0,63 mm	55,9 ± 3,5	40,5 ± 2,2	25,6 ± 2,2
0,63 - 2,0 mm	2,6 ± 0,7	0,7 ± 0,1	2,4 ± 0,5
Soil Type	Sand (S)	loamy Sand (l'S)	loamy Sand (lS)
Particle Sizes According to USDA (%)			
< 0,002 mm	3,6 ± 1,2	8,0 ± 1,1	8,5 ± 1,4
0,002 - 0,05 mm	9,5 ± 2,2	14,9 ± 2,6	29,2 ± 3,2
0,05 - 2,0 mm	86,8 ± 1,9	77,1 ± 3,1	62,3 ± 4,1
Soil Type	Sand	loamy Sand	sandy Loam
Water Holding Capacity (g/100 g)	34,8 ± 5,3	48,6 ± 4,1	35,2 ± 3,4
Weight per Volume (g/1000 ml)	1390 ± 40	1148 ± 40	1335 + 85

Sassafras field reference soil

The US standard field soil "Sassafras field reference soil" was used in this ring test (Lab O8). The properties of this reference soil are shown in Table 3.

Table 3: Typical characteristics of Sassafras field reference soil; texture: sandy loam.

Sieved 5mm; air dried				Collected from the field: 15 Oct 2003					
FIELD ID code	**pH**	**OM**	**Sand**	**Silt**	**Clay**	**CEC**	**Conductivity**	**Mg**	
		%	%	%	%	cmol/kg	mmhos/cm	mg/kg	
SSL1	4.9	0.7	73.9	10.6	15.5	6.9	0.09	120	
SSL2	5.4	0.7	74.1	9.4	16.6	7.0	0.09	122	
SSL3	5.4	0.7	74.4	9.3	16.3	6.9	0.08	120	
SSL4	5.4	0.7	74.9	8.0	17.1	7.2	0.09	122	
MEAN	**5.3**	**0.7**	**74.3**	**9.3**	**16.4**	**7.0**	**0.09**	**121.0**	
STD ERR	0.13	0.00	0.22	0.53	0.34	0.07	0.003	0.58	
	P	**K**	**Ca**	**N (NO_3)**	**N (NH_4)**	**Mn**	**Zn**	**Cu**	**Fe**
	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
SSL1	6	98	639	1.5	5.31	3.1	2.65	1.51	79.5
SSL2	4	99	643	1.4	5.50	2.0	2.01	0.88	73.7
SSL3	4	101	629	1.4	4.44	1.7	2.09	0.88	72.1
SSL4	5	101	645	3.0	6.68	1.8	2.87	0.88	72.8
MEAN	**4.8**	**99.8**	**639.0**	**1.8**	**5.5**	**2.2**	**2.4**	**1.0**	**74.5**
STD ERR	0.48	0.75	3.56	0.39	0.46	0.32	0.21	0.16	1.69
Collected from the field: 17 Aug 2004									
Field ID code	**pH**	**OM**	**Sand**	**Silt**	**Clay**	**CEC**	**Conductivity**	**Mg**	
		%	%	%	%	cmol/kg	mmhos/cm	mg/kg	
1BINFRONT	4.6	2.4	67.1	18.4	14.5	10.0	0.48	78	
2BINMID	4.4	1.9	70.8	16.2	13.0	9.5	0.49	75	
3BINREAR	4.5	2.0	67.4	18.4	14.2	9.8	0.50	78	
4DRUMLEFT	4.3	1.9	72.9	13.7	13.3	10.0	0.48	73	
5DRUMMID	4.3	1.9	71.6	15.2	13.2	9.4	0.48	73	
6DRUMRIGHT	4.3	2.0	71.8	14.3	13.9	9.1	0.44	71	
MEAN	**4.4**	**2.0**	**70.3**	**16.0**	**13.7**	**9.6**	**0.48**	**75**	
STD ERR	0.05	0.08	0.99	0.82	0.25	0.15	0.01	1.2	
Field ID	**P**	**K**	**Ca**	**N (NO_3)**	**N (NH_4)**	**Mn**	**Zn**	**Cu**	**Fe**
	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
1BINFRONT	9	125	779	88.6	10.2	10.97	8.29	6.59	111.94
2BINMID	11	121	571	83.4	13.2	10.26	8.39	5.69	93.34
3BINREAR	10	125	635	87.4	12.6	10.7	8.58	5.61	93.9
4DRUMLEFT	11	112	555	85.6	12.7	11.63	8.69	7.71	103.86
5DRUMMID	11	115	557	82.1	12.9	11.53	8.44	7.29	102.67
6DRUMRIGHT	10	106	501	78.2	12.8	11.39	9.06	7.59	101.87
MEAN	**10.3**	**117.3**	**599.7**	**84.2**	**12.4**	**11.1**	**8.6**	**6.7**	**101.3**
STD ERR	0.3	3.1	39.9	1.6	0.4	0.2	0.1	0.4	2.8

Mediterranean field soils

A subset of test runs (Lab O9) was conducted with two mediterranean field soils (LIT and LUV). The characteristics of these soils are shown in Table 4.

Table 4: Typical characteristics of the Mediterranean field soils in comparison with parameters measured for OECD artificial soil.

Method	LIT	LUV	OECD soil	Units
pH (H_20)	5.23	5.5		pH (H_2O)
pH (KCl)	4.55	4.35	6.1	pH(KCl)
Organic matter	4.2	2	5.8	%
P	25	90	n.d.	ppm P_2O_5
K	122	78	n.d.	ppm K_2O
Ca ext.(ammonium acetate)	453	515	n.d.	ppm Ca
Mg ext.(ammonium acetate)	95	115	n.d.	ppm Mg
Coarse sand	41.90	29.78	n.d.	%
Fine sand	24.82	38.23	n.d.	%
Sand	66.72	68.01	86.60	%
Silt	21.55	20.29	2.70	%
Clay	11.73	11.28	10.69	%
Texture	Sandy-Loam	Sandy-Loam	Loamy sand	
Mineral N	49	18	n.d.	ppm
Total N	0.16	0.08	n.d.	%
C/N	15.25	14.50	n.d.	
Exch. Ca	2.98	3.23	n.d.	meq Ca/100 g
Exch. Mg	0.99	1.18	n.d.	meq Mg/100 g
Exch. K	0.21	0.12	n.d.	meq K/100 g
Exch. Na	0.05	0.23	n.d.	meq Na/100 g
Cation Exchange Capacity (CEC)	8.64	9.92	n.d.	meq/100 g
Sat.	48.9	48.7	n.d.	%sat
Na	73	15	n.d.	ppm Na
Total Cu	40	23	20	ppm Cu
Total Fe	46370	30900	2730	ppm Fe
Total Mn	211	392	12	ppm Mn
Total Zn	67	54	15	ppm Zn
Total Cd	<5.6	<5.6	0.1	ppm Cd
Total Cr	21	24	8	ppm Cr
Total Pb	16	19	10	ppm Pb
Total Co	<24	<24	0.12	ppm Co
Total Ni	48	28	3	ppm Ni
Organic Carbon	2.44	1.16	3.92	%
WHC	42.38	32.05	61.3	%

4.4 Determination of Soil pH, Moisture and Organic Carbon Content (TOC)

A defined quantity of soil was dried at room temperature for at least 12 h. The pH was measured according to ISO (1994).

Samples of control soil were analysed for total organic carbon content (TOC) by the following procedures, which are based on DIN (1985) and ISO (1993). Four aliquots of control soil (approx. 10 ± 2 g wet weight) were to be weighed to the nearest 0.1 g, and dried overnight at 105 ± 2°C in a drying cabinet. After cooling in a desiccator for 1 - 2 h, the samples were weighed again.

The water loss was calculated by subtracting the dry weight of the sample from the wet weight of the sample. The relative moisture was calculated by dividing the water loss by the sample dry weight and expressed in % of soil dry weight [1].

$$[1] \qquad \text{Relative Moisture [\% of soil dw]} = \frac{\text{water loss of sample [g]}}{\text{dry weight of sample [g]}} \cdot 100$$

Subsequently, the TOC was determined, e.g. by the following procedure, which is based on DIN (1985): A portion of each of the dried soil samples was transferred to pre-heated, pre-weighed porcelain crucibles, and weighed. HCl (4 mol/L) was mixed in excess with the specimens to remove carbonate bound carbon. After 2 - 4 h the specimens were dried overnight at 60 ± 2°C in a drying cabinet. The specimens were weighed after cooling in a desiccator for 1 - 2 h and ashed in a muffle furnace at 550 ± 10°C for 1 - 2 h. After cooling in a desiccator for 1 - 2 h, the specimens were weighed again.

The weight loss (loss on ignition, LOI) of the soil was calculated by subtracting the ash weight of the specimen from the dry weight of the specimen [2] and reported in percent of soil dry weight.

$$[2] \qquad \text{LOI [g]} = \text{dry weight of specimen [g]} - \text{ash weight of specimen [g]}$$

The weight loss was corrected for kaolin-bound structural water (typically 10 - 12% of kaolin dry weight) being evaporated during combustion. The structural water content of the batch of kaolin actually used in the test was used for this correction.

$$[3] \qquad \text{Kaolin - bound structural water [g]} = \text{dry weight kaolin [g]} \cdot \text{fraction of structural water}$$

The resulting corrected LOI [4] was divided by the dry weight of the specimen, and multiplied by 100 to result in the corrected LOI in percent of dry weight of the specimen [5].

$$[4] \qquad \text{Corrected LOI [g]} = \text{LOI [g]} - \text{kaolin - bound structural water [g]}$$

$$[5] \qquad \text{Corrected LOI [\%]} = \frac{\text{Corrected LOI [g]}}{\text{dry weight of specimen [g]}} \cdot 100$$

The TOC of the specimen was calculated by dividing the corrected LOI in percent of dry weight by the factor 1.72.

$$[6] \qquad \text{TOC [\%]} = \frac{\text{Corrected LOI [\%]}}{1.72}$$

The TOC is reported in % of soil dry weight.

4.5 Determination of Soil Water Holding Capacity (WHC)

Samples of control soil were used to determine the water holding capacity (WHC) according to ISO (1992). The water holding capacity of the soil was calculated and reported in percent of dry weight.

$$WHC = \frac{\text{water content in soil [g] (difference between wet and dry soil)}}{\text{dry soil [g]}} * 100$$

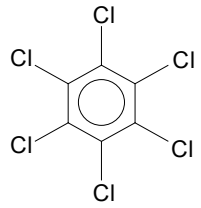
5 TEST SUBSTANCES

5.1 Hexachlorobenzene (HCB)

Hexachlorobenzene (HCB) was chosen as a test substance in this ring test. It served as a model of chlorinated hydrocarbons and nonpolar narcotics. Its lipophilicity (log K_{ow}: 5.73; De Bruijn et al., 1989) and its resistance to be metabolised in biota were the main reasons for using HCB as a model substance in this present study. Furthermore, a large ecotoxicological data-set exists for HCB (see e.g. IUCLID, ECB 2000). Since this ring test was a performance-based project which focused on the test method itself, the selection of HCB was deemed to minimise the occurrence of unexpected results elicited by the test substances properties. The ^{14}C-labelled test compound was obtained from Institute of Isotopes Co., Ltd., 1121 Budapest, Hungary.

The information below was provided by the supplier if not referenced otherwise.

- Chemical name (IUPAC): UL-^{14}C-hexachlorobenzene
- CAS number: 118-74-1
- Structure:



- Empirical formula: C_6Cl_6
- Molecular weight: 284.78
- Batch No.: CC-357
- Radiochemical Purity: 98.96%
- Specific radioactivity 24.49 mCi/mmol
- Appearance: white crystalline solid
- Solubility: 0.006 mg/L in water at 20°C (Verschueren 1996)
- Henry coefficient (20°C) 10.32 Pa*m^3/mol (ARS/U.S.DA, 1995)
- K_{oc} (Sediment) 10800 - 1200000 (Rippen, 1991)
- log K_{ow} 5.73 (De Bruijn et al., 1989)

5.1.1 Method of Spiking

A stock solution of ^{14}C-HCB was prepared by dissolving an appropriate aliquot of the original solution in cyclohexane. The stock solution was used immediately or stored in a gas-tight container in the freezer.

The stock solution was spiked to an appropriate portion of the quartz sand or natural soil (10% of the total amount of quartz sand or natural soil needed). The solvent-volume used for application was 0.25 mL per g quartz sand or natural soil (e.g. for 500 g total amount of quartz sand, 50 g quartz sand were spiked with 12.5 mL of stock solution). The concentration of the stock solution was chosen to achieve a final nominal concentration of approx. 15000 dpm/g soil dw (dpm: disintegrations per minute). The solvent was evaporated to dryness under a fume hood before mixing the spiked fraction into the soil. Total ^{14}C-radioactivity was determined in the quartz sand after evaporation of the solvent by liquid scintillation counting (LSC).

The spiked soil fraction (quartz sand or natural soil) was then mixed with the soil to achieve the desired concentration level. The spiked soil was thoroughly mixed to ensure that the test chemical added to soil is evenly distributed within the soil. The control soil and the soil for the elimination phase received a corresponding amount of quartz sand before use. The concentration of the test chemical based on total ^{14}C-radioactivity was determined in the spiked soil according to section 0. Sampling procedures are described in section 0.

The spiked soil was stored during the equilibration period (see section 0).

5.1.2 Test Apparatus

When testing ^{14}C-HCB, contamination of ambient air by evaporating radioactivity should be avoided. In order to minimise contamination of ambient air, the replicate test chambers were placed under a fume hood, or incubated in a closed box equipped with borings for air supply and for exhaust air (see example in 0). In this latter case, a vacuum pump removed the exhaust air from the incubation box and subsequently pass it through a set of ice-cooled washing bottles. The first bottle was empty to retain evaporated water. The following two bottles contained triethylene glycol (TEG) for trapping evaporated volatiles. Two washing bottles were filled with KOH or NaOH (10%) to absorb $^{14}CO_2$ that might be produced from metabolic breakdown of the test chemical.

5.1.3 Radio-chemical Analysis

Samples were to be taken and analysed during the application procedure for each batch of spiked soil:

- from the stock solution
- from the application solution
- from the spiked soil fraction (quartz sand or natural soil): after solvent has evaporated (only for ^{14}C-HCB)
- from the spiked soil: approximately 1 h after application
- from the control soil: approximately 1 h after application

The quantification limit (LOQ) for ^{14}C Hexachlorobenzene was approximately equivalent to twice the background values for each sample matrix.

Soil Samples

The dried soil sample of each replicate was thawed, placed into a cellulose combustion cone (e.g. CombustoCone, PerkinElmer Life Sciences) and combusted in a sample oxidizer to comprise extractable and non-extractable ^{14}C-activity, and measured by LSC.

Worm Samples

Enchytraeids: For determination of total ^{14}C-activity, worm samples were thawed, mixed with 1 mL of tissue solubiliser (e.g. Soluene, PerkinElmer Life Sciences), heated for approx. 1 hour at 60°C and thereafter cooled down to room temperature, neutralised with 100 µL HCl, mixed with LSC-cocktail (e.g. Hionic Fluor, PerkinElmer Life Sciences), and measured by LSC.

Earthworms: Worm samples of each replicate were thawed, placed into a cellulose combustion cone (e.g. CombustoCone, PerkinElmer Life Sciences) and combusted in a sample oxidizer to comprise extractable and non-extractable ^{14}C-activity; 14CO2 was trapped in an absorbing agent (CarboSorb, PerkinElmer Life Sciences), mixed with LSC-cocktail (e.g. Permafluor, PerkinElmer Life Sciences), and measured by LSC.

Trapping Solutions

Water and TEG samples were mixed with LSC-cocktail (e.g. Ultima Gold, PerkinElmer Life Sciences). KOH or NaOH samples were mixed with LSC-cocktail (e.g. Hionic Fluor). Afterwards, they were analysed for total ^{14}C-activity by LSC. The amount of trapped ^{14}C-activity was calculated for the total volume of the content in the washing bottles.

Recovery Samples

In order to assure the validity of the analytical results, control test soil and worm samples were fortified with the test substance in the range between the quantification limit and the test concentration, and analysed once before start of analytical procedures.

The average recovery in fortified samples was to be within the range of 90 - 110% with a standard deviation of less than 20% to be considered as acceptable. At least two recoveries were performed per sample matrix.

Quantification of parent compound in worm and soil extracts

Additionally, samples of spiked, aged OECD soil, and earthworms from a selected test run (O5) were extracted with n-hexane, and analysed for the percentage of radioactivity associated with parent compound (PRP) by thin layer chromatography (TLC) and subsequent radioactivity scan (Berthold Digital Autoradiograph, Laboratorium Dr. Berthold, Wildbad, Germany).

Table 5: Extraction methods and TLC conditions for ^{14}C-HCB in worm and soil samples.

worm samples	**soil samples**
– solvent: hexane – four times extraction with 10 mL using ultra turrax	solvent : hexane double extraction with 10 mL using vortex mixer
– centrifugation, determination of volume and activity in the supernatant – evaporation of hexane using nitrogen stream	centrifugation, determination of volume and activity in the supernatant evaporation of hexane using nitrogen stream
– filtration of the extract using PTFE-filter	
TLC conditions	
TLC-plate Silica gel 60 F254 size 20*20 cm glass sheet with concentration zone	
solvent system: chloroform/acetone (80/20 v/v)	

5.2 Cadmium

Cadmium was selected as a representative of heavy metals. Cadmium chloride hemipentahydrate was obtained by Sigma-Aldrich, Taufkirchen, Germany at the following specifications.

- Chemical name (IUPAC): cadmium chloride hemipentahydrate
- CAS number: 7790-78-5
- Empirical formula: $CdCl_2$ x 2.5 H_2O
- Molecular weight: 228.36
- Batch No.: 448298/1
- Purity: 100%
- Appearance: colourless crystalline solid
- Solubility in water: 140 g/100 mL

5.2.1 Method of Spiking

The artificial soil or field soil was prepared according to section 0 taking into account the amount of water required for spiking the test chemical (e.g. approx. half of the final water content or 10% of the soil dry weight). The target final water content was 40% to 60% of the maximum WHC. The stock solution of the test chemical was spiked to the pre-moistened soil. The concentration of the stock solution was chosen to achieve a final nominal concentration of 5 mg cadmium /kg dry soil. An aliquot of the stock solution was stored as a reserve; the concentration of this stock solution was only determined if later measurements showed unexpected results.

The spiked soil was thoroughly mixed to ensure even distribution of the test chemical within the soil. Thereafter, samples were taken for determination of Cd concentration in the spiked soil according to section 0.

The spiked soil was stored during the equilibration period (see section 0).

5.2.2 Chemical Analysis

Samples from the tests were analysed for total amount of cadmium by Atomic Absorption Spectometry (AAS). One lab used inductively-coupled plasma mass spectrometry (ICO-MS) for Cd-analysis. The following samples were taken according to the ring test protocol:

- stock solution (reserve)
- spiked soil: approximately 1 h after application
- control soil: approximately 1 h after application
- worm and soil samples during uptake and elimination phase.

In general, the samples were processed and analysed for metal concentration according to the methods available to analysing laboratories. The cadmium residues in each sample were determined by comparison to a calibration curve consisting of at least 3 points (duplicate determination) or 5 points (single determination). The lowest point of the calibration curve was to correspond to 70% or less of the determination limit of the method.

Demonstration of the validity of the analytical method and results were performed by each participating laboratory. For example, control test soil and worm samples were fortified with the test item in the range of the quantification limit to the highest test concentration of the biological part, and analysed with each set of samples.

The average recovery should be within the range of 70 - 110% with a standard deviation of less than 20 % to be considered as acceptable. At least two recoveries were performed per sample matrix.

Soil and Worm Samples

The soil and worm samples for metal analysis should be stored dried (105°C) or freeze-dried until analysis according to the ring test protocol. Note: Weighing of dried worms appeared to be problematic especially for enchytraeid samples because of hygroscopic weight increase and electrostatic charge. Additionally, worm tissue tended to stick to vessel walls and was difficult to remove when dried. Worms should therefore be stored frozen before analysis, not dried in an oven.

Samples for analysis were digested using a mixture of concentrated HNO_3 and HCl (4:1) for soil and earthworm samples, and a mixture of concentrated HNO_3 and $HClO_4$ (7:1) for enchytraeid samples.

Analysis was performed by Atomic Absorption Spectrometry (AAS) at a wavelength of 228.9 nm. The quantification limit (LOQ) for cadmium in soil was 7 µg/l or 0.32-0.7 µg/g dry soil as reported by lab O15. For earthworm samples, the LOQ was 7 µg/l or approx. 1 µg/g dry earthworm tissue. The detection limit for enchytraeid samples was 0.224 µg/l or 0.025 µg/g dry sample.

5.3 Equilibration and Addition of Food

After spiking, the spiked soil was stored in one container for equilibration. The equilibration period for ^{14}C-HCB-spiked soil was 4 days, while cadmium-spiked soil was equilibrated for 3 weeks. The equilibration period ended on day 0 of the uptake phase.

The container with the spiked soil was incubated for equilibration under test conditions (i.e., at 20 ± 2°C, and a light regime of 16:8 (light:dark) with a light intensity of 100 - 1000 lx). After the equilibrium period the

moisture of the soil was controlled by reweighing the vessel without test organisms and food, and - if necessary - adjusted to the initial value by adding deionised water.

The control soil was treated in the same manner as the spiked soil.

After the equilibration period, the food source was added to the soil. The worms were fed with the same type of food as in the cultures.

Example: For earthworms, 20 mg dried dung per g soil dry weight were recommended; for enchytraeids, 4 - 5 mg of ground oat flakes per g soil dry weight were recommended.

If other food sources were to be used in the test, the concentration of food per g dry soil was to be chosen so that the biomass of the test organisms did not decrease, and that the food or substrate did not get moldy.

The food source was thoroughly and evenly distributed within the soil. The type and quantity of food applied (in g/kg dry soil) was reported by each participating laboratory.

Soil samples were taken (3 spiked and 4 control samples) for analysis of the concentration of the test chemical in soil after equilibration.

The spiked, equilibrated soil (with food source) was then distributed to the replicate test vessels.

5.4 Measurement Endpoints

Parameters to be determined were the concentrations of the test chemical (^{14}C-HCB and Cd) in soil and worms over the time course of uptake and elimination phase. Endpoints to be determined were the bioaccumulation factor (BAF) as the ratio of the concentration of the test chemical in the test organisms and the concentration of the test chemical in the soil at the end of the uptake phase, and the concentration of the test chemical in the test organisms at the end of the elimination phase compared to the concentration of the test chemical in the test organisms at the end of the uptake phase. Additionally, the kinetics of uptake and elimination including the corresponding rate constants were to be calculated.

6 EXPERIMENTAL PROCEDURES AND METHODS

6.1 General Aspects: Test Vessels, Loading, Replicates

The test was conducted in glass, plastic or stainless steel vessels. The vessels were to be appropriately covered to prevent escaping of the worms, while allowing sufficient air supply (e.g. by a perforated aluminium foil). The target amount of soil in the vessels was a layer of 2 - 3 cm (enchytraeids) or 4 - 5 cm (earthworms) of soil prepared as described above.

For example, for *Eisenia* sp., a loading rate of 3 - 5 mg of worm tissue (wet weight) per gram of wet soil was recommended. This corresponds to a loading rate of 1 mg of worm tissue (dry weight) per 1.25 g of soil (dry weight) calculated for *Eisenia* sp. and artificial soil. Values for the other species were to be selected accordingly.

In general, a minimum of 3 replicates containing spiked soil was prepared for each sampling date.

At least 3 x 4 control replicates were prepared for sampling at the start and end of the uptake phase, and at the end of the elimination phase, respectively.

In cases where worms with and without gut content were analysed at the end of the uptake phase and/or at start of the elimination phase (e.g. lab O5), the corresponding number of additional replicate test vessels was prepared.

If dry weight and lipid content were to be determined additionally at the end of the uptake phase, the corresponding number of additional control vessels was prepared.

A set of at least 3 replicates containing spiked soil was to be prepared as a reserve.

All vessels were weighed on day 0 after addition of worms.

6.2 Test Set-up

For tests with ^{14}C-HCB, the vessels were assembled either under a fume hood, or in a gas-tight box, or covered by a lid. Figure 2 shows an example of a test setup used for radiolabelled test compounds.

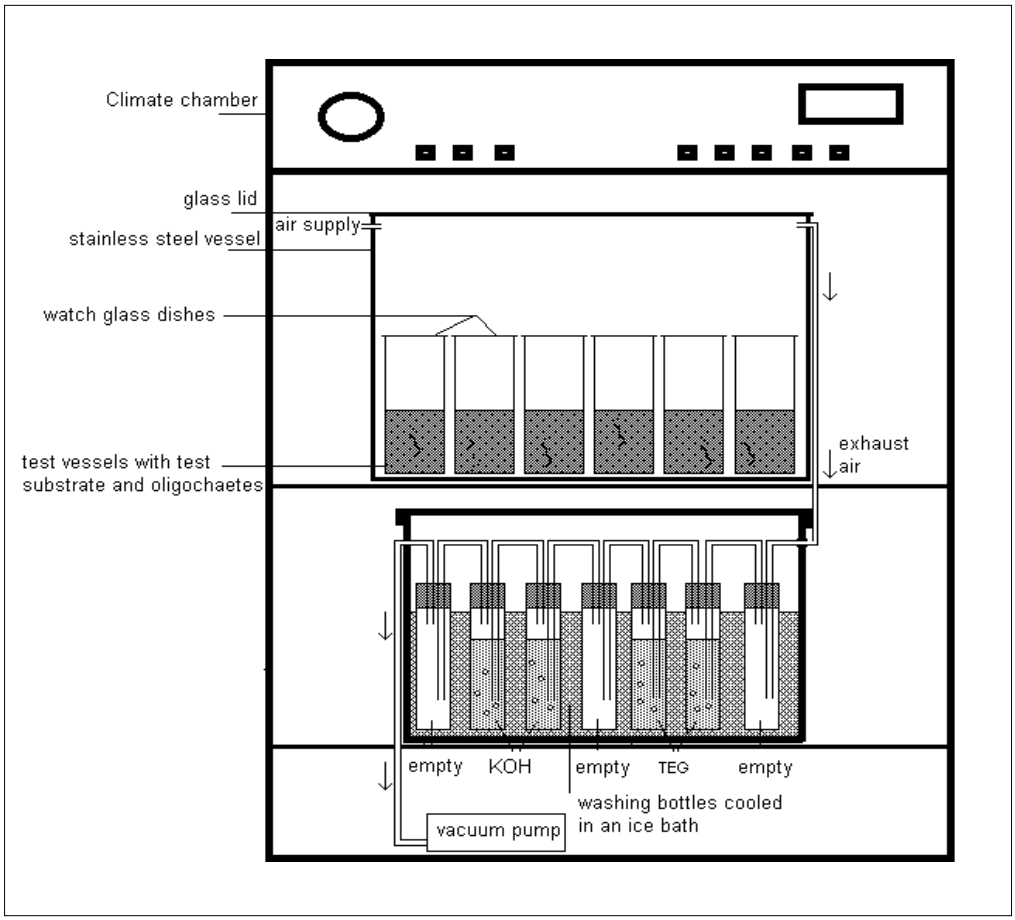


Figure 2: Test apparatus, schematic setup (from Bruns et al. 2001a).

For tests with cadmium chloride, a specific test set-up was not required.

6.3 Addition of the Test Organisms

The test organisms were separated from the cultures (24 - 72 h) before start of the uptake phase for acclimation in control soil under test conditions.

Adding the test organisms (day 0 of the study):

Earthworms	**Enchytraeids**
Adult animals with clitellum of similar weight (250 - 600 mg), from the same source were separated from the acclimatisation chamber. The worms were rinsed with water, and excess water was removed from the worms by gently blotting them dry on moist filter paper using soft steel forceps. The worms were weighed to the nearest 1 mg and were distributed randomly to pre-weighed test vessels; each test vessel typically contained one worm.	Adult animals with clitellum of similar weight/size from the same source were separated from the acclimatisation chamber. The worms were distributed randomly to vessels (e.g. petri dishes) containing e.g. reconstituted water by adding groups of 2 worms to each vessel, until each vessel contained the desired number of worms (typically 20 worms per vessel). Each of these groups of worms was randomly transferred to separate test vessels.

The test vessels including worms were weighed to the nearest 1 mg for later moisture control. The test vessels were subsequently incubated under test conditions. The test vessels, and the controls were observed on a regular basis in order to assess visually any behavioural differences (e.g. burrowing behaviour/soil avoidance) of the test organisms compared with the control animals.

6.4 Duration of Uptake and Elimination Phase

The uptake phase was run for 21 days with earthworms, and for 14 days with enchytraeids.

The elimination phase was started on the day the uptake phase ended. Then, exposed worms from the remaining spiked replicates were transferred to control soil. The elimination phase was terminated after 21 d for earthworms, and after 14 d for enchytraeids. A typical sampling schedule is shown in Table 6.

Examples of activity schedules for the uptake and elimination phase are given in the draft guideline in the Annex.

Table 6: Typical sampling schedule for earthworms and enchytraeids.

Earthworms			samples			
			controls		treated	
uptake		day	worms	soil	worms	soil
Mo	0	**(0)**	4	4		3
Tu	1	**1**			3	3
Wed	2	**2**			3	3
Thu	3	3				
Fr	4	**4**			3	3
Sat	5	5				
Sun	6	6				
Mo	7	**7**			3	3
Tu	8	8				
Wed	9	9				
Thu	10	**10**			3	3
Fr	11	11				
Sat	12	12				
Sun	13	13				
Mo	14	**14**			3	3
Tu	15	15				
Wed	16	16				
Thu	17	**17**			3	3
Fr	18	18				
Sat	19	19				
Sun	20	20				
Mo	21	**21**	4	4	3	3
elimination						
Mo	21.25	**0.25**			3	3
Tu	22	**1**			3	3
Wed	23	**2**			3	3
Thu	24	3				
Fr	25	**4**			3	3
Sat	26	5				
Sun	27	6				
Mo	28	**7**			3	3
Tu	29	8				
Wed	30	9				
Thu	31	**10**			3	3
Fr	32	11				
Sat	33	12				
Sun	34	13				
Mo	35	14				
Tu	36	**15**			3	3
Wed	37	16				
Thu	38	17				
Fr	39	18				
Sat	40	19				
Sun	41	20				
Mo	42	**21**	4	4	3	3
samples			**12**	**12**	**48**	**51**
test vessels				**8**		**48**

			samples			
Enchy-traeids			controls		treated	
uptake		day	worms	soil	worms	soil
Mo	0	**(0)**	4	4		3
Tu	1	**1**			3	3
Wed	2	**2**			3	3
Thu	3	3				
Fr	4	**4**			3	3
Sat	5	5				
Sun	6	6				
Mo	7	**7**			3	3
Tu	8	8				
Wed	9	**9**			3	3
Thu	10	10				
Fr	11	**11**			3	3
Sat	12	12				
Sun	13	13				
Mo	14	**14**	4	4	3	3
elimination						
Mo	14.25	**0.25**			3	3
Tu	15	**1**			3	3
Wed	16	**2**			3	3
Thu	17	3				
Fr	18	**4**			3	3
Sat	19	5				
Sun	20	6				
Mo	21	**7**			3	3
Tu	22	8				
Wed	23	9				
Thu	24	**10**			3	3
Fr	25	11				
Sat	26	12				
Sun	27	13				
Mo	28	**14**	4	4	3	3
samples			**12**	**12**	**42**	**45**
test vessels				**8**		**42**

Note: Bold figures represent sampling dates. On day 0 of the uptake phase, spiked and control soil samples (for confirmation of test concentration, analytical background, and for dry weight and TOC measurements) and control worm samples (for analytical background, dry weight, and lipid measurements) were taken. For the samples taken on day 0 of the uptake phase, no test vessels are necessary.

In selected test runs, worms with and without gut content were analysed at the end of the uptake phase; in these cases, a corresponding number of additional replicate vessels was prepared.

6.4.1 Maintenance during the exposure period

Daily on weekdays
Worm behaviour (e.g. burrowing behaviour/soil avoidance) was checked visually. If a closed test system was used, aeration was checked, and adjusted, if necessary.

Once per week
The moisture of the soil was controlled by re-weighing each test vessel and if necessary adjusted to the initial value with deionised water.

Measurement of at least the following parameters was required:
- temperature in test room (at least at start and end of uptake and elimination phase, preferably online measurement at least once daily)
- control and adjustment of test vessel weight (at start of uptake and elimination phase, and once per week)
- soil pH (at start and at end of uptake and elimination phase)
- water holding capacity (for each batch of soil)
- soil dry matter and soil moisture (for each batch of soil, and at start of uptake and elimination phase)
- soil total organic carbon (for each batch of soil)
- worm dry weight to wet weight ratio (at least at start of uptake phase)
- lipid in worms (at least at start of uptake phase).

6.4.2 Sampling Procedures

Worms
In general, the worms were rinsed immediately after sampling to remove soil particles. The adhering water was removed carefully without injuring the worms, e.g. by blotting them on a pre-moistened filter prior to weighing.

The contents of each test chamber were poured onto a stainless steel tray; the worms including any cocoons and juveniles were collected with soft steel forceps, counted and transferred to a petri dish containing e.g. reconstituted water.

Earthworms	**Enchytraeids**
were rinsed, placed on a moist filter paper to allow gut-purging (overnight). Thereafter the worms were transferred to pre-weighed containers (LSC-vials), weighed (to the nearest 1 mg), and killed by freezing.	were rinsed, placed on a pre-moistened paper tissue to remove excess water, transferred to pre-weighed LSC-vials, weighed (to the nearest 1 mg), and killed by freezing.

The worm samples for metal analysis were stored dried (105°C) or freeze-dried until analysis. The worm samples for analysis of ^{14}C-HCB were stored frozen until analysis.

Soil
After collection of the worms, the soil of a given replicate was mixed, and for each replicate subsamples of approximately 0.5 g soil wet weight were taken (approximately 2 g soil wet weight for cadmium analysis). The samples were transferred to pre-weighed LSC-vials, and weighed to the nearest 1 mg to determine the wet weight; afterwards the weighed samples were air-dried at room temperature overnight under a fume hood, weighed (to the nearest 1 mg) and processed or stored in the freezer. The soil samples for metal

analysis were stored dried (105°C) or freeze-dried in appropriate containers (e.g. paper envelopes) until analysis.

When required by the schedule, soil samples for pH measurement were also taken.

Trapping solutions (for ^{14}C-HCB test with closed system only)
At least at the end of the uptake phase, the total volume of solution was recorded for each washing bottle. 2-5 mL samples of each of the trap contents were taken and directly processed as described in section 0.

6.4.3 Worm Dry Weight

Samples of acclimatised control worms were used to determine the dry weight of the test organisms. At least 3 (preferably 4) aliquots of control worms were transferred to dried, pre-weighed and labelled weigh boats (one weigh boat per replicate), weighed to the nearest 1 mg, killed e.g. by deep-freezing, and dried overnight at 105 ± 2°C in a drying cabinet. After cooling in a desiccator for 1 - 2 h, the samples were weighed again to determine the worm dry weight.

The worm dry-to-wet weight ratio and the water content of the worms were calculated for each aliquot:

dry-to-wet weight ratio = dry weight of sample [mg] / wet weight of sample [mg]

$$\text{water content in \% of wet weight} = \frac{(\text{wet weight - dry weight})\,[\text{g}]}{\text{wet weight}\,[\text{g}]} * 100$$

6.4.4 Worm Lipid Content

At least 3 (preferably 4) separate samples of gut-purged control worms designated for dry weight (dw), and at least 3 (preferably 4) separate samples of control worms designated for lipid measurements were prepared (approx. 1 g wet weight (ww) per sample) and used for determination of dry weight and lipid content of the worms.

The method according to De Boer *et al.* (1999) was recommended for determination of lipid content (F_{lipid}) of the test organisms, using non-chlorinated solvents for extraction of lipids.

After sample processing, the resulting lipid content was calculated and expressed in per cent of worm wet weight. The lipid content (F_{lipid}) was calculated according to:

$$\frac{\text{(lipid weight [g]) x 100}}{\text{sample ww [g]}} = F_{lipid} \text{ in \% ww}$$

Using the previously measured dry weight to wet weight ratio, the lipid content could then be converted to per cent of worm dry weight.

7 DATA ASSESSMENT AND STATISTICAL EVALUATION

This section gives an overview of the assessment and statistical evaluation of the kinetic parameters measured in the single test runs, and on the statistical assessment of the ring test results.

7.1 General Evaluations

The concentration in soil and worm samples was expressed in dpm/g (^{14}C-HCB) and mg/kg (cadmium) based on worm and soil wet and dry weight, respectively. They were obtained by dividing the amount of test compound in the sample (dpm: corrected for background activity) by sample weight in g. The concentration in worm samples was then divided by the concentration in the soil of the corresponding sampling vessel to result in the bioaccumulation factor (BAF) for each specific sampling date of the uptake phase.

Body residues in earthworms worms with and without gut content were analysed using one-way Analysis of Variance (ANOVA; $p \leq 0.05$). Since the enchytraeids were not allowed to purge their gut contents after sampling, the measured body concentration includes residues bound to soil present in the gastro-intestinal tract. Information on the influence of gut-associated residues on the total body concentration in the worms can be obtained from the concentration in worms after the initial 4 - 6 hours of the elimination phase. The difference between the concentration in the worms at the end of the uptake phase and the concentration in the worms after the initial 4 - 6 hours of the elimination phase can be assigned to gut-soil bound residues. In addition, a comparison was made between purged and non-purged worms sampled at the end of the uptake phase of selected test runs.

The main parameters evaluated statistically were the uptake and elimination kinetics (see sections 0 and 0). The software package SPSS 11.5 was used for the statistical calculations.

The Non-Eliminated Residues (NER) were calculated as a secondary endpoint by multiplying the ratio of the average concentration in the worms (C_a) at the end of the elimination phase and the average concentration in the worms (C_a) at the end of uptake phase by 100:

$$\text{NER [\%]} = \frac{C_a \text{ at end of elimination (average)} * 100}{C_a \text{ at end of uptake (average)}}$$

7.2 Evaluation of Uptake Kinetics

All kinetic calculations are based on total ^{14}C- or Cd-concentrations in worm and soil, respectively. The uptake curve of the test chemical was obtained by plotting the average bioaccumulation factor (BAF) for each specific sampling date during the uptake phase against time on arithmetic scales. The BAF was calculated from:

$$\frac{C_a \text{ at end of uptake (mean)}}{C_s \text{ at end of uptake (mean)}}$$

where C_a is the concentration of the test chemical in the test organisms (in dpm/g), and C_s is the concentration of the test chemical in the soil (in dpm/g).

If the curve had reached a plateau, that is, become approximately parallel to the time axis, the BAF at the end of the uptake phase is the steady state BAF.

The results of the last three sampling dates of the uptake phase were evaluated for confirmation of steady state. Several criteria for steady state were checked:

a) difference between minimum and maximum BAF of the last 3 sampling means ≤ 40% of total mean;

b) ANOVA & pair-wise multiple comparisons (Bonferroni t-test) of the BAFs on the last 3 sampling dates: no significant difference ($p \leq 0.05$);

c) uptake curve (nonlinear regression) approx. parallel to x axis (≤ 90%).

As a general approach, nonlinear regression analysis was performed based on the models described by Spacie & Hamelink (1982) with the average accumulation factors (C_a, mean values of each sampling date/C_s, mean values of each sampling date = AF) of the uptake phase. For ^{14}C-HCB, wet weight-based concentrations were used for evaluation, while dry weight based concentrations were used for cadmium. The determination of kinetic parameters of uptake, and of the uptake curve was performed using the following model equation:

$$BAF(t) = BAF*(1-e^{k_e*t}) \qquad 0 < t < t_c$$

$$BAF(t) = BAF*(e^{-k_e(t-t_c)} - e^{-k_e t}) \qquad t > t_c$$

where BAF(t) is the ratio of concentration in worms and the concentration in soil in the soil at any given time point of the uptake phase, k_e is the elimination rate constant, and t_c is the time in days at the end of the uptake phase.

The kinetic bioaccumulation factor (BAF_K) and the elimination rate constant k_e (expressed in d^{-1}) were determined from nonlinear regression analysis. As the BAF_K is defined as the ratio of the soil uptake rate constant k_s (expressed in kg soil kg^{-1} of worm d^{-1}) and the elimination rate constant k_e ($BAF_K = k_s/k_e$), the uptake rate constant k_s was then calculated given k_e and BAF_K which is derived from the uptake curve:

$$k_s = BAF_K * k_e.$$

For ^{14}C-HCB, the Biota-Soil Accumulation Factor (BSAF) was calculated by normalising the BAF for lipid content in the test organisms and total organic carbon content of the soil.

A calculation of the Biota-soil Accumulation Factor (BSAF) will be given in Annex 2 of the draft guideline as follows:

$$BSAF = BAF_K * \frac{f_{oc}}{f_{lip}}$$

where f_{oc} is the fraction of soil organic carbon, and f_{lip} is the fraction of worm lipid, both based either on dry weight or on wet weight, respectively.

7.3 Evaluation of Elimination Kinetics

After a 10 day elimination phase, the elimination rate constant k_e (expressed in d^{-1}) was determined by nonlinear regression analysis (e.g. SPSS for Windows). The following models based on the models described by Spacie & Hamelink (1982) were used and fitted to the elimination data (C_a, mean values, n = 3 per sampling date):

1-compartment model: $C_a * \exp^{(-k_e * t)}$

2-compartment model: $C_{a(A)} * \exp^{(-k_a * t)} + C_{a(B)} * \exp^{(-k_b * t)}$

where C_a is the concentration in the animals at a given time point of the elimination phase, t is the time in days, k_e is the elimination constant in the 1-compartment model. A and B represent the two compartments (e.g. in per cent of mean concentration in worms at end of uptake phase), while k_a and k_b are the elimination constants for compartments A and B, resp., in the 2-compartment model. The data of the end of the uptake phase (mean measured value of the total radioactive concentration in the worms) were included representing the concentration in the worms at time point 0 of elimination.

The following criteria were used for model selection: Best model fit (coefficient of determination, r^2) and/or display of 1 or 2 straight lines on logarithmic scale.

The software package SPSS 11.5 was used for calculation of kinetics.

If a two-compartment model is applied to the elimination data, it is possible to calculate the soil uptake rate constant k_s from the following equation (Nagel 1988, Zok et al. 1991):

$$k_s = \frac{(A*k_a + B*k_b)*BAF}{A + B}$$

This model may also be considered for inclusion in the respective annex of the draft guideline.

7.4 Alternative Model Calculations

Using the model equations above, the kinetics (k_s and k_e) may also be calculated in one run by applying the first order kinetics model to all data from both the uptake and elimination phase together. For a description of a method that may allow for such a combined calculation of uptake and elimination rate constants, Janssen et al. (1991), Van Brummelen & Van Straalen (1996) and Sterenborg et al. (2003) may be consulted.

In the ring test, the following equation was applied in addition to the approaches described in sections 0 and 0:

$$C_a = \frac{k_s}{k_e}\left(C_s(1-e^{-k_e t})*(m=1)\right) + \left(\frac{k_s}{k_e} * C_s(e^{-k_e(t-tc)} - e^{-k_e t})*(m=2)\right)$$

m = 1 for uptake phase and 2 for elimination phase

Examples of kinetics evaluated according to this equation are shown in section 0.

7.5 Tests for normal-distribution and variance homogeneity

Normal-distribution of the data was tested with the Kolmogorov-Smirnov goodness-of-fit test, or - in case of less than 4 replicates per treatment - with the range-to-standard-deviation test (two-sided, $p \leq 0.05$). If $\geq$ 50% of the treatments passed the normality test, data were regarded normally distributed. Otherwise the data were transformed prior to hypothesis testing. Cochran's test (two-sided, $p \leq 0.05$) was used to test variance homogeneity.

7.6 Validity Criteria in the Ring Test

The following, preliminary validity criteria were to be fulfilled according to the test protocol used in the ring test:

- The overall mortality during uptake and elimination phase or other adverse effects/diseases in both control and treated worms should be less than 10% (earthworms) or 20% (enchytraeids) at the end of the test (at the end of the elimination phase).

In case of significant deviations from these validity criteria the test was not regarded as valid. The validity criteria as given in the test protocol were revised on the basis of the ring test results.

The preliminary validity criteria (above) were discussed and refined by the ring test participants after evaluation of the ring test results. According to the outcome of these discussions, the following validity criteria were adopted for the draft guideline:

- At the end of the test, the overall mortality during uptake and elimination phase should not exceed 10% (earthworms) or 20% (enchytraeids) of the total number of the introduced worms.

- For earthworms, the mean mass loss as measured at the end of the uptake and at the end of the elimination phase should not exceed 20% compared to the initial fresh weight (fw) for each phase.

7.7 Number of Tests

The number of evaluated tests per species, soil type and test substance is shown in Table 7.

Table 7: Overview of the evaluated data sets.

	Eisenia sp.		*Enchytraeus* sp.		Total
	OECD soil	Field soil	OECD soil	Field soil	
^{14}C-HCB	6	3	2	3	14
$CdCl_2$	8	3	5	3	19
Total	14	6	5	3	33

8 RESULTS

8.1 Hexachlorobenzene

8.1.1 Kinetics in Earthworms

OECD Soil

Table 8: Overview of the HCB uptake parameters: Earthworms in spiked OECD soil.

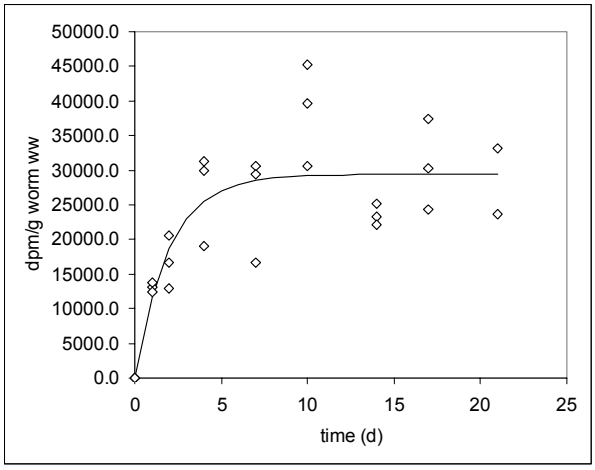
Species	Lab-Code		Parameter Estimate	Asymptotic Std. Error	Asymptotic Confidence Lower	95% Interval Upper	Coefficient of r^2 replicate data	Determination mean data
E. andrei	O4	BAF	**1.7**	0.1	1.5	1.9	0.80	0.93
		k_s	1.0					
		k_e	0.62	0.14	0.30	0.94		
E. fetida	O5	BAF	**3.4**	0.3	2.8	4.0	0.77	0.84
		k_s	1.6					
		k_e	0.47	0.16	0.09	0.86		
E. andrei	O6	BAF	**1.0**	0.1	0.7	1.2	0.45	0.63
		k_s	1.2					
		k_e	1.22	0.89	-0.88	3.31		
E. fetida	O9	BAF	**1.4**	0.2	1.1	1.8	0.54	0.66
		k_s	1.2					
		k_e	0.85	0.51	-0.36	2.06		
E. andrei	O10	BAF	**6.9**	0.4	5.9	7.8	0.79	0.91
		ks	3.6					
		k_e	0.53	0.14	0.20	0.85		
E.fetida	O11	BAF	**3.6**	0.1	3.3	3.9	0.96	0.97
		k_s	1.6					
		k_e	0.43	0.06	0.30	0.57		

BAF: bioaccumulation factor in kg soil/kg worm; k_s: soil uptake rate constant in kg soil/kg worm/day; k_e: elimination rate constant in 1/d.

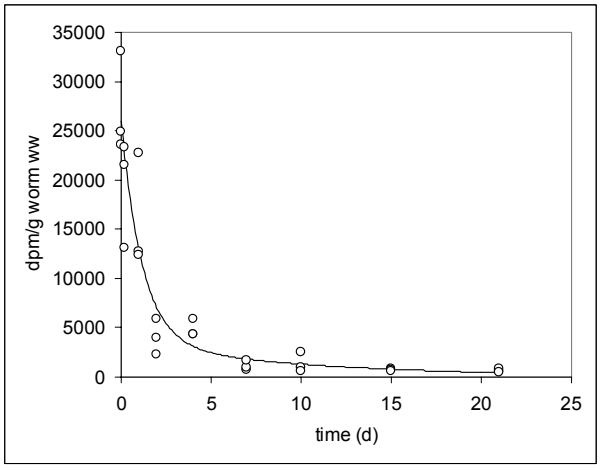
Table 9: Overview of the HCB elimination parameters: Earthworms in spiked OECD soil; parameters in dpm/g wet weight.

Species	Lab code	Parameter	Parameter Estimate	Asymptotic Std. Error	Asymptotic 95% Confidence Interval		NER (%)	Coefficient of Determination r^2	
					Lower	Upper		replicate data	mean data
E. andrei	O4	A	7024.7	643.9	5369.4	8679.9			
		k_a	4.2	0.8	2.2	6.2			
		B	10277.0	580.2	8785.5	11768.5			
		k_b	0.3	0.0	0.2	0.3	2.9	0.94	1.00
E. fetida	O5	A	22410.9	7402.3	3382.6	41439.2			
		k_a	0.8	0.4	-0.3	2.0			
		B	3510.2	7540.9	-15874.3	22894.7			
		k_b	0.1	0.2	-0.5	0.7	2.3	0.89	0.96
E. andrei	O6	A	4561.8	767.4	2589.2	6534.4			
		k_a	0.7	0.3	-0.1	1.4			
		B	140.5	533.2	-1230.1	1511.0			
		k_b	-0.1	0.2	-0.6	0.5	11.7	0.59	0.89
E. fetida	O9	A	6207.1	1463.9	2444.1	9970.1			
		k_a	0.5	0.3	-0.3	1.2			
		B	175.0	1311.2	-3195.5	3545.6			
		k_b	0.0	0.4	-1.2	1.1	4.4	0.84	0.86
E. andrei	O10	A	23654.0	1224.9	20505.4	26802.7			
		k_a	0.3	0.0	0.2	0.4			
		B	601.2	1261.1	-2640.4	3842.9			
		k_b	0.0	0.1	-0.3	0.2	6.6	0.90	1.00
E.fetida	O11	A	3619.8	320.6	2795.6	4444.0			
		k_a	0.1	0.0	0.1	0.1			
		B	1212.1	426.9	114.7	2309.6			
		k_b	3.4	3.0	-4.3	11.1	8.6	0.87	0.97

A and B: compartments (in dpm/g worm); k_a and k_b: elimination constants for compartments A and B (in 1/d); NER: non-eliminated residues



uptake



elimination

Figure 3: Example of HCB kinetics in earthworms calculated conventionally according to sections 0 & 0; HCB in *E. fetida* in OECD soil (lab code O5).

An example calculation was done using the alternative modelling approach described in section 0, using the HCB kinetics in *E. fetida* in OECD soil. Figure 4 shows the integrated uptake and elimination curves, Table 10 summarises the resulting kinetic parameters.

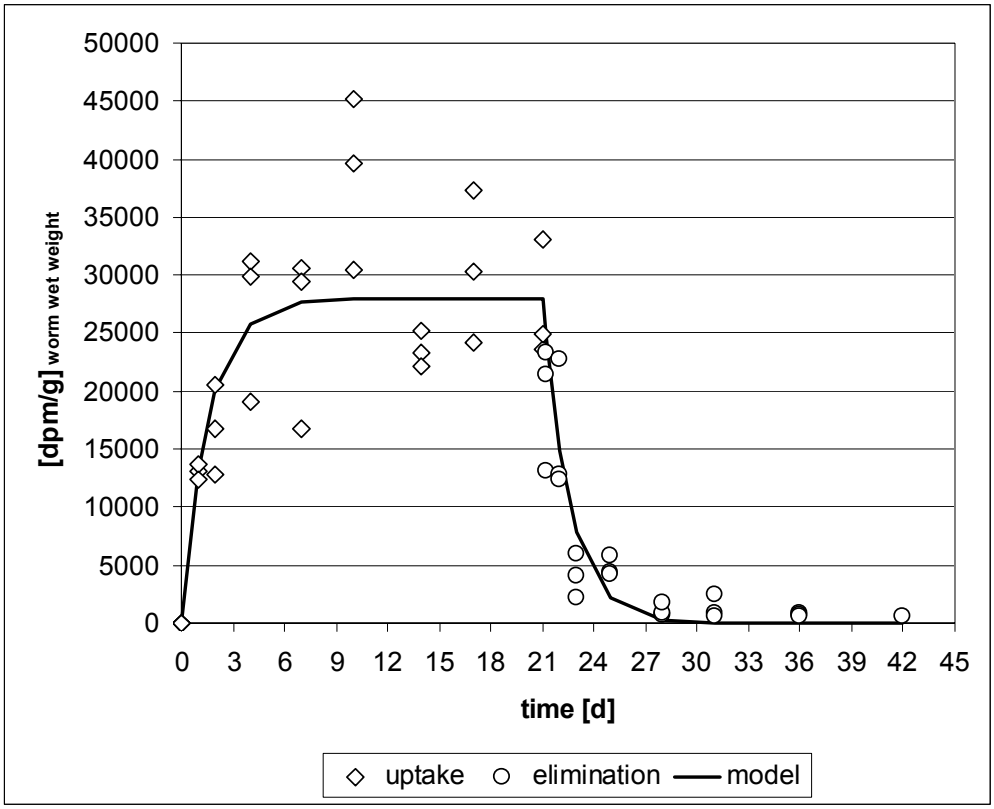


Figure 4: Example of HCB kinetics in earthworms calculated according to section 0; HCB in *E. fetida* in OECD soil (lab code O5).

Table 10: Example of HCB kinetics in earthworms calculated according to section 0: parameters are based on soil and worm wet weight.

Species	Lab Code		Parameter Estimate	Asymptotic Std. Error	Asymptotic Confidence Lower	95% Interval Upper	Coefficient of r^2 replicate data
E. fetida	O5	BAF	**3.3**				
OECD soil		k_s	2.0	0.3	1.4	2.6	0.85
		k_e	0.6	0.1	0.5	0.8	

The differences between the parameters calculated by the conventional and alternative models appear to be relatively small (see Table 8 for comparison with conventionally modelled parameters).

Field Soils

Table 11: Overview of the HCB uptake parameters: Earthworms in spiked field soils.

Species	Soil	Lab-Code		Parameter Estimate	Asymptotic Std. Error	Asymptotic Confidence Lower	95% Interval Upper	Coefficient of r^2 replicate data	Determination mean data
E. fetida	*LUFA*	O5	BAF	**4.8**	0.3	4.1	5.5	0.81	0.86
			k_s	4.1					
			k_e	0.86	0.30	0.16	1.56		
E. andrei	*Lit*	O10	BAF	**12.3**	0.3	11.5	13.1	0.90	0.98
			k_s	5.7					
			k_e	0.46	0.06	0.33	0.59		
E. andrei	*LUV*	O10	BAF	**12.6**	0.3	11.9	13.3	0.90	0.98
			k_s	5.3					
			k_e	0.42	0.04	0.32	0.53		

BAF: bioaccumulation factor in kg soil/kg worm; k_s: soil uptake rate constant in kg soil/kg worm/day; k_e: elimination rate constant in 1/d.

Table 12: Overview of the HCB elimination parameters: Earthworms in spiked field soils; parameters in dpm/g wet weight.

Species	Lab code		Parameter Estimate	Asymptotic Std. Error	Asymptotic 95% Confidence Interval Lower	Upper	NER (%)	Coefficient of Determination r^2 replicate data	mean data
E.fetida	O5	A	40752.2	2296.3	34849.4	46654.9			
		k_a	0.5	0.1	0.3	0.7			
LUFA		B	251.5	1673.4	-4050.0	4553.0			
		k_b	-0.1	0.4	-1.0	0.9	2.6	0.89	0.99
E. andrei	O10	A	24453.3	6755.1	7088.7	41817.8			
		k_a	0.1	0.0	0.0	0.2			
Lit		B	33953.2	6655.0	16845.9	51060.5			
		k_b	0.9	0.3	0.2	1.6	8.1	0.93	0.99
E. andrei	O10	A	40938.7	2752.8	33862.5	48015.0			
		k_a	0.1	0.0	0.1	0.1			
LUV		B	31016.4	3843.9	21135.3	40897.5			
		k_b	4.2	1.4	0.7	7.7	6.0	0.76	0.99

A and B: compartments (in dpm/g worm); k_a and k_b: elimination constants for compartments A and B (in 1/d); NER: non-eliminated residues

8.1.2 Kinetics in Enchytraeids

OECD Soil

Table 13: Overview of the HCB uptake parameters: Enchytraeids in spiked OECD soil.

Species	Soil	Lab Code		Parameter Estimate	Asymptotic Std. Error	Asymptotic Confidence Interval Lower	95% Interval Upper	Coefficient of r^2 replicate data	Determination mean data
E. albidus	*OECD*	O5	BAF	13.3	0.6	11.8	14.8	0.95	0.96
			k_s	5.8					
			k_e	0.43	0.08	0.24	0.63		
E. albidus	*OECD*	O10	BAF	9.5	0.4	8.5	10.5	0.69	0.94
			k_s	9.9					
			k_e	1.05	0.24	0.45	1.65		

BAF: bioaccumulation factor in kg soil/kg worm; k_s: soil uptake rate constant in kg soil/kg worm/day; k_e: elimination rate constant in 1/d.

Table 14: Overview of the HCB elimination parameters: Enchytraeids in spiked OECD soil; parameters in dpm/g wet weight.

Species	Lab code		Parameter Estimate	Asymptotic Std. Error	Asymptotic 95% Confidence Interval Lower	Upper	NER (%)	Coefficient of Determination r^2 replicate data	mean data
E. albidus	O5	A	7902.1	26178.1	-64779.9	80584.1			
		k_a	0.01	0.3	-0.8	0.8			
		B	88030.3	25158.5	18179.1	157881.5			
		k_b	0.4	0.2	-0.1	0.9	7.1	0.96	0.98
E. albidus	O10	A	15249.1	18615.1	-36434.7	66933.0			
		k_a	0.3	0.4	-0.7	1.3			
		B	41955.6	18354.8	-9005.5	92916.7			
		k_b	1.9	1.1	-1.2	5.0	1.5	0.89	0.98

A and B: compartments (in dpm/g worm); k_a and k_b: elimination constants for compartments A and B (in 1/d); NER: non-eliminated residues

Field Soils

Table 15: Overview of the HCB uptake parameters: Enchytraeids in spiked field soils.

Species	Soil	Lab Code		Parameter Estimate	Asymptotic Std. Error	Asymptotic 95% Confidence Interval Lower	Asymptotic 95% Confidence Interval Upper	Coefficient of Determination r^2 replicate data	Coefficient of Determination r^2 mean data
E. albidus	*LUFA*	O5	BAF	25.6	1.0	23.2	27.9	0.97	0.98
			k_s	9.2					
			k_e	0.36	0.05	0.24	0.48		
E. crypticus	Lit	O10	BAF	15.0	1.2	12.2	17.8	0.64	0.78
			k_s	225.7					
			k_e	15.02	1.15	12.21	17.84		
E. crypticus	LUV	O10	BAF	30.0	7.0	12.9	47.1	0.47	0.36
			k_s	38.8					
			k_e	1.29	1.88	-3.30	5.89		

BAF: bioaccumulation factor in kg soil/kg worm; k_s: soil uptake rate constant in kg soil/kg worm/day; k_e: elimination rate constant in 1/d.

Table 16: Overview of the HCB elimination parameters: Enchytraeids in spiked field soils; parameters in dpm/g wet weight.

Species	Lab code		Parameter Estimate	Asymptotic Std. Error	Asymptotic 95% Confidence Interval Lower	Asymptotic 95% Confidence Interval Upper	NER (%)	Coefficient of Determination r^2 replicate data	Coefficient of Determination r^2 mean data
E. albidus	O5	A	207644.9	6611.4	191467.4	223822.4			
LUFA		k_e	0.2	0.0	0.2	0.3	7.2	0.93	0.99
E. crypticus	O10	A	28625.4	620.2	26903.5	30347.3			
Lit		k_a	2.0	0.1	1.6	2.3			
		B	616.5	432.1	-583.3	1816.3			
		k_b	0.0	0.1	-0.2	0.2	2.8	0.94	1.00
E. crypticus	O10	A	67718.9	6124.6	52732.6	82705.1			
LUV		k_e	0.6	0.2	0.3	1.0	1.0	0.70	0.94

A and B: compartments (in dpm/g worm); k_a and k_b: elimination constants for compartments A and B (in 1/d); NER: non-eliminated residues

8.1.3 Quantification of Radioactive Residues in Worms and Soil

At the end of the uptake phase (21 days after start of exposure), worm and soil samples were extracted as described in section 0. Extraction efficiency was good for worm tissue and for fortified soil, while less than 10% of the applied radioactive total radioactive residues (TRR) could be extracted from the stored soil

samples. Recovery of TRR following combustion was 93%. The following table shows the extraction efficiencies for HCB-spiked soil and earthworms.

Table 17: Extraction efficiency for worm and soil samples spiked with ^{14}C-HCB.

	Recovery of extracts	Mean value	Standard deviation
worms	extracts before filtration	94.3[1]	14.2
	extracts after filtration	88.8[1]	20.0
soil	fortification soil samples	106.5[2]	12.1
	extracts of stored soil samples	8.4[3]	0.4

[1] (n = 4); recovery based on TRR at end of uptake phase
[2] (n = 2); recovery based on TRR in fortification spike
[3] (n = 2); recovery based on TRR at end of uptake phase

In the TLC runs and subsequent TLC scan, the worm extracts showed > 95% of the total radioactivity in one spot. This spot was assigned to HCB by co-chromatography with ^{14}C-HCB standard solution. Other radioactive spots were not detected by the TLC scan.

Since the extraction efficiency and thus the radioactive concentration for the soil extract was low, the available radioactivity was only slightly higher than the baseline noise. As for the worm extracts, only one radioactive spot was observed, which was also confirmed by co-chromatography with ^{14}C-HCB standard. The radioactivity associated with this spot was only 0.9% of the total signals encountered in the scan.

Consequently, it can be stated that only negligible metabolism of ^{14}C-HCB had taken place in the worms, and the measured TRR can be assigned to the parent compound, HCB. The same could be concluded for the soil-associated TRR, but less than 10% of the applied TRR were extractable. Therefore, a reliable statement, whether HCB was metabolised in the spiked OECD soil or not, cannot be made based on the information gained during this study.

8.2 Cadmium

8.2.1 Kinetics in Earthworms

OECD Soil

Table 18: Overview of the Cd uptake parameters: Earthworms in spiked OECD soil based on residues in mg/kg dry weight.

Species	Lab Code		Parameter Estimate	Asymptotic Std. Error	Asymptotic Confidence Lower	95% Interval Upper	Coefficient of r^2 replicate data	Determination mean data
E. fetida	O1	BAF	29.3	6.5	13.8	44.7	0.89	0.98
OECD		k_s	1.6					
		k_e	0.05	0.02	0.01	0.10		
E. fetida	O2	BAF	132.9	76.4	-47.8	313.6	0.95	0.99
OECD		k_s	2.2					
		k_e	0.02	0.01	-0.01	0.04		
E. andrei	O3	BAF	29.91	16.36	-8.77	68.59	0.90	0.96
OECD		k_s	1.0					
		k_e	0.03	0.02	-0.02	0.09		
E. fetida	O5	BAF	57.9	58.8	-81.2	197.0	0.85	0.97
OECD		k_s	1.0					
		k_e	0.02	0.02	-0.03	0.07		
E. andrei	O6	BAF	-13.9	12.6	-43.8	15.9	0.91	0.94
OECD		k_s	0.6					
		k_e	-0.04	0.03	-0.10	0.02		
E. fetida	O8	BAF	-18.2	18.3	-61.5	25.2	0.89	0.92
OECD		k_s	0.9					
		k_e	-0.05	0.03	-0.12	0.03		
E. andrei	O13	BAF	481	16953	-39607	40569	0.79	0.89
OECD		k_s	0.5					
		k_e	0.001	0.04	-0.09	0.09		
E. fetida	O15	BAF	39.9	15.3	3.7	76.2	0.88	0.97
OECD		k_s	1.6					
		k_e	0.04	0.02	-0.01	0.09		

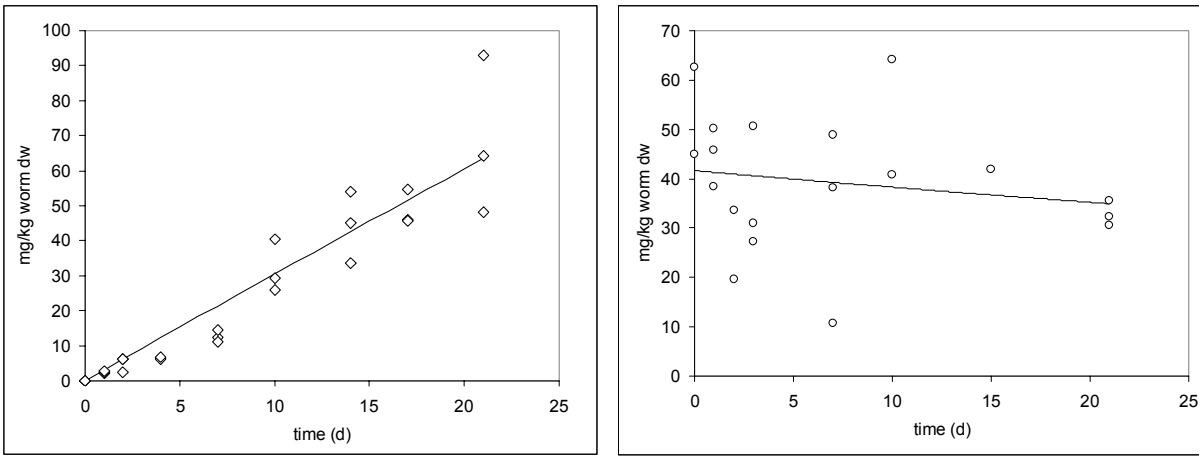
BAF: bioaccumulation factor in kg soil/kg worm; k_s: soil uptake rate constant in kg soil/kg worm/day; k_e: elimination rate constant in 1/d.

Table 19: Overview of the Cd elimination parameters: Earthworms in spiked OECD soil; parameters in mg/kg dry weight.

Species	Lab code	Parameter Estimate		Asymptotic Std. Error	Asymptotic 95% Confidence Interval		NER (%)	Coefficient of Determination r^2	
					Lower	Upper		replicate data	mean data
E. fetida OECD	O1	A	81.3	11.1	55.1	107.5	99.1	0.03	0.07
		k_e	-0.009	0.013	-0.040	0.021			
E. fetida OECD	O2	A	39.8	45.4	-76.9	156.4	70.3	0.29	0.55
		k_a	0.3	0.7	-1.5	2.2			
		B	118.8	47.2	-2.5	240.1			
		KB	0.00	0.02	-0.06	0.06			
E. andrei OECD	O3	A	78.8	4.5	69.5	88.1	67.2	0.29	0.47
		k_e	0.022	0.007	0.007	0.038			
E. fetida OECD	O5	A	9.1	13.1	-24.7	42.8	92.0	0.07	0.22
		k_a	4.1	17.5	-40.9	49.2			
		B	77.5	7.3	58.7	96.3			
		k_b	0.004	0.009	-0.018	0.027			
E. andrei OECD	O6	A	27.0	11.5	-2.5	56.6	37.4	0.48	0.80
		k_a	9.6	12.0	-21.3	40.5			
		B	59.0	6.5	42.3	75.7			
		k_b	0.03	0.01	-0.01	0.06			
E. fetida OECD	O8	A	15.8	0.6	14.3	17.4	79.5	0.02	0.08
		k_e	-0.004	0.006	-0.019	0.01			
E. andrei OECD	O13	A	41.9	5.4	28.5	55.2	61.1	0.03	0.03
		k_e	0.01	0.01	-0.03	0.04			
E. fetida OECD	O15	A	98.14	6.66	82.40	113.88	46.3	0.34	0.56
		k_e	0.02	0.01	0.00	0.05			

A and B: compartments (in mg/kg worm); k_a and k_b: elimination constants for compartments A and B (in 1/d); k_e: elimination constant; NER: non-eliminated residues

The cadmium kinetics followed a different pattern than uptake and elimination of ^{14}C-HCB. Figure 5 shows an example test run where the uptake takes place linearly, and the elimination does not show any clear trend.



uptake phase elimination phase

Figure 5: Example of Cd kinetics in earthworms calculated conventionally according to sections 0 & 0; Cd in *E. fetida* in OECD soil (lab code O13).

In some cases negative BAF were modelled for Cd, and in most of the test runs no considerable elimination of the accumulated residues was observed. Therefore, an example calculation was done using the alternative modelling approach described in section 0, using the Cd kinetics in *E. fetida* in OECD soil (lab code O13). Figure 4 shows the integrated uptake and elimination curves, Table 10 summarises the resulting kinetic parameters.

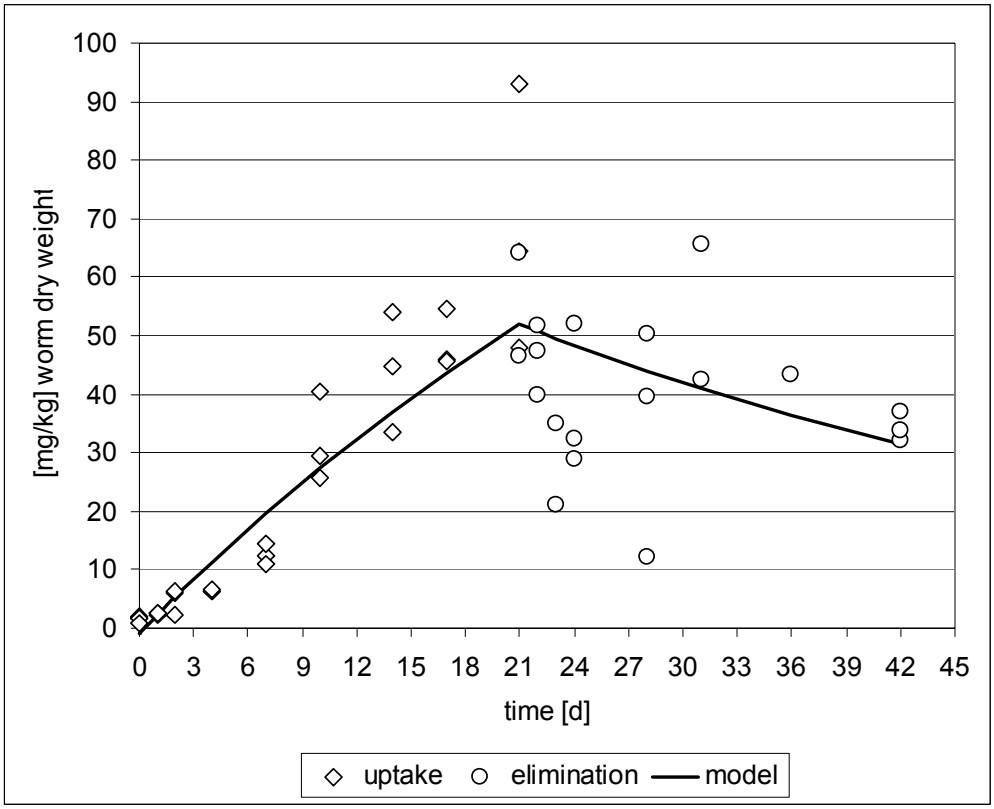


Figure 6: Example of Cd kinetics in earthworms calculated according to section 0; Cd in *E. fetida* in OECD soil (lab code O13).

Table 20: Example of Cd kinetics in earthworms calculated according to section 0: parameters are based on soil and worm dry weight.

Species	Lab-Code		Parameter Estimate	Asymptotic Std. Error	Asymptotic Confidence Lower	95% Interval Upper	Coefficient of r^2 replicate data
E. andrei	O13	BAF	**25.2**				0.72
		k_s	0.580	0.08	0.41	0.75	
		k_e	0.023	0.01	0.01	0.04	

While the conventional modelling (see Table 18 and Table 19 for comparison) did not lead to reasonable kinetic parameters, the results gained with the alternative model calculation are in good agreement with the values expected from the kinetic plot (see Figure 6).

Field Soils

Table 21: Overview of the Cd uptake parameters: Earthworms in spiked field soil based on residues in mg/kg dry weight.

Species	Lab-Code		Parameter Estimate	Asymptotic Std. Error	Asymptotic Confidence Lower	95% Interval Upper	Coefficient of r^2 replicate data	Determination mean data
E. fetida	O2	BAF	29.3	8.7	8.6	49.9	0.90	0.98
LUFA		k_s	1.3					
		k_e	0.04	0.02	0.00	0.09		
E. fetida	O5	BAF	35.2	17.9	-7.2	77.6	0.88	0.94
LUFA		k_s	1.5					
		k_e	0.04	0.03	-0.03	0.11		
E. fetida	O8	BAF	-12.7	6.3	-27.6	2.1	0.76	0.97
SSL		k_s	0.7					
		k_e	-0.06	0.02	-0.10	-0.02		

BAF: bioaccumulation factor in kg soil/kg worm; k_s: soil uptake rate constant in kg soil/kg worm/day; k_e: elimination rate constant in 1/d.

Table 22: Overview of the Cd elimination parameters: Earthworms in spiked OECD soil; parameters in mg/kg dry weight.

Species	Lab code	Parameter Estimate	Asymptotic Std. Error	Asymptotic 95% Confidence Interval		NER (%)	Coefficient of Determination r^2	
				Lower	Upper		replicate data	mean data
E. fetida	O2	A	12.72	0.00	12.72	12.72	71.97	0.20	0.55
LUFA		k_a	78.17	7.32	59.35	96.99			
		B	69.63	0.01	69.62	69.64			
		k_b	0.01	3.44	-8.84	8.85			
E. fetida	O5	A	32.31	10.93	1.97	62.66	76.04	0.21	0.87
LUFA		k_a	0.75	0.55	-0.79	2.29			
		B	90.99	10.54	61.72	120.27			
		k_b	-0.001	0.01	-0.03	0.03			
E. fetida	O8	A	93.75	8.81	72.92	114.59	100.16	0.01	0.00001
SSL		k_e	0.0001	0.01	-0.02	0.02			

A and B: compartments (in mg/kg worm); k_a and k_b: elimination constants for compartments A and B (in 1/d); k_e: elimination constant; NER: non-eliminated residues

8.2.2 Kinetics in Enchytraeids

OECD Soil

Table 23: Overview of the Cd uptake parameters: Enchytraeids in spiked OECD soil based on residues in mg/kg dry weight.

Species	Lab Code		Parameter Estimate	Asymptotic Std. Error	Asymptotic Confidence Lower	95% Interval Upper	Coefficient of r^2 replicate data	Determination mean data
E. albidus	O1	BAF	9.1	10.6	-16.9	35.1	0.91	0.98
OECD		k_s	0.2					
		k_e	0.02	0.03	-0.04	0.08		
E. albidus	O2	BAF	4.2	0.7	2.5	5.9	0.90	0.98
OECD		k_s	0.5					
		k_e	0.11	0.03	0.03	0.19		
E. albidus	O5	BAF	6.7	1.1	3.9	9.4	0.98	1.00
OECD		k_s	0.3					
		k_e	0.05	0.01	0.02	0.08		
E. albidus	O8	BAF	5.4	1.0	2.9	8.0	0.96	0.97
OECD		k_s	0.6					
		k_e	0.11	0.04	0.02	0.20		
E. crypticus	O15	BAF	-8.9	1.9	-13.6	-4.3	0.99	1.00
OECD		k_s	0.8					
		k_e	-0.09	0.01	-0.12	-0.06		

BAF: bioaccumulation factor in kg soil/kg worm; k_s: soil uptake rate constant in kg soil/kg worm/day; k_e: elimination rate constant in 1/d.

Table 24: Overview of the Cd elimination parameters: Enchytraeids in spiked OECD soil; parameters in mg/kg dry weight.

Species	Lab code	Parameter	Parameter Estimate	Asymptotic Std. Error	Asymptotic 95% Confidence Interval		NER (%)	Coefficient of Determination r^2	
					Lower	Upper		replicate data	mean data
E. albidus	O1	A	6.16	1.57	1.82	10.51	41.37	0.62	0.90
OECD		k_a	0.89	0.53	-0.58	2.36			
		B	3.84	1.46	-0.22	7.91			
		KB	-0.01	0.04	-0.11	0.10			
E. albidus	O2	A	15.22	1.18	12.34	18.10	61.20	0.48	0.64
OECD		k_e	0.05	0.02	0.01	0.09			
E. albidus	O5	A	3.06	0.68	1.16	4.96	121.98	0.71	0.96
OECD		k_a	9.02	5.85	-7.23	25.26			
		B	12.49	0.36	11.48	13.50			
		k_b	-0.03	0.00	-0.04	-0.02			
E. albidus	O8	A	2.00	1.73	-2.79	6.80	103.69	0.06	0.31
OECD		k_a	1.97	4.52	-10.56	14.51			
		B	15.00	1.26	11.51	18.49			
		k_b	-0.01	0.01	-0.04	0.02			
E. crypticus	O15	A	104.65	19.30	51.07	158.23	31.52	0.63	0.91
OECD		k_a	0.36	0.20	-0.19	0.91			
		B	5.91	18.92	-46.61	58.44			
		k_b	-0.12	0.24	-0.79	0.56			

A and B: compartments (in mg/kg worm); k_a and k_b: elimination constants for compartments A and B (in 1/d); k_e: elimination constant; NER: non-eliminated residues

Field Soils

Table 25: Overview of the Cd uptake parameters: Enchytraeids in spiked field soil based on residues in mg/kg dry weight.

Species	Lab-Code		Parameter Estimate	Asymptotic Std. Error	Asymptotic Confidence Lower	95% Interval Upper	Coefficient of r^2 replicate data	Determination mean data
E. albidus	O2	BAF	6.0	12.5	-24.7	36.6	0.48	0.70
LUFA		k_s	0.3					
		k_e	0.05	0.14	-0.30	0.40		
E. albidus	O5	BAF	-0.9	0.6	-2.5	0.6	0.90	0.95
LUFA		k_s	0.1					
		k_e	-0.13	0.04	-0.24	-0.03		
E. albidus	O8	BAF	19.2	13.0	-12.6	51.0	0.93	0.98
SSL		k_s	0.6					
		k_e	0.03	0.02	-0.03	0.09		

BAF: bioaccumulation factor in kg soil/kg worm; k_s: soil uptake rate constant in kg soil/kg worm/day; k_e: elimination rate constant in 1/d.

Table 26: Overview of the Cd elimination parameters: Enchytraeids in spiked field soil; parameters in mg/kg dry weight.

Species	Lab code	Parameter Estimate		Asymptotic Std. Error	Asymptotic 95% Confidence Interval		NER (%)	Coefficient of Determination r^2	
					Lower	Upper		replicate data	mean data
E. albidus LUFA	O2	A	2.00	0.14	1.65	2.35	66.06	0.27	0.57
		k_e	0.04	0.01	0.003	0.07			
E. albidus LUFA	O5	A	16.91	1.65	13.46	20.35	62.89	0.29	0.22
		k_e	0.07	0.02	0.02	0.12			
E. albidus SSL	O8	A	11.32	0.00	11.32	11.32	84.73	0.76	0.95
		k_a	76.41	1.39	72.56	80.26			
		B	14.75	0.01	14.73	14.76			
		k_b	-0.03	0.65	-1.84	1.78			

A and B: compartments (in mg/kg worm); k_a and k_b: elimination constants for compartments A and B (in 1/d); k_e: elimination constant; NER: non-eliminated residues

8.3 Synopsis

Table 27: Overview of the HCB uptake parameters: Parameters based on residues in wet worm and soil.

Species	Lab code	Soil	BAF_K (kg soil kg^{-1} worm)	k_s (kg soil kg^{-1} worm d^{-1})	k_e (d^{-1})	BSAF (kg soil OC kg^{-1} worm lipid content)
E. fetida / E. andrei	O4	OECD	1.7	1.0	0.62	3.0
	O5	OECD	3.4	1.6	0.47	3.9
	O6	OECD	4.8	4.1	0.86	3.4
	O9	OECD	1.0	1.2	1.22	1.0
	O10	OECD	1.4	1.2	0.85	5.7
	O11	OECD	6.9	3.6	0.53	7.8
	O5	LUFA	12.3	5.7	0.46	13.1
	O10	Lit	12.6	5.3	0.42	10.4
	O10	LUV	3.6	1.6	0.43	3.6
Enchytraeus sp.	O5	OECD	13.3	5.8	0.43	73.6
	O10	OECD	9.5	9.9	1.05	n.r.
	O5	LUFA	25.6	9.2	0.36	n.r.
	O10	Lit	15.0	225.7	15.02	n.r.
	O10	LUV	30.0	38.8	1.29	n.r.

n.r.: not reported to date

Table 28: Overview of the HCB elimination parameters: Parameters in % of eliminated residues.

Species	Lab code	Soil	A (%)	k_a (d^{-1})	B (%)	k_b (d^{-1})	NER (%) (end of test)
E. fetida / E. andrei	O4	OECD	40.6	4.17	59.4	0.27	2.9
	O5	OECD	86.5	0.84	13.5	0.10	2.3
	O6	OECD	97.0	0.65	3.0	-0.06	11.7
	O9	OECD	97.3	0.46	2.7	-0.04	4.4
	O10	OECD	97.5	0.27	2.5	-0.05	6.6
	O11	OECD	74.9	0.09	25.1	3.38	8.6
	O5	LUFA	99.4	0.47	0.6	-0.08	2.6
	O10	Lit	56.9	0.09	43.1	4.20	6.0
	O10	LUV	41.9	0.10	58.1	0.86	8.1
Enchytraeus sp.	O5	OECD	8.2	0.01	91.8	0.4	7.1
	O10	OECD	26.7	0.30	73.3	1.9	1.5
	O5	LUFA	100.0	0.22	--	--	7.2
	O10	Lit	97.9	1.99	2.1	-0.02	2.8
	O10	LUV	100.0	0.63	--	--	1.0

Table 29: Overview of the cadmium uptake parameters: Parameters based on residues in dry worm and soil.

Species	Lab code	Soil	BAF_K (kg soil kg^{-1} worm)	k_s (kg soil kg^{-1} worm d^{-1})	k_e (d^{-1})
E. fetida / E. andrei	O1	OECD	29.3	1.6	0.05
	O2	OECD	132.9	2.2	0.02
	O3	OECD	29.9	1.01	0.03
	O5	OECD	57.9	1.0	0.02
	O6	OECD	-13.9	0.6	-0.04
	O8	OECD	-18.2	0.9	-0.05
	O15	OECD	39.9	1.6	0.04
	O2	LUFA	29.3	1.3	0.04
	O5	LUFA	35.2	1.5	0.04
	O8	SSL	-12.7	0.7	-0.06
Enchytraeus sp.	O1	OECD	9.1	0.2	0.0
	O2	OECD	4.2	0.5	0.1
	O5	OECD	6.7	0.3	0.0
	O8	OECD	5.4	0.6	0.1
	O15	OECD	-8.9	0.8	-0.1
	O2	LUFA	6.0	0.3	0.1
	O5	LUFA	-0.9	0.1	-0.1
	O8	SSL	19.2	0.6	0.0

Table 30: Overview of the cadmium elimination parameters: Parameters based on residues in dry worm and soil.

Species	Lab code	Soil	A (mg/kg)	k_a (d^{-1})	B (mg/kg)	k_b (d^{-1})	NER (%) (end of test)
E. fetida / E. andrei	O1	OECD	81.3	-0.01			99.1
	O2	OECD	39.8	0.34	118.8	0.0004	70.3
	O3	OECD	78.8	0.02			67.2
	O5	OECD	9.1	4.13	77.5	0.004	92.0
	O6	OECD	27.0	9.61	59.0	0.03	37.4
	O8	OECD	15.8	-0.004			79.5
	O13	OECD	41.9	0.01			61.1
	O15	OECD	98.1	0.02			46.3
	O2	LUFA	12.7	78.17	69.6	0.01	72.0
	O5	LUFA	32.3	0.7	91.0	-0.001	76.0
	O8	SSL	93.8	-0.0001			103.7
Enchytraeus sp.	O1	OECD	6.2	0.89	3.8	-0.01	41.4
	O2	OECD	15.2	0.05			61.2
	O5	OECD	3.1	9.02	12.5	-0.03	122.0
	O8	OECD	2.0	1.97	15.0	-0.01	103.7
	O15	OECD	104.7	0.36	5.9	-0.12	31.5
	O2	LUFA	2.0	0.04			66.1
	O5	LUFA	16.9	0.07			62.9
	O8	SSL	11.3	76.4	14.7	-0.03	84.7

8.4 Test Validity

8.4.1 Mortality

Earthworm mortality was kept well below 10% during the ring test runs; only in one case, 10% was reached. The validity criterion is therefore considered acceptable. Enchytraeid mortality was - as expected - somewhat higher than earthworm mortality. In the test runs where enchytraeid mortalities were reported, the values range from 1% to 38% of the initial number of worms, which exceeds the preliminary validity criterion of 20%. This high mortality was reported mostly where *E. albidus* was tested in OECD soil; reported mortalities for the other soil types and enchytraeid species were in general much lower.

8.4.2 Weight Loss (Earthworms)

In general, the weight loss of the earthworms measured as the difference between the initial wet weight, and the wet weight at date of sampling was less than 20% of the initial wet weight. Therefore, a weight loss of less than 20% of the initial weight can be considered as an indication of appropriate test conditions and is included as a critical value in the validity criteria.

8.5 Gut-purging: Impact on Body Residue

The difference in body residues of purged and non-purged worms was in general not statistically significant at p 0.05 (see Table 31). Also for enchytraeid body residues, gut content did not play a significant role (data not shown). An exception can be observed for Cd residues after exposure in LUFA soil, where the concentration in purged worms was significantly higher than in non-purged worms. This indicates that Cd may have been eliminated during voiding of the gut when spiked OECD soil was used, while in Lufa soil, most of the accumulated Cd remained in the tissue after gut purging.

Table 31: Comparison of body residues in purged and non-purged earthworms.

		HCB/OECD	HCB/LUFA	Cd/OECD	Cd/LUFA*
		dpm/g worm (d.w.)		µg/g worm (d.w.)	
non-purged	Mean	101432	132403	75.4	66.3
	N	3	3	2	4
	Std. Deviation	19272	30883	--	20.1
	Minimum	87990	98968	73.6	47.0
	Maximum	123512	159860	77.3	86.2
	Std. Error of Mean	11127	17830	--	10.0
purged	Mean	93552	132910	85.6	124.5
	N	4	4	3	3
	Std. Deviation	13322	24687	27.1	18.1
	Minimum	79484	118901	63.1	112.7
	Maximum	111475	169872	115.6	145.4
	Std. Error of Mean	6661	12344	15.6	10.5

* significant at p = 0.01 (ANOVA)

In general the removal of gut-contained soil did not significantly influence the body concentration in the worms, therefore non-purged worms may be used for calculating the bioaccumulation factor. In order to minimise the risk of underestimating the bioaccumulation factor if non-purged worms are used, it is recommended to allow for an additional set of worm samples (e.g. 4 replicates) to be purged at the end of the uptake phase. Body concentrations in these samples can be used to indicate any influence of gut-associated test substance, and, if necessary, to correct body concentrations in non-purged worms accordingly.

9 DISCUSSION

9.1 Test Method

9.1.1 Practicability

No major difficulties were reported by the ring rest participants. Some participants reported losses of ^{14}C-HCB after evaporation of the solvent during coating of the quartz sand. In order to minimise losses, it is recommended to keep the time allowed for evaporation of the solvent to a minimum, and to mix the coated quartz sand into the soil immediately after the quartz sand is dry.

9.1.2 Test Species

In general, both earthworms and enchytraeids appeared suitable as test organisms. Due to their size, earthworms were easier to handle and to weigh than the much smaller enchytraeids.

Mortalities were low in the majority of test runs. Problems were evident when testing enchytraeids in OECD soil. In some cases high mortalities occurred in treatment and in controls, which were most probably associated with the moisture and/or pH of the soil. This indicates that the soil parameters as described in section 0 need to be met closely and monitored during the test; earthworms appeared more tolerant towards slight variations of the mentioned soil parameters.

9.2 Non-eliminated Residues (NER)

In the ring test, ^{14}C-HCB was lost from the worm tissue nearly completely. The non-eliminated residues were 5.2% of the accumulated residues on average, and did not exceed 11.7%.

With Cadmium, elimination was highly variable, and in most cases the accumulated residues were not lost from the worms during the elimination phase. The average NER was 73.5%; the lowest NER was 31.5%.

9.3 Kinetic Modelling

In this ring test, two modelling approaches were applied. In the conventional approach, uptake and elimination kinetics are modelled separately (see sections 0 and 0). In the alternative approach, both uptake and elimination kinetics are integrated in one model equation (see section 0). It was shown that for the HCB kinetics, which followed a first order model, both approaches led to similar results.

Cadmium did not show the uptake and elimination pattern found for HCB. In many cases a linear increase of body residues over time was observed during the uptake phase. Also, most of the accumulated Cd residues remained in the worms during the elimination phase. Thus, the conventional one- or two compartment uptake and elimination models did not lead to reasonable results. In the case of Cadmium the alternative, combined model described the time series of data points much better, reflecting the measured values more accurately than the conventional models. It is therefore considered appropriate to use the combined model where the conventional models fail, e.g. for kinetics of metals.

10 CONCLUSIONS

The ring test has demonstrated that the test method was practicable and produced comparable results in the different laboratories. The mathematical tools available for evaluation of the test results proved to be appropriate. On the basis of the ring test protocol, the results obtained, and the discussions at the final ring test workshop, the earlier version of the draft test guideline was revised and refined according to OECD format.

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Van Brummelen, T.C. & Van Straalen, N.M. (1996). Uptake and elimination of benzo(a)pyrene in the terrestrial isopod *Porcellio scaber*. Arch. Environ. Contam. Toxicol. 31: 277-285.

Zok, S., Görge, G., Kalsch, W. & Nagel, R. (1991). Bioconcentration, metabolism and toxicity of substituted anilines in the zebrafish (*Brachydanio rerio*). Sci. Total Environment 109/110: 411-421.

12 ANNEXES

Annex 1: Minutes of the final workshop: Ring Test of the Test Method: “Bioaccumulation: Soil Test using terrestrial Oligochaetes” held at Coimbra/Curia, Portugal, May 17 - 18, 2007

Annex 2: Draft Test Guideline

Annex 1: Minutes of the final workshop: Ring Test of the Test Method: "Bioaccumulation: Soil Test using terrestrial Oligochaetes" held at Coimbra/Curia, Portugal, May 17 - 18, 2007

Minutes of the

F I N A L W O R K S H O P

Ring Test of the Test Method:

"Bioaccumulation: Soil Test using terrestrial Oligochaetes"

held at

Coimbra/Curia, Portugal, May 17 - 18, 2007

Sponsored by the

Federal Environmental Agency (Umweltbundesamt, UBA, Dessau, Germany)

Organised by

ECT Oekotoxikologie GmbH (Floersheim / Main, Germany)

and

Instituto do Ambentie e Vida
Dep. Zoologia da
Universidade de Coimbra
(Coimbra Portugal)

PARTICIPANTS IN THE WORKSHOP

M. Amorim	(Universidade de Aveiro, PT)
S. Chelinho	(Universidade de Coimbra, PT)
D. Gilberg	(ECT GmbH, DE)
R. Kuperman	(U.S. Army, USA)
R. Lanno	(Ohio State University, USA)
T. Leicher	(Bayer AG, DE)
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C. Norr	(BBA, DE)
S. Novais	(Universidade de Aveiro, PT)
J. Princz	(Environment Canada, CDN)
P. Robidoux	(NRC, CDN)
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G. Stephenson	(Stantec, CDN)
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R. Verweij	(Vrije Universiteit Amsterdam, NL)

Venue: Hotel Termas da Curia, Curia, 3780-541 Tamengos, Portugal

Minutes: Philipp Egeler & Adam Scheffczyk

WORKSHOP AGENDA

Thursday, May 17, 2007

Welcome, organisationals

Short introduction of the participants (who is who?)

Introduction and presentation of workshop agenda (Joerg Roembke)

Method Summary of Cd analysis in soil & worms (Kees van Gestel & Rudo Verweij)

Presentation of the ring test results: Cadmium (Joerg Roembke)
Kinetics:
Uptake and elimination
Ring test comparisons

Questions/Discussion

Method Summary of ^{14}C-HCB analysis in soil & worms (Daniel Gilberg)

Presentation of the ring test results: Hexachlorobenzene (Joerg Roembke)
Kinetics:
Uptake and elimination parameters
Ring test comparisons

Questions/Discussion

Summary of test conditions (Adam Scheffczyk)

Test system performance, discussion of:
Biological parameters (survival, reproduction, biomass)
Pre-exposure phases, choice of food source

Discussion of the concept of data evaluation (e.g. kinetics modelling, statistics)

Selection of relevant endpoints

Friday, May 18, 2007

Discussion of results, of advantages and drawbacks of the method (e.g. experiences, practical aspects), and possible modifications of the method

Summary of discussions

Implementation of agreed items into the draft guideline (I)

Implementation of agreed items into the draft guideline (II)

Summary of issues incorporated in the draft guideline

Discussion of further steps:

- Draft report of the ring test
- Submission of draft guideline to OECD

- Dissemination of ring test results (publications): Who, where and when?
- Future research to improve/refine the method

Résumé & end of the workshop

OPENING CONSIDERATIONS, INTRODUCTION OF PARTICIPANTS AND APPROVAL OF AGENDA

Welcome, short introduction of the participants

Introduction and workshop agenda, history of the project, legal requirements (Chemicals EC 2003; REACH RIP 3.3), key topics of the ring test were presented by J. Römbke. Introductory slides are shown in Annex I.

METHOD SUMMARY OF CD ANALYSIS IN SOIL & WORMS (R. VERWEIJ)

Samples from the tests were analysed for total amount of Cadmium by Atomic Absorption Spectometry (AAS). One lab used inductively-coupled plasma mass spectrometry (ICO-MS) for Cd-analysis. The following samples were taken according to the ring test protocol:

- stock solution (reserve)
- spiked soil: approximately 1 h after application
- control soil: approximately 1 h after application
- worm and soil samples during uptake and elimination phase.

Drying or freeze-drying of soil and worm samples was recommended in the ring test protocol.

Summary/slides on sample processing and Cd analysis as presented at the workshop (R. Verweij & C.A.M. van Gestel) are shown in Annex I.

Discussion
Weighing of dried worms appeared to be problematic especially for enchytraeids because of hygroscopic weight increase and electrostatic charge. Additionally, worm tissue tended to stick to vessel walls and was difficult to remove when dried. Worms should therefore be stored frozen before analysis, not dried in an oven.

Measuring of samples: For metal analyses it was recommended to analyse each sample twice or more often, at least for problematic heavy metals.

PRESENTATION OF THE RING TEST RESULTS: CADMIUM (J. RÖMBKE)

The presented Cd kinetics can be summarised in general: No steady state (linear uptake) and no elimination of Cd was found, which was considered most likely due to accumulation of Cd in certain organs. The slides shown at the workshop are shown in the Annex.

Discussion
Uptake and elimination phase can be assessed by two separate models, as was done to date with limited success, or with one model integrating both phases. The latter would assess the uptake and elimination

kinetics in one combined, more extensive model. An example of the kinetics calculation is given in the ring test report. This model was provided by K. van Gestel.

The following relevant references could be included in the revised draft guideline:

- Janssen, M.P.M., A. Bruins, T.H. De Vries & Van Straalen, N.M. (1991). Comparison of cadmium kinetics in four soil arthropod species. Arch. Environ. Contam. Toxicol. 20: 305-312.
- Van Brummelen, T.C. & Van Straalen, N.M. (1996). Uptake and elimination of benzo(a)pyrene in the terrestrial isopod *Porcellio scaber*. Arch. Environ. Contam. Toxicol. 31: 277-285.
- Sterenborg, I., Vork, N.A., Verkade, S.K., Van Gestel, C.A.M. & Van Straalen, N.M. (2003). Dietary zinc reduces uptake but not metallothionein binding and elimination of cadmium in the springtail *Orchesella cincta*. Environ. Toxicol. Chemistry 22: 1167-1171.

In the guideline, one model should be given. Options should be allowed but modified models should be named/referenced. Models should be modified according to the substance.

In the model proposed by K. van Gestel, a blank value of Cd (i.e. the background concentration of Cd in the worms on day zero) was used to calculate the BAF to account for different Cd concentration in different peat batches. The model shows a steady state after approx. 50 days.

In cases where a steady state is reached before the default end of the uptake phase (14 d for enchytraeids, and 21 d for earthworms, respectively), the elimination phase can be started earlier. This option is included in the revised draft guideline.

In case of a linear uptake, it was proposed to report the AF measured at the end of the uptake phase as BAF.

Non-eliminated residues (NER) should be expressed in per cent of the latest measured body concentration of the uptake phase.

Presentation of models and results by R. Kuperman and P.Y. Robidoux (to be added in the final version); implications of different statistics software; discussion of curves (O8, U.S. Army; O4, NRC).

Tests with *E. crypticus* were done with a different batch of peat (result O8, U.S. Army).

PRESENTATION: METHOD SUMMARY OF ^{14}C-HCB ANALYSIS IN SOIL & WORMS (D. GILBERG)

A summary of the methods, and the available results can be found in Annex I.

Discussion
All earthworm tests were performed with gut purging. Earthworm analysis was done by combusting. Enchytraeids were either combusted, or dissolved in soluene.

PRESENTATION OF THE RING TEST RESULTS: ^{14}C-HCB (J. RÖMBKE)

The presented kinetics can be summarised in general: A steady state was reached after less than 12 days of exposure. The radioactive residues were eliminated from the worms by the end of the elimination phase (approx. 90% of the activity at start of elimination). The slides shown at the workshop are shown in Annex I.

Discussion
Steady state was reached, and elimination of ^{14}C-HCB was done with both test systems. The *E. albidus* test in OECD soil performed in lab O5 will be re-calculated since the water content of the animals appears considerably higher than all other ring test data.

The tests with OECD soil done at Universidade de Coimbra were performed with a different peat (5.8% organic matter; 3.9% total organic carbon content). The respective results will be recalculated and normalised to lipid/total organic carbon content (D. Gilberg). Universidade de Coimbra will start another test with enchytraeids (natural soil).

A decrease of BAF between day 14 and 21 was noted by two labs (O9, O11). This finding led to the question whether possible malnutrition could be a reason for decreasing chemical uptake, and to the discussion of an additional validity criterion for earthworm tests (upper limit for weight decrease). The ring test data will be evaluated with respect to decreasing accumulation towards the end of the uptake phase.

Mortality in the enchytraeids tests was in general below 20%, indicating that the validity criteria were fulfilled. In lab O5, enchytraeid mortality exceeded 20%. Possible reasons are still under discussion.

SUMMARY OF TEST CONDITIONS (A. SCHEFFCZYK)

The general test conditions were summarised. The corresponding slides are shown in Annex I.

Discussion

Soil
Comment:
Artificial soil with 5% peat should be included into the guideline as a worst-case option regarding adsorption of the tested substance. It was stated by several participants that artificial soil with 5% peat works well for *E. fetida* and for enchytraeids. Less than 3% peat may however lead to decreased reproduction in earthworm tests.

Related references:

EPPO (2003). Environmental risk assessment scheme for plant protection products. Chapter 8. Soil organisms and functions. Bull. OEPP/EPPO Bull. 33; 195 – 209.

van Gestel CAM (1992). The influence of soil characteristics on the toxicity of chemicals for earthworms; a review. In: Ecotoxicology of Earthworms (Ed. Becker, H, Edwards, PJ, Greig-Smith, PW & Heimbach, F). Intercept Press, Andover (GB).

In lab O10 (Universidade de Coimbra), two mediterranean field soils were also tested. The following table describes the characteristics of these soils.

	OECD soil	LIT	LV	Units
ph_H_20		5.23	5.5	pH (H_2O)
ph_KCl	6.1	4.55	4.35	pH(KCl)
Organic matter	5.8	4.2	2	%
P		25	90	ppm P_2O_5
K		122	78	ppm K_2O
Ca ext.(ammonium acetate)		453	515	ppm Ca
Mg ext.(ammonium acetate)		95	115	ppm Mg
Coarse sand		41.90	29.78	%
Fine sand		24.82	38.23	%
Sand	86.60	66.72	68.01	%
Silt	2.70	21.55	20.29	%
Clay	10.69	11.73	11.28	%
Texture	Loamy sand	Sandy-Loam	Sandy-Loam	
Mineral N		49	18	ppm
Total N		0.16	0.08	%
C/N		15.25	14.50	
Exch. Ca		2.98	3.23	meq Ca/100 g
Exch. Mg		0.99	1.18	meq Mg/100 g
Exch. K		0.21	0.12	meq K/100 g
Exch. Na		0.05	0.23	meq Na/100 g
CEC		8.64	9.92	meq/100 g
Sat.		48.9	48.7	%sat
Na		73	15	ppm Na
Total Cu	20	40	23	ppm Cu
Total Fe	2730	46370	30900	ppm Fe
Total Mn	12	211	392	ppm Mn
Total Zn	15	67	54	ppm Zn
Total Cd	0.1	<5,6	<5,6	ppm Cd
Total Cr	8	21	24	ppm cr
Total Pb	10	16	19	ppm Pb
Total Co	0.12	<24	<24	ppm Co
Total Ni	3	48	28	ppm Ni
Organic Carbon	3.9	2.44	1.16	%
WHC	61.3	42.38	32.05	%

It was argued that ageing of Lufa soil appeared critical (fungal growth), resulting in higher observed mortality. Suggestions for appropriate storage conditions and/or maximum storage period should be given in the revised draft guideline.

At one participating laboratory (O9, BayerCropScience) soil avoidance of the earthworms was observed. It was suggested to see for appropriate covering of the test vessels.

Validity Criteria

It was proposed to include an additional validity criterion for tests with earthworms: The biomass loss in earthworms should not exceed 20% by the end of the test. A weight loss of up to 20% of the initial wet weight was considered acceptable to demonstrate that test conditions and constitution of the test organisms are appropriate. To assess the weight loss, the worms will have to be weighed before and after gut purging.

DRAFT GUIDELINE REVISION

The agreed items were implemented into the draft guideline. The changes are visible for commenting and supplementing in the revised draft document (Annex III).

OUTLOOK

Workshop: Bioaccumulation in Terrestrial Oligochaetes Coimbra, Portugal, May 17-18, 2007

Outlook

Data under evaluation:

-Biota-to-soil Accumulation Factors (BSAF)

-Details of test conditions in individual tests

-Detailed evaluation of nominal & actual test concentrations

-Biological performance of the test species

-Evaluation of field soil influence on bioaccumulation

-Evaluation of ring test variability/reproducibility

The ring test report will be available in spring 2008.

Further Tasks:

The type and quantity of food applied (in g/kg dry soil; feeding frequency) will be reported by each participating laboratory if not already done.

The origin of the test animals, and the confirmation of species identity will be recorded in the raw data, reported in detail by the respective participating laboratory, and summarised in the report.

Manuscripts covering parts of the ring test are being prepared.

Annex I: Slides shown at the workshop

Introduction (J. Römbke)

"Bioaccumulation with Terrestrial Oligochaetes - an International Ring Test"

Final Workshop

Coimbra, Portugal, May 17-18, 2007

Funded by the Federal Environmental Agency
(Umweltbundesamt, UBA, Dessau, Germany)

Organised by ECT Oekotoxikologie GmbH
(Floersheim / Main, Germany)

and

Instituto do Ambiente e Vida
Dep. Zoologia da Universidade de Coimbra
(Coimbra, Portugal)

Workshop: Bioaccumulation in Terrestrial Oligochaetes Coimbra, Portugal, May 17-18, 2007

"Bioaccumulation with Terrestrial Oligochaetes - an International Ring Test"

Final Workshop

Coimbra, Portugal, May 17-18, 2007

Introduction to the Workshop

Jörg Römbke & Philipp Egeler

Organised by ECT Oekotoxikologie GmbH
(Floersheim / Main, Germany)

Workshop: Bioaccumulation in Terrestrial Oligochaetes Coimbra, Portugal, May 17-18, 2007

1. Legal requirements for soil bioaccumulation tests I

Chemicals (EC 2003):
Bioaccumulation in soil organisms is part of the assessment of secondary poisoning via the food chain but is less relevant than in aquatics. Tests can (rarely) be asked for depending on the substance properties. Experimental data are preferred but not required.

REACH RIP 3.3 (EU 2007):
Besides in the context of secondary poisoning potentially bioaccumulation testing may be required in decisions on long-term soil organism toxicity

Depending on the amount manufactured or imported (> 10 tpa/1000 tpa), test data on earthworm bioaccumulation may be needed.

Workshop: Bioaccumulation in Terrestrial Oligochaetes Coimbra, Portugal, May 17-18, 2007

1. Legal requirements for soil bioaccumulation tests II

REACH RIP 3.3 (EU 2007):
Integrated Test Strategy (ITS) for soil bioaccumulation in the context of Chemical Safety Assessment (hierarchy of data):

1. BCF determined in standard laboratory tests (ASTM 2004; OECD ?)
2. Assessment based on read-across information (i.e. using sediment tests with *Lumbriculus variegatus*) – not from fish.
3. Usage of information from field studies (rarely available)
4. Prediction of bioaccumulation based on QSAR
5. First assessment of bioaccumulation: Based on octanol-water partition coefficient (Kow)

In reality, the risk assessment will probably be performed just the other way around.

Workshop: Bioaccumulation in Terrestrial Oligochaetes Coimbra, Portugal, May 17-18, 2007

1. Legal requirements for soil bioaccumulation tests III

No real change since the Kick-off Workshop in 2005

Pesticides (EU 1991, 2003):
In contrast to the assessment for the **aquatic compartment**, the testing of bioaccumulation in soil can be required in the context of **secondary poisoning** in the soil food web.

Biocides (EU 1998) and drugs (EMEA 2005):
No specific requirement for terrestrial bioaccumulation.

Soil quality assessment:
Despite some examples, not an issue in Europe or North America

Workshop: Bioaccumulation in Terrestrial Oligochaetes Coimbra, Portugal, May 17-18, 2007

2. History of this project

1993:	OECD included a soil bioaccumulation test in its "Test Developmental Program"
1996 - 1998:	Literature review on soil bioaccumulation supported by UBA
1999 – 2001:	UBA sponsored the standardisation of a test method
2002:	Germany proposed a new test guideline at OECD
2004:	UBA sponsors a ring test using the draft protocol
2005:	Kick-off workshop of the ring test (Rüsselsheim, Germany)
2005 – 2007:	Ring test performance
2007:	Final workshop of the ring test (Coimbra, Portugal)
2008:	**Submission of draft test guideline to OECD?**

Workshop: Bioaccumulation in Terrestrial Oligochaetes Coimbra, Portugal, May 17-18, 2007

3. Structure of the ring test group I

Number of institutions present at the Kick-off Workshop:	15
Number of institutions participating in the ring test:	12
Institutions from Academia, Government or Industry:	5 / 5 / 2
Countries represented:	7

Canada, Czech Republic, Germany, Netherlands, Portugal, Russia, USA

Number of institutions present at this Final Workshop:	13
Institutions from Academia, Government or Industry:	5 / 5 / 3
Countries represented:	6

Canada, Czech Republic, Germany, Netherlands, Portugal, USA

Workshop: Bioaccumulation in Terrestrial Oligochaetes Coimbra, Portugal, May 17-18, 2007

3. Structure of the ring test group II

Testing activities planned:	**HCB**	**Cadmium**	**Total**
with earthworms	**5**	**11**	**16**
with enchytraeids	**3**	**10**	**13**
Sum	**8**	**21**	**29**

Additional activities planned:

- Bioaccumulation of selenium in soil oligochaetes (2 partner)
- Additional concentrations (2 partner)
- Additional soil (LUFA) (3 partner)
- Comparison of gut-purging (1 partner)
- **Metal analysis (1 partner)**

4. Key topics of the ring test performance

4.1 Suitable test organisms for soil bioaccumulation tests

Earthworms and enchytraeids were selected because:

- They fulfilled criteria like ecological relevance, easy handling, relevant exposure, practicability
- Ecotoxicological toxicity test guidelines are available
- High number of papers published on oligochaete bioaccumulation
- The suitability of the two enchytraeid species has to be discussed.

Despite some work with isopods and snails there is no obvious alternative……

4. Key topics of the ring test performance

4.2 Selection of "good" model substances

Hexachlorobenzene and cadmium were selected because:

- They are representatives of persistent organics and heavy metals
- Both have widely been used in ecotoxicological toxicity tests as well as in bioaccumulation studies with oligochaetes
- Analytical methods are available
- Both are easily and (relatively) cheap

In hindsight, and despite potential alternatives, no better substances became obvious during the project……

4. Key topics of the ring test performance

4.3 Selection of appropriate test substrates and test conditions

OECD Artificial Soil (optional LUFA St. 2.2 Soil) and the test conditions as in other OECD standard tests were selected because:

- They are relevant for current legal requirements (e.g. 91/414/EC for pesticide registration)
- Available experience shows that they work
- Usage of OECD requirements allows global comparability

As it will be shown later during this workshop, these decisions have been backed up by the experiences during the ringtest.

5. Status of deliverables

	^{14}C-HCB			
	Eisenia sp.		*Enchytraeus* sp.	
data sets	OECD soil	Field soil	OECD soil	Field soil
evaluated	5	3	3	3
delivered	6	3	3	3
expected	6	3	3	3

	$CdCl_2$			
	Eisenia sp.		Enchytraeus sp.	
data sets	OECD soil	Field soil	OECD soil	Field soil
evaluated	7	3	5	3
delivered	7	3	7	3
expected	13	7	13	8

6. Summary

Before going into details of ring test results and recommending a draft guideline the following statements can be made:

Legal requirements:

Slowly, the need for a soil bioaccumulation test becomes more obvious

Selection of key topics:

With the knowledge of today, the decisions made would probably be the same as back in 2005.

Participation in the ring test:

The interest of partners as well as their commitments to deliver data were mostly fulfilled.

Finally, we would like to thank those who made the ring test as well as this workshop possible:

- **The German Umweltbundesamt and in particular Ms. Gabriele Stuhlinger for their support.**
- **The colleagues from the University of Coimbra for the preparation of this event.**
- **Philipp Egeler**

Slides on sample processing and Cd analysis as presented at the workshop (R. Verweij & C.A.M. van Gestel).

Cadmium analysis in soil, earthworms and enchytraeids

Rudo Verweij & Kees van Gestel

Department of Animal Ecology
Institute of Ecological Science,
Vrije Universiteit, Amsterdam

General principle of methods

1. Drying of samples by freeze drying or in oven
2. Precisely weighing of samples and transfer into digestion tube
3. Digestion of samples in mixture of concentrated acids
4. Analysis of samples by Atomic Absorption Spectrometry (AAS) at wavelength of 228.9 nm
5. Quality control: analysing certified reference materials

Soil samples

1. 100 -150 mg dry soil sample in teflon digestion bomb
2. 2 ml of mixture of concentrated HNO_3 and HCl (4:1)
3. Digestion of samples: 7 h in a stove at 140 ℃
4. Water added to make volume of 7 or 10 ml
5. Analysis of samples by flame AAS at wavelength of 228.9 nm
6. Detection limit: 7 µg/l or 0.32-0.7 µg/g dry soil

Soil samples (continued)

1. Quality control: certified reference soils
   BCR 145: Sewage Sludge
2. Problem:
   Control soils always below detection limit for flame AAS

Which samples analysed:
Uptake phase: treated and control soils (6 samples)
Elimination phase: = control soil (6 samples)

Earthworm samples

1. Individual dry earthworms in teflon digestion tubes

→ → → → 2 ml of mixture of concentrated HNO_3 and HCl (4:1)

1. Digestion of the samples in a stove (140℃)
2. Adding water to make volume of 7 ml
3. Analysis of samples by flame AAS at wavelength of 228.9 nm
4. Detection limit: 7 µg/l or approx. 1 µg/g dry earthworm

Earthworm samples (continued)

1. Quality control: certified reference material
   BCR 145: Sewage Sludge
2. Problems:
   Should have taken organic reference sample e.g. DOLT2 (Dog Fish Liver)

   All samples analyzed

Enchytraeid samples

1. Pooled dry enchytraeids from one replicate test container (sampling) in 1 ml pyrex digestion tube
2. 500 µl of mixture of concentrated HNO_3, $HClO_4$ (7:1)
3. Digestion of samples on heating block
4. Evaporation to dryness
5. Residue taken up in 500 µl or 1 ml 0.1 M HNO_3.
6. Analysis of samples by graphite furnace AAS with Zeeman correction.

Enchytraeid samples (continued)

1. Detection limit: 0.224 µg/l or 0.025 µg/g dry sample
2. Quality control: certified reference material

   Dog Fish Liver (DOLT2)

3. Problems:

   Difficulties with weighing due to static electricity and hygroscopic nature of material

Methods and results for evaluation of Cadmium kinetics (D. Gilberg & J. Römbke)

Session 1: Cadmium

Method Summary of Cd analysis in soil & worms
(Kees van Gestel & Rudo Verweij)

Presentation of the ring test results:
(Joerg Roembke)
- Kinetics:
  - Models
  - Uptake and elimination
  - Ring test comparisons

Questions/Discussion

Statistical evaluation (non-linear regression analysis)

Uptake Model: $BAF*(1-(exp^{(-ke*days)}))$

Evaluated data: concentration in worm dry weight,
AF (dw/dw) single and mean values per sampling

Steady state?:
difference between min and max of last 3 sampling means:
< 40% of total mean

Elimination Models:

1-compartment model: $c_a*EXP^{(-ke*days)}$

2-compartment model: $c_a*exp^{(-ka*t)}+c_b*exp^{(-kb*t)}$

Criterion: fit (r^2) and/or display of 2 lines on log scale

Evaluated data: conc. (dw) single and mean values per sampling,
conc. (dw) in % of residues at end of uptake

Ring test results: Cadmium

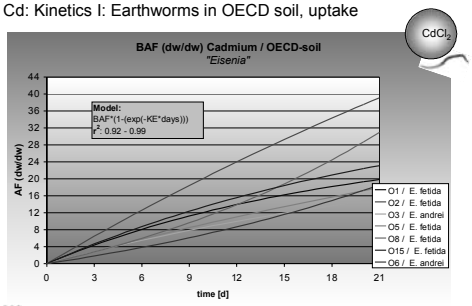
Examples of uptake and elimination

Ring test comparisons

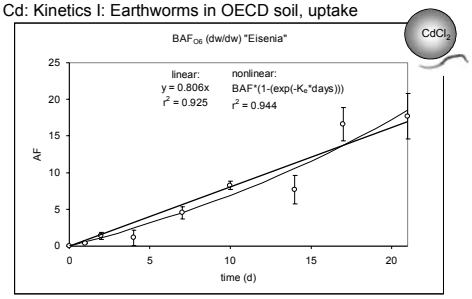
(Joerg Roembke)

$CdCl_2$

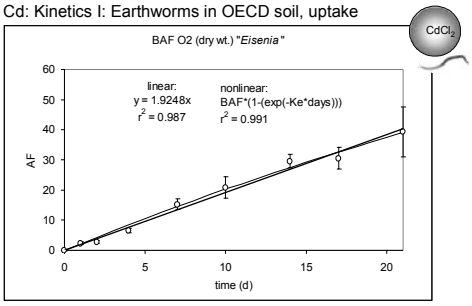
Cd: Kinetics I: Earthworms in OECD soil, uptake



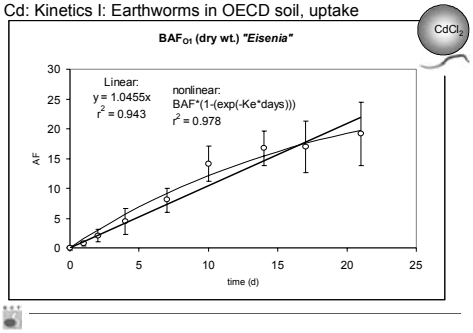
Cd: Kinetics I: Earthworms in OECD soil, uptake



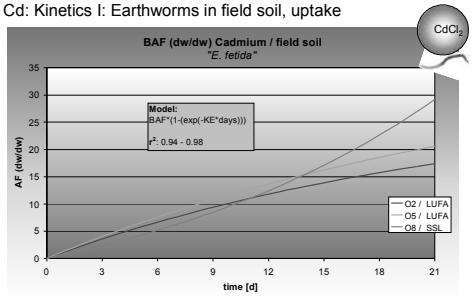
Cd: Kinetics I: Earthworms in OECD soil, uptake



Cd: Kinetics I: Earthworms in OECD soil, uptake

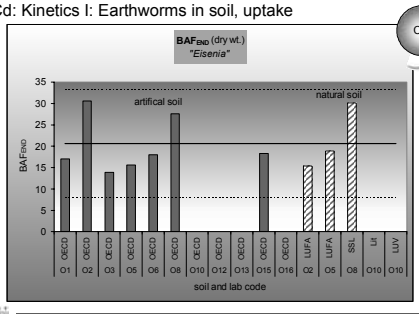


Cd: Kinetics I: Earthworms in field soil, uptake



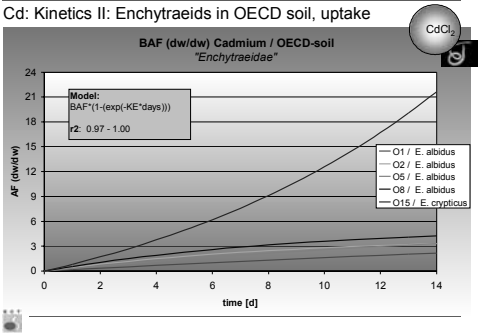
Workshop: Bioaccumulation in Terrestrial Oligochaetes Coimbra, Portugal, May 17-18, 2007

Cd: Kinetics I: Earthworms in soil, uptake



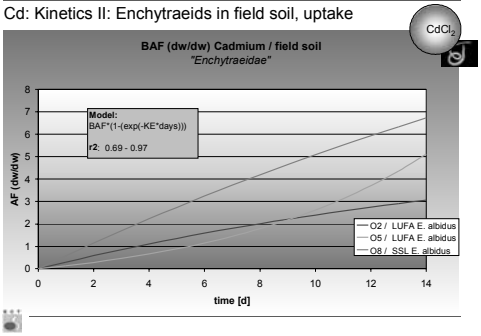
Workshop: Bioaccumulation in Terrestrial Oligochaetes Coimbra, Portugal, May 17-18, 2007

Cd: Kinetics II: Enchytraeids in OECD soil, uptake



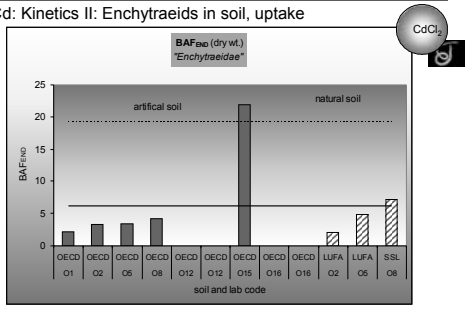
Workshop: Bioaccumulation in Terrestrial Oligochaetes Coimbra, Portugal, May 17-18, 2007

Cd: Kinetics II: Enchytraeids in field soil, uptake



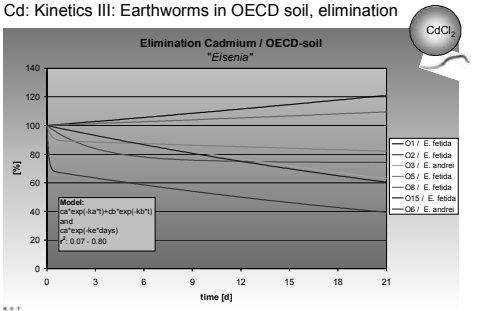
Workshop: Bioaccumulation in Terrestrial Oligochaetes Coimbra, Portugal, May 17-18, 2007

Cd: Kinetics II: Enchytraeids in soil, uptake



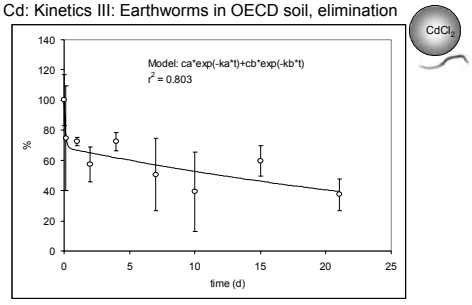
Workshop: Bioaccumulation in Terrestrial Oligochaetes Coimbra, Portugal, May 17-18, 2007

Cd: Kinetics III: Earthworms in OECD soil, elimination



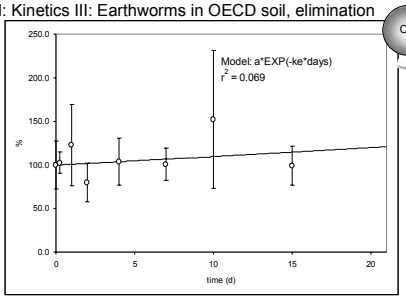
Workshop: Bioaccumulation in Terrestrial Oligochaetes Coimbra, Portugal, May 17-18, 2007

Cd: Kinetics III: Earthworms in OECD soil, elimination



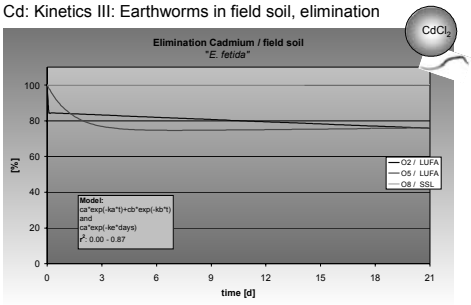
Workshop: Bioaccumulation in Terrestrial Oligochaetes Coimbra, Portugal, May 17-18, 2007

Cd: Kinetics III: Earthworms in OECD soil, elimination

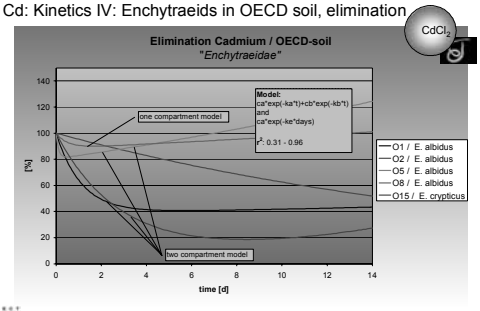


Workshop: Bioaccumulation in Terrestrial Oligochaetes Coimbra, Portugal, May 17-18, 2007

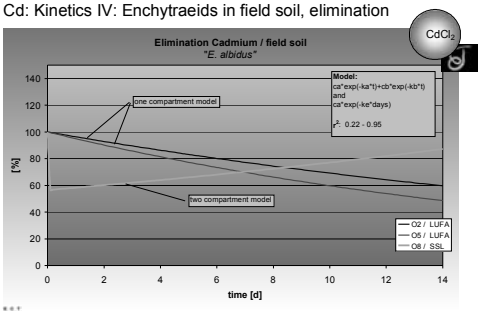
Cd: Kinetics III: Earthworms in field soil, elimination



Cd: Kinetics IV: Enchytraeids in OECD soil, elimination



Cd: Kinetics IV: Enchytraeids in field soil, elimination



Cd: Kinetics : BAF Earthworms / Enchytraeids

	Eisenia; OECD				*Eisenia*; field soil			
Parameter	Mean	SD	Range	N	Mean	SD	Range	N
$BAF_{end; uptake}$	22.5	8.5	13.8 – 39.3	7	21.9	7.2	16.7 – 30.1	3
	Enchytreidae; OECD				*Enchytreidae*; field soil			
Parameter	Mean	SD	Range	N	Mean	SD	Range	N
$BAF_{end; uptake}$	7.0	8.4	2.2 – 21.9	5	4.7	2.6	2.1 – 7.2	3

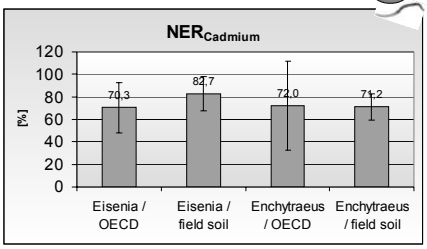
Cd: Kinetics : Elimination & NER (%)
Earthworms

E. fetida	OECD	O1	99,1
E. fetida	OECD	O2	70,3
E. andrei	OECD	O3	67,2
E. fetida	OECD	O5	92,0
E. andrei	OECD	O6	37,4
E. fetida	OECD	O8	79,5
E. fetida	OECD	O15	46,3
E. fetida	LUFA	O2	72,0
E. fetida	LUFA	O5	76,0
E. fetida	SSL	O8	100,2

Cd: Kinetics : Elimination & NER (%)
Enchytraeids

E. albidus	OECD	O1	41,4
E. albidus	OECD	O2	61,2
E. albidus	OECD	O5	122,0
E. albidus	OECD	O8	103,7
E. crypticus	OECD	O15	31,5
E. albidus	LUFA	O2	66,1
E. albidus	LUFA	O5	62,9
E. albidus	SSL	O8	84,7

Cd: Kinetics : Elimination & NER
Earthworms / Enchytraeids



Presentation of the methods and ring test results for ^{14}C-HCB (D. Gilberg & J. Römbke)

Session 2: ^{14}C-HCB

Method Summary of ^{14}C-HCB analysis in soil & worms (Daniel Gilberg)

Presentation of the ring test results: (Joerg Roembke)
- Kinetics:
  - Models
  - Uptake and elimination
  - Ring test comparisons

Questions/Discussion

Statistical evaluation (non-linear regression analysis)

Uptake Model: $BAF*(1-(exp^{(-ke*days)}))$

Evaluated data: concentration in worm dry weight,
AF (dw/dw) single and mean values per sampling

Steady state?:
difference between min and max of last 3 sampling means:
< 40% of total mean

Elimination Models:

1-compartment model: $c_a*EXP^{(-ke*days)}$

2-compartment model: $c_a*exp^{(-ka*t)}+c_b*exp^{(-kb*t)}$

Criterion: fit (r^2) and/or display of 2 lines on log scale

Evaluated data: conc. (dw) single and mean values per sampling,
conc. (dw) in % of residues at end of uptake

Summary of ^{14}C-HCB analysis (Total radioactive residues)

Test solution(s), trapping solutions:

- Test start
  -Application solutions (hexane)
- Test end
  - TEG Triethylenclycol for volatile organic compounds (3-5mL)
  - NaOH/KOH for CO_2–trapping (3-5mL)

direct measurement by Liquid Scintillation Counting (LSC)

Summary of ^{14}C-HCB analysis (Total radioactive residues)

Soil: combustion in sample oxidizer
- 250 – 1000 mg soil wet weight
- placed in combusto cone
- dried over night at room temperature
- combusted under an oxygen stream
- CO_2 catched with an liquid adsorber

Liquid Scintillation Counting (LSC)

Summary of ^{14}C-HCB analysis (Total radioactive residues)

Earthworms: combustion in sample oxidizer
- wet earthworms
- placed in pre-weight LSC-vials
- placed in a freezer
- thawed and placed in combusto cones
- combusted under an oxygen stream
- CO_2 catched with an liquid adsorber

Liquid Scintillation Counting (LSC)

Summary of ^{14}C-HCB analysis (Total radioactive residues)

Enchytraeids: tissue solubiliser
- wet enchytraeids
- placed in pre-weight LSC-vials
- placed in a freezer
- thawed and topped with tissue solubiliser
- heating (60°C, approx. 1h)
- neutraliesed with HCl

Liquid Scintillation Counting (LSC)

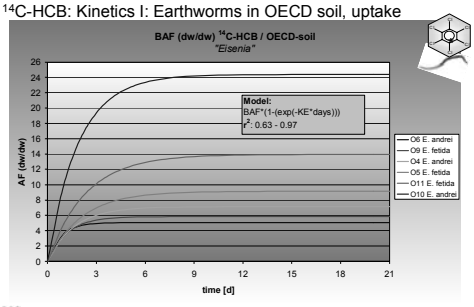
Ring test results: ^{14}C-HCB

Uptake and elimination

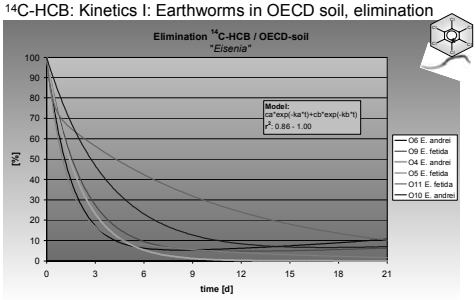
Ring test comparisons

(Joerg Roembke)

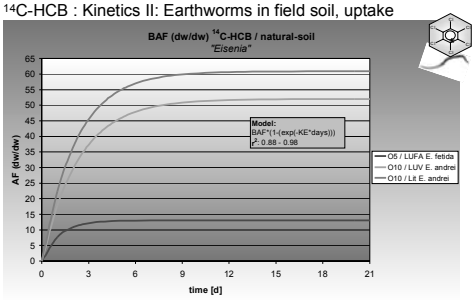
^{14}C-HCB: Kinetics I: Earthworms in OECD soil, uptake



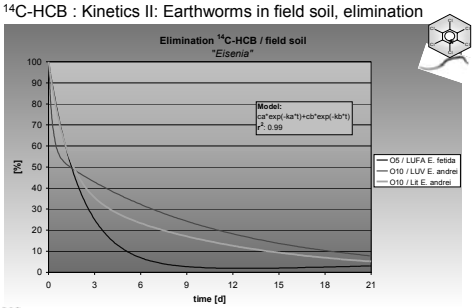
^{14}C-HCB: Kinetics I: Earthworms in OECD soil, elimination



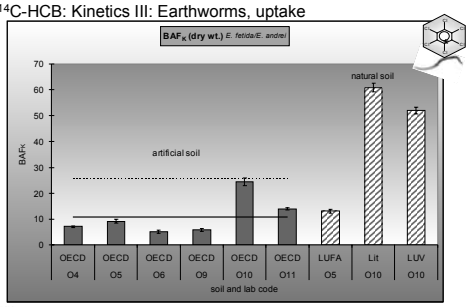
^{14}C-HCB : Kinetics II: Earthworms in field soil, uptake



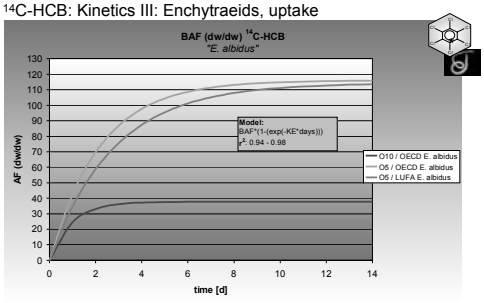
^{14}C-HCB : Kinetics II: Earthworms in field soil, elimination



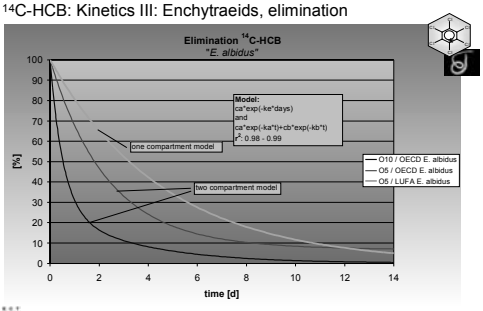
^{14}C-HCB: Kinetics III: Earthworms, uptake



^{14}C-HCB: Kinetics III: Enchytraeids, uptake



^{14}C-HCB: Kinetics III: Enchytraeids, elimination



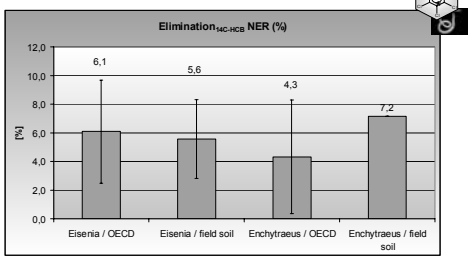
Kinetic Parameters: Uptake

Species	Lab code	Soil	BAF_K (dw/dw) (g soil g^{-1} worm)	Std. Error of BAF_K	k_s (g soil g^{-1} worm d^{-1})	k_s (d^{-1})	Std. Error of k_s
E. fetida / E. andrei	O4	OECD	7.1	0.3	4.4	0.62	0.13
	O5	OECD	9.1	0.7	4.4	0.48	0.17
	O6	OECD	5.1	0.6	6.2	1.22	0.89
	O9	OECD	5.8	0.6	4.9	0.83	0.46
	O10	OECD	24.4	1.4	12.9	0.53	0.14
	O11	OECD	13.9	0.4	6.0	0.43	0.06
	O5	LUFA	13.1	0.7	11.6	0.89	0.27
	O10	Lit	60.9	1.7	27.9	0.46	0.06
	O10	LUV	52.0	1.3	22.0	0.42	0.04
E. albidus	O5	OECD	116.1	5.0	53.31	0.46	0.08
	O10	OECD	37.8	1.6	39.6	1.05	0.24
	O5	LUFA	114.3	4.4	41.3	0.36	0.05

Kinetic Parameters: Elimination

Species	Lab code	Soil	A (%)	k_a (d^{-1})	B (%)	k_b (d^{-1})	NER (%) (end of test)
E. fetida / E. andrei	O4	OECD	40.6	4.17	59.4	0.27	2.9
	O5	OECD	86.5	0.84	13.5	0.10	2.3
	O6	OECD	97.0	0.65	3.0	-0.06	11.7
	O9	OECD	97.3	0.46	2.7	-0.04	4.4
	O10	OECD	97.5	0.27	2.5	-0.05	6.6
	O11	OECD	74.9	0.09	25.1	3.38	8.6
	O5	LUFA	99.4	0.47	0.6	-0.08	2.6
	O10	Lit	56.9	0.09	43.1	4.20	6.0
	O10	LUV	41.9	0.10	58.1	0.86	8.1
E. albidus	O5	OECD	8.2	0.01	91.8	0.4	7.1
	O10	OECD	26.7	0.30	73.3	1.9	1.5
	O5	LUFA	100.0	0.22	--	--	7.2

Kinetic Parameters: Elimination / NER (%)



Summary of general test conditions (Adam Scheffczyk)

Session 3: Test system performance

Summary of test conditions (Adam Scheffczyk)

Discussion of test system performance

Biological parameters (survival, reproduction, biomass)
Pre-exposure phases, choice of food source

Discussion of data evaluation (e.g. kinetics modelling, statistics)

Selection of relevant endpoints

Test organisms

Earthworms	**Enchytraeids**
Eisenia fetida/andrei	*Enchytraeus albidus* *Enchytraeus crypticus*

Test substrates:

➢ artificial soil:	➢ field soils:
according to OECD guideline 207	Lufa soil 2.2, US soil (Sassafras) mediterran. soils (LIT, LUV)

Test conditions

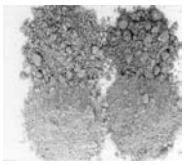
Temperature: **18 - 22 °C**
Light conditions: **16 : 8 (light : dark) at 100 - 1000 lx**
Moisture: **35 – 55% of dry wt.** (depending on water holding capacity)
Feeding: as in culture once at start of each phase

Test vessels:
glass/plastic beakers with lid
50 mL/18 - 20 g dry soil (enchytraeids)
250 mL/33 - 39 g dry soil (earthworms)

Test substrates:

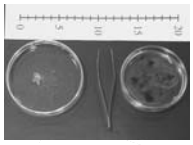
Parameter	Artificial soil	Natural soil
	OECD guideline 207	Lufa soil 2.2
pH-value	6.0 ± 0.5	5.7
Org. Carbon	5.6 %	2.2 %
Moisture	40 - 60% of WHC	40 -60% of WHC
Sand content	70 %	73 %

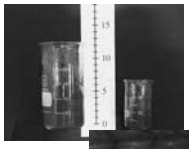
Additional field soils



Artificial and natural field soil (moist and dry mixtures)

Test organisms **Test vessels**





E. fetida

E. albidus

photo: T. Natal da Luz

Validity criteria:

overall mortality during uptake and elimination phase:

-earthworms up to 10%

-enchytraeids up to 20%

Annex II: Workshop Participants

Figure 7: Workshop Participants.

Address list of workshop participants

Monica	Amorim	Departamento de Biologia, Universidade de Aveiro, 3810-193 Aveiro	Portugal
Sonia	Chelinho	Instituto do Ambiente e Vida, Dep. Zoologia da Universidade de Coimbra, Lg. Marquês de Pombal, 3004-517 Coimbra	Portugal
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Sara	Novais	Departamento de Biologia, Universidade de Aveiro, 3810-193 Aveiro	Portugal
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Tiago	Natal da Luz	Instituto do Ambiente e Vida, Dep. Zoologia da Universidade de Coimbra, Lg. Marquês de Pombal, 3004-517 Coimbra	Portugal
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Juliska	Princz	Biological Methods Division, Environmental Technology Centre, Environment Canada, 335 River Road, Ottawa, Ontario, Canada, K1A 0H3	Canada
Pierre Yves	Robidoux	National Research Council (NRC), Biotechnology Research Institute, 6100 Royalmount Ave., Montreal Quebec, H4P 2R2	Canada
Jörg	Römbke	ECT Oekotoxikologie GmbH, Böttgerstr. 2-14, D-65439 Flörsheim/Main	Germany
Adam	Scheffczyk	ECT Oekotoxikologie GmbH, Böttgerstr. 2-14, D-65439 Flörsheim/Main	Germany
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Michael	Simini	U.S. Army Edgewood Chemical Biological Center, AMSRD-ECB-RT-TE E5641, 5183 Blackhawk Road, Aberdeen Proving Ground MD 21010-5424	USA
Markus	Simon	Fraunhofer Institut fuer Molekularbiologie und Angewandte Oekologie, (Fraunhofer-IME), D-57392 Schmallenberg	Germany
Ben	Smith	Stantec Consulting Ltd., 361 Southgate Dr., Guelph, ON, N1G 3M6	Canada
Paulo	Sousa	Instituto do Ambiente e Vida, Dep. Zoologia da Universidade de Coimbra, Lg. Marquês de Pombal, 3004-517 Coimbra	Portugal
Gladys	Stephenson	Stantec Consulting Ltd., 361 Southgate Dr., Guelph, ON, N1G 3M5	Canada
Kees	van Gestel	Dept. Animal Ecology, Institute of Ecological Science, Vrije Universiteit, De Boelelaan 1085, 1081 HV Amsterdam	The Netherlands
Rudo	Verweij	Dept. Animal Ecology, Institute of Ecological Science, Vrije Universiteit, De Boelelaan 1085, 1081 HV Amsterdam	The Netherlands

Annex 2: Draft Test Guideline

Second Draft

December 2008

PROPOSAL FOR A NEW GUIDELINE

for

OECD GUIDELINES FOR THE TESTING OF CHEMICALS

BIOACCUMULATION IN TERRESTRIAL OLIGOCHAETES

Second Draft

December 2008

TABLE OF CONTENTS

INTRODUCTION

1. Within the OECD test guideline programme, adopted standardised bioaccumulation guidelines exist for fish and sediment oligochaetes bioaccumulation tests up to now (OECD 1996; OECD 2007). However, the extrapolation of aquatic bioaccumulation data to terrestrial organisms like earthworms is difficult, if possible at all. Therefore, model calculations based on a compound's lipophilicity (e.g., Connell & Markwell 1990) are currently used for the assessment of bioaccumulation of chemicals in the soil ecosystem, as e.g. in the EU Technical Guidance Document (EC 2003). The need for a compartment-specific test method was already addressed more than a decade ago (e.g., Phillips 1993). Such a method is especially important for the evaluation of secondary poisoning in terrestrial food chains (Beek et al. 2000). Several national guidelines address the issue of bioaccumulation in organisms other than fish (e. g., ASTM 2000, and U.S. EPA 2000). More importantly, a guideline describing the measurement of bioaccumulation from contaminated soils in earthworms (*Eisenia fetida*, Savigny) and potworms has recently been adopted (ASTM 2004). The development, standardisation and implementation of an internationally accepted, standardised method for the experimental determination of bioaccumulation in spiked soil can therefore considerably improve the risk assessment for chemicals in terrestrial ecosystems (e.g., EPPO 2003, Füll et al. 2003).

2. Soil-ingesting invertebrates are subject to high exposure to soil bound substances and should therefore be given preferential attention. Among these animals, terrestrial oligochaetes play an important role for the structure and the function of the soil ecosystem (Didden 1993; Edwards & Bohlen 1996). They live in the soil and, partly, at the soil surface (especially the litter layer) and often represent the most abundant species if considered in terms of biomass (Petersen & Luxton 1982). By bioturbation of the soil and by serving as prey these animals can have a strong influence on the bioavailability of such substances to other organisms like invertebrate (e.g. predatory mites and beetles; e.g. Schlosser & Riepert (1992)) or vertebrate (e.g. foxes and gulls) predators (Romijn et al. 1993; Dietrich et al. 1995). Some species of terrestrial oligochaetes that are currently used in ecotoxicological testing are described in Annex 5.

3. The ASTM Standard Guide for Conducting Laboratory Soil Toxicity or Bioaccumulation Tests with the Lumbricid Earthworm *Eisenia fetida* and the Enchytraeid Potworm *Enchytraeus albidus* (ASTM 2004) provides many essential and useful details for the performance of the presented soil bioaccumulation method. Further documents that are referred to in this draft guideline are the OECD Guideline 305: Bioconcentration: Flow-through Fish Test (OECD 1996) and the OECD Draft Guideline: Bioaccumulation: Sediment test using benthic oligochaetes (OECD 2007). Practical experience with soil bioaccumulation studies and their standardisation, and details from literature (e.g., Belfroid et al. 1994; Füll 1996; Widianarko & Van Straalen 1996; Marinussen et al. 1997; Khalil 1990; Posthuma et al. 1996; Römbke et al. 1998; Amorim 2000; Bruns et al. 2001, Jager et al 2003b, Van Straalen et al. 2005, Vijver et al 2005) are also major sources of information for drawing up this draft.

4. The method described is sufficiently detailed so that the test can be carried out whilst allowing for adaptations in the experimental design depending on the varied characteristics of test items and the

conditions in particular laboratories. It is mostly applicable to stable, neutral organic chemicals, which tend to adsorb to soils. The testing of bioaccumulation of soil-associating, stable metallo-organic compounds may also be possible with this method. It is also applicable to metals and other trace elements.

PREREQUISITE AND GUIDANCE INFORMATION

5. Tests for bioaccumulation, i.e. the increase in concentration of a substance in terrestrial oligochaetes relative to the surrounding medium (in this case the soil), have been performed with heavy metals (see e.g. Sample et al. (1999)) and persistent, organic substances having log K_{ow} values between 3.0 and 6.0 (e.g. Jager et al 2003b). Such tests can also be applied to

- substances that show a log K_{ow} of more than 6.0 (superlipophilic substances),
- substances which belong to a class of organic substances known to have the potential to bioaccumulate in living organisms, e.g. surface active or highly adsorptive substances,
- substances that indicate the potential for bioaccumulation from structural features, e.g. analogues of substances with known bioaccumulation potential.
- Metals (excluding essential metals, which are biologically regulated in organisms).

6. Information on the test substance such as safety precautions, proper storage conditions and analytical methods should be obtained before beginning the study. Before carrying out a test for bioaccumulation with terrestrial oligochaetes, the following information about the test compound should be known:

(a) solubility in water;
(b) octanol-water partition coefficient, K_{ow};
(c) soil-water partition coefficient, expressed as K_{oc};
(d) vapour pressure;
(e) degradability;
(f) known metabolites.

Other information on the test item such as common name, chemical name (preferably IUPAC name), structural formula, CAS registry number, purity should be known before beginning the study.

7. In order to mimic environmentally relevant exposure concentrations, to test at non-toxic concentrations, and to facilitate the analysis of the test item and its major metabolites in soil and worm samples, it is recommended to use radiolabelled organic test items. The method described here may have to be modified, e.g., to provide sufficient biomass, for measuring non-radiolabelled organic test items or metals. If total radioactive residues are measured (e.g. by liquid scintillation counting following extraction, combustion or tissue solubilisation), the bioaccumulation factor (BAF) is based on the parent compound including any retained metabolites. It is also possible to combine a metabolism study with a bioaccumulation study by analysis and quantification of the percentage of parent compound and its

metabolites in samples taken at the end of the uptake phase or at the peak level of bioaccumulation. In any case, the BAF calculation should be based on the concentration of the parent compound in the organisms and not only on total radioactive residues.

8. In addition to the knowledge of the properties of the test item given in paragraphs 5 - 6 other information required is the toxicity to the species to be used in the test, such as an effect concentration (EC_x) or lethal concentration (LC_x) for the time required for the uptake phase (e.g. EC 2003**)**. This is to ensure that selected exposure concentrations are much lower than toxic levels. If available, preference should be given to toxicity values derived from long-term studies on sublethal endpoints (OECD 2004a & b). If such data are not available, an acute toxicity test under conditions identical with the bioaccumulation test conditions may provide useful information, see e.g. Elmegaard & Jagers op Akkerhuis (2000).

9. An appropriate analytical method of known accuracy, precision, and sensitivity for the quantification of the substance in the test solutions, in the soil, and in the biological material must be available, together with details of sample preparation and storage as well as material safety data sheets. Analytical detection limits of the test item in soil and worm tissue should also be known. If a ^{14}C-labelled test item is used, the specific radioactivity (i.e. e.g., Bq mol^{-1}) and the percentage of radioactivity associated with impurities must be known. The specific radioactivity of the test compound should be as high as possible in order to detect test concentrations as low as possible.

10. The test can be performed with artificial soil (see paragraph 21 and Annex 4) or with field soils (see paragraph 21). Information on characteristics of the test soil (e.g. origin of soil or its constituents, pH, organic carbon content, particle size distribution (percent sand, silt, and clay), and water holding capacity (WHC)), should be acquired before the start of the test (OECD 1984; ASTM 2004).

PRINCIPLE OF THE TEST

11. The parameters which characterise the bioaccumulation of a substance include first of all the bioaccumulation factor (BAF), the uptake rate constant (k_u) and the elimination rate constant (k_e). Detailed definitions of these parameters are provided in Annex 1.

12. The test consists of two phases; the uptake (exposure) phase and the elimination (post-exposure) phase. During the uptake phase, replicated groups of worms are exposed to soil which has been spiked with the test item (for preparation of a suitable artificial soil see Annex 4; guidance on application of test item is given in paragraph 22). In addition to the animals exposed to the test item, groups of control worms are held under identical conditions without the test item. The absence of any adverse effects of the test item towards the test organisms in the bioaccumulation test can then be confirmed by matching the control group. Furthermore, the dry weight and the lipid content of the test organisms should be measured. This can be done using worms of the control group. Analytical background values (blank) can be obtained by analysing samples of the control worms and soil. For the elimination phase, the worms are transferred to a soil free of the test item. An elimination phase is necessary to gain information on the rate at which the test substance is excreted by the test organisms (e.g. Franke et al. 1994). An elimination phase is always

required unless uptake of the test item during the exposure phase has been insignificant (e.g. there is no statistically significant difference between the concentration of the test item in test and control worms). If a steady-state (see Annex 1 for definition) has not been reached during the uptake phase, the determination of the kinetic results (BAF_k, uptake and elimination rate constant(s)) should be based on the results of the uptake and elimination phase. The change of the concentration of the test item in/on the worms is monitored throughout both phases of the test. In case a radiolabelled test item is used, results of total radioactive concentration in soil and worm samples can be obtained within less than 2 days after sampling.

13. During the uptake phase, measurements are taken by time-series sampling for 14 days (enchytraeids) or 21 days (earthworms) to reach steady-state as described e.g. by Sousa et al., (2000), Bruns et al. (2001). The steady state occurs when a plot of the concentration in the worms against time becomes parallel to the time axis and three successive analyses of concentrations made on samples taken at intervals of at least two days varying no more than ± 20% of each other based on statistical comparisons (e.g., analysis of variance, regression analysis).

14. The elimination phase is started by transferring exposed oligochaetes to vessels containing the same substrate without the test substance. During the elimination phase, measurements are taken by time-series sampling for 14 days (enchytraeids) or 21 days (earthworms) unless analytical determination at earlier dates showed absence of the test item. The concentration of the test item in the worms at the end of the elimination phase is reported as an additional endpoint. The bioaccumulation factor (BAF_{ss}) is calculated preferably both as the ratio of concentration in the worms (C_a) and in the soil (C_s) at apparent steady state, and as a kinetic bioaccumulation factor, BAF_K, as the ratio of the rate constant of uptake from soil (k_s) and the elimination rate constant (k_e) (see Annex 1 for definitions) assuming first-order kinetics (see Annex 2 for calculations). If first-order kinetics are obviously not applicable, other models should be employed.

15. The uptake rate constant, the elimination rate constant (or constants, where other models are involved), the kinetic bioaccumulation factor (BAF_K), and where possible, the confidence limits of each of these parameters are calculated from computerised model equations (see Annex 2 for possible models). The goodness of fit of any model can be determined from e.g., the correlation coefficient or the coefficient of determination (coefficients close to 1 indicate a good fit).

16. To reduce variability in test results for those substances with high lipophilicity, bioaccumulation factors can be expressed additionally in relation to lipid content (expressed in kg soil OC kg^{-1} worm lipid content). This approach is based on experiences and theoretical correlations, where - for some chemical classes - there is a clear relationship between a substance´s potential for bioaccumulation and its lipophilicity, which has been well established for fish as model organisms (e.g., Nendza 1991). There is also a corresponding relationship between the lipid content of the test fish and the observed bioaccumulation of such substances. For benthic organisms, similar correlations have been found (e.g., Gabric et al. 1990, Landrum 1989). Also for terrestrial oligochaetes this correlation has been utilised (e.g., Connell & Markwell 1990, Belfroid et al. 1993, 1994, 1995). If sufficient worm tissue is available, the lipid content of the test animals may be determined on the same biological material as is used to determine the concentration of the test item. Alternatively, control animals may be used to measure the lipid content, which can then be used to normalise BAF values.

VALIDITY OF THE TEST

17. For a test to be valid the following criteria should be fulfilled for both controls and treatments:

- At the end of the test, the overall mortality during uptake and elimination phase should not exceed 10% (earthworms) or 20% (enchytraeids) of the total number of the introduced worms.
- For *Eisenia fetida and Eisenia andrei*, the mean mass loss as measured at the end of the uptake and at the end of the elimination phase should not exceed 20% compared to the initial fresh weight (fw).

DESCRIPTION OF THE METHOD

Test species

18. Several species of terrestrial oligochaetes can be recommended for bioaccumulation testing. The most commonly used species *Eisenia fetida* or *Eisenia andrei* (Lumbricidae), or *Enchytraeus albidus*, *Enchytraeus crypticus*, *Enchytraeus luxuriosus* (Enchytraeidae)), one of which should be used in the method described, are listed in Annex 5.

Apparatus

19. Care should be taken to avoid the use of materials, for all parts of the equipment, that can dissolve, absorb test items or leach other substances and have an adverse effect on the test animals. Standard rectangular or cylindrical vessels, made of chemically inert material and of suitable capacity can be used in compliance with the loading rate, i.e. the number of test worms, (see also paragraph 32**)**. Stainless steel, plastic or glass should be used for any equipment having contact with the test media. The test vessels should be appropriately covered to prevent escaping of the worms, while allowing sufficient air supply. For substances with high adsorption coefficients, such as synthetic pyrethroids, silanised glass may be required. In these situations the equipment will have to be discarded after use (OECD 1996). Radiolabelled test items and volatile chemicals should be prevented from escaping. Traps (e.g. glass gas washing bottles) should be employed containing suitable absorbents to retain any residues evaporating from the test vessels.

Soil

20. The soil to be used must be of a quality that will allow the survival and preferably the reproduction of the test organisms for the duration of the acclimation and test periods without them showing any abnormal appearance or behaviour. The worms should burrow in the soil.

21. The artificial soil described in the OECD Guideline 207 (OECD 1984) is recommended for use as the substrate in the tests. Preparation of the artificial soil for use in the bioaccumulation tests and recommendations for the storage of artificial soil are given in Annex 4. Air-dried artificial soil may be stored at room temperature until use.

However, natural soils from unpolluted sites may serve as test and/or culture soil (e.g. the German standard soil Lufa). Natural soils should be characterised at least by origin (collection site), pH, organic carbon content, particle size distribution (percent sand, silt, and clay), maximum water holding capacity (WHC_{max}), and percent water content (ASTM 2004). Analysis of the soil or its constituents for micropollutants prior to use might provide useful information. If field soil from agricultural land is used,

this must not have been treated (e.g. with pesticides and fertilizers) for 5 months prior to use in a test. Manipulation procedures for natural soils prior to use in ecotoxicological tests with oligochaetes in the laboratory are described in ASTM (2004). For natural soils the storage time in the laboratory should be kept as short as possible.

Application of the test item

22. The test item should be incorporated into the soil. The physicochemical properties of the test item should be taken into consideration. Water soluble test items are mixed directly into the soil. The spiking procedure for poorly water soluble test items recommended for the artificial soil involves coating of one or more of the soil constituents with the test item. For example, the quartz sand, or a portion thereof, can be soaked with a solution of the test item in a suitable solvent, which is then slowly evaporated. The coated fraction can then be mixed into the wet soil. The major advantage of this procedure is that no solvent is introduced to the soil. When using natural soil, the test chemical may be added by spiking a dried portion of the soil as described above for the artificial soil, or by stirring the test item into the wet soil, with subsequent evaporating of any solubilising agent used. In general, the contact of wet soil with solvents should be minimised. According to ASTM (2004), the following considerations are necessary. If a solvent other than water is used, it should be one that is water-miscible and/or can be driven off (for example, evaporated), leaving only the test chemical on the soil. If a solvent control is used, then no negative control needs to be applied (see paragraph 39). The solvent control must contain the highest concentration of solvent added to the soil and must use solvent from the same batch used to make the stock solution. Toxicity and volatility of the solvent, and the solubility of the test item in the chosen solvent should be the main criteria for the selection of a suitable solubilising agent.

23. For substances that are poorly soluble in water and organic solvents, 2.0 - 2.5 g of finely ground quartz sand per test vessel can be mixed with the quantity of test substance to obtain the desired test concentration. This mixture of quartz sand and test substance is added to the pre-moistened soil and thoroughly mixed after adding an appropriate amount of de-ionised water to obtain the required moisture content. The final mixture is distributed to the test vessels. The procedure is repeated for each test concentration, and an appropriate control with 2.0 - 2.5 g of finely ground quartz sand per test vessel is also prepared.

24. The concentration of the test item in the soil must be determined after spiking. The homogenous distribution of the test item in the soil should be confirmed before introducing the test organisms. The method used for spiking, and the reasons for choosing a specific spiking procedure should be reported (Environment Canada 1995).

25. An equilibrium between the soil and the soil pore water phase should ideally be established within a suitable period of time. For many poorly water soluble organic chemicals the time required to reach true equilibrium between adsorbed and dissolved fractions may range from days to months. Therefore, an arbitrary time period of 4 d at 20°C is recommended for ageing the spiked soil. Depending on the purpose of the study, e.g., when environmental conditions are to be mimicked, the spiked soil may be aged for a longer period (e.g. for metals 3 weeks at 20°C).

Culturing of the test organisms

26. In order to have a sufficient number of worms for conducting bioaccumulation tests the worms have to be kept in permanent laboratory culture. Summaries of laboratory culture methods for *Eisenia fetida* and *Eisenia andrei*, *Enchytraeus albidus* and *Enchytraeus luxuriosus* are outlined in Annex 5. For details of culturing these species see e.g. OECD (1984) or OECD (2004a & b).

27. To ensure that the tests are performed with animals of the same species, the establishment of single species cultures is strongly recommended. Ensure that the cultures and especially the worms used in the tests are free from observable diseases and abnormalities.

PERFORMANCE OF THE TEST

28. The test organisms are exposed to the test item during the uptake phase. The uptake phase should be run for up to 14 (enchytraeids) or 21 (earthwoms) days unless it can be demonstrated earlier that steady state has been reached.

29. For the elimination phase, the worms are transferred to a soil free of the test item. The first sample should be taken at 4 - 24 h after start of elimination phase. Examples of sampling schedules for a 21 d uptake phase and a 21 d elimination phase are given in Annex 3.

Test organisms

30. The number of worms per sample must provide a mass of worm tissue such that the mass of test item per sample at the beginning of the uptake phase and at the end of the elimination phase, respectively, is significantly higher than the substance´s analytical detection limit in biological material. This refers to the experience that in bioaccumulation tests, in the mentioned stages of uptake and elimination phase the concentration in the test animals is usually relatively low. Since the individual weight in many species of terrestrial enchytraeids is very low (e.g. 5 - 10 mg wet weight per individual for *Enchytraeus albidus* or even less for *Enchytraeus crypticus* or *Enchytraeus luxuriosus*), the worms of each single replicate test vessel may be pooled for weighing and chemical analysis. The preferred number of enchytraeid worms per replicate is 20. If the analytical detection limit of the test item is high, a higher number of worms may be necessary. For test species with higher individual weight (*Eisenia fetida* and *Eisenia andrei*), replicates containing one individual may be used.

31. The earthworms to be used in a test should be of similar weight (e.g. *Eisenia fetida* and *Eisenia andrei* should have an individual weight of 250 – 600 mg). Enchytraeids (e.g. *Enchytraeus albidus*) should have a length of approximately 1 cm. All worms used in a particular test should come from the same source, and should be adult animals with clitellum (see Annex 5). Since weight and age of an animal sometimes appears to have a significant effect on BAF-values (e.g. due to varying lipid content and/or presence of eggs), these parameters should be recorded accurately. It is recommended that a sub-sample of the worms to be used is weighed before the test in order to estimate the mean wet and dry weight.

32. Use a high soil-to-worm ratio in order to minimise the decrease of test item concentration in the soil during the uptake phase. For *Eisenia fetida* and *Eisenia andrei* a minimum amount of 50 g DW of soil per worm, and for enchytraeids, a minimum of 10 – 20 g DW of soil per test vessel are recommended. The vessels should contain a soil layer of 2 – 3 cm (enchytraeids) or 4 – 5 cm (earthworms).

33. The worms to be used in a test are removed from the culture (e.g. enchytraeids by using jeweller's tweezers). Adult animals are transferred to non-treated test soil for acclimation. If the test conditions differ from the culture conditions, an acclimation phase of 24 – 72 h should be sufficient to adapt the worms to the test conditions. After acclimatisation, earthworms are transferred to glass dishes (e.g. petri dishes) containing clean water, and subsequently weighed before adding them to the test soil. Prior to weighing, excess water should be removed from the worms by gently touching them against the edge of the dish or by blotting them cautiously dry by using a slightly moistened paper towel.

34. Burrowing behaviour can have an influence on the exposure of the test organisms. Therefore, burrowing behaviour of the test organisms should be observed and recorded. In tests with earthworms, the animals (control and treatments) should burrow in the soil within a period of 24 h after addition to the test vessels. If the earthworms fail to burrow in the soil (more than 10%), this indicates that either the test conditions are not appropriate or the test organisms are not healthy. In such a case the test should be stopped and repeated at improved conditions. For enchytraeids, the exposure situation is different from the exposure situation for earthworms. Enchytraeids mainly live in the interstitial pores of the soil, and frequently their integument may be only partly in contact with the surrounding substrate. Therefore, exposure of burrowing and non-burrowing enchytraeids is assumed to be virtually equal, and non-burrowing of the enchytraeids does not necessarily require the repetition of the test.

Feeding

35. Feeding should be considered when a soil with low total organic carbon is used. Food should then be added to the soil before the test is started. When artificial soil according to OECD (1984) is used, a weekly rate for earthworms of 10 mg of dried dung and for enchytraeids of 2 - 2.5 mg of ground oat flakes per g soil dry weight is recommended (Bruns et al. 2001a). Preferably the same type of food as in the cultures should be used.

Light and temperature

36. The tests should be carried out under a controlled 16:8 hours light/dark cycle (preferably 400 to 800 lx in the area of the test vessels) (ASTM 2004). The test temperature should be 20 ± 2°C throughout the test.

Test concentrations

37. A single concentration is sufficient for determining the bioaccumulation potential. If required, evaluation of a possible concentration dependency of bioaccumulation will require testing more concentrations. For metals, the concentration should also be above the background levels in tissue and soil. If the effect concentration of the test item is close to the analytical detection limit, the use of radiolabelled test item with high specific radioactivity is recommended (see also paragraphs 8 and 9).

Replicates

38. The minimum number of replicates should be three per sampling point.

39. If any solubilising agent is used for application of the test item, a solvent control containing all constituents except for the test item should be run in addition to the treated replicates (4 replicates should be sampled at start, 4 at the end of the uptake phase, and 4 at the end of the elimination phase. As an option, 4 replicates of a negative control (no solvent) may also be provided for sampling at the end of the uptake phase. These replicates can be compared biologically with the solvent control in order to gain information on a possible influence of the solvent on the test organisms. In case no solvent other than water is used, at least 12 replicates of a negative control (4 sampled at start, 4 at end of uptake and 4 at end of elimination) should be provided for biological (dry-to-wet weight ratio, lipid content) and background analysis. It is recommended to establish a sufficient number of reserve control replicates (e.g. 8).

Frequency of soil quality measurements

40. Soil pH, soil moisture content and the temperature (continuously) in the test chamber should be measured at the start and the end of the uptake and the elimination phase. Once per week the soil moisture content should be controlled by reweighing the test vessels and comparing actual weights with initial weights at test start. Water losses should be compensated by adding deionised water.

Sampling and analysis of worms and soil

41. Examples of activity schedules for uptake and elimination phase in earthworm and enchytraeid tests are given in Annex 3.

42. Sample the soil from the test vessels for determination of test item concentration before inserting the worms, and during both uptake and elimination phases. During the test the concentrations of test item are determined in the worms, and soil in order to monitor the distribution of the test item in the compartments of the test system.

43. Sample the worms and soil on at least six occasions during the uptake as well as the elimination phase. If the stability of a test item can be demonstrated, the number of soil analyses may be reduced. However, it is recommended to analyse at least three replicates at the beginning and the end of the uptake phase in order to show homogeneity of test item distribution. In addition, soil samples from other dates should be kept until the end of the study.

Remove the worms of a given replicate from the soil at each sampling time (e.g., after spreading the soil of the replicate on a shallow tray and picking the worms using soft jewellers' tweezers), rinse them quickly with water in a shallow glass or steel tray. Remove the excess water as described in paragraph 33. Transfer the worms carefully to a pre-weighed vessel, weigh them instantly and kill them immediately thereafter, using the most appropriate method (e.g., using liquid nitrogen, or freezing at -20 ± 2°C).

The earthworms should be allowed to purge their gut for 24 h before being killed. The weight of the worms must be determined before and after purging in order to assess a possible decrease in biomass during the

test (see validity criterion in paragraph 17). Enchytraeids are not allowed to purge their gut before weighing and tissue analysis.

44. During the elimination phase, the worms purge their gut in clean soil. This means, measurements immediately before the elimination phase include contaminated gut soil. For aquatic oligochaetes it is assumed that after the initial 4 - 24 h of the elimination phase, most of the contaminated gut content has been replaced by clean sediment (e.g., Mount et al. 1999). Similar findings have been reported for earthworms in studies on the accumulation of radiolabelled cadmium and zinc (Vijver et al. 2005). The concentration in the worms of this first sample of the elimination phase may then be considered as the tissue concentration after gut purge. To account for dilution of the test item concentration by uncontaminated soil during the elimination phase, the weight of the gut content may be estimated from worm wet weight/worm ash weight or worm dry weight/worm ash weight ratios.

45. Preferably analyse the soil and worm samples immediately (i.e. within 1 - 2 d) after removal in order to prevent degradation or other losses, and to calculate the approximate uptake and elimination rates as the test proceeds. Failing immediate analysis, the samples should be stored by an appropriate method, e.g. by deep-freezing (≤ -18 C). Obtain information on the proper storage conditions for the particular test item - for example, duration and temperature of storage, extraction procedures, etc. - before beginning the study.

46. Since the whole procedure is governed essentially by the accuracy, precision and sensitivity of the analytical method used for the test item, check experimentally that the precision and reproducibility of the chemical analysis, as well as the recovery of the test item from soil and worm samples are satisfactory for the particular method. Also, check that the test item is not detectable in the control vessels in concentrations higher than background. When C_a is > 0 in the control worms, this has to be included in the calculation of kinetic parameters (see Annex 2). Handle all samples throughout the test in such a manner so as to minimise contamination and loss (e.g. resulting from adsorption of the test item on the sampling device).

47. The overall recovery and the recovery of test item in worms, soil, and, if employed, in traps containing absorbents to retain evaporated test item, should be recorded and reported.

48. Since the use of radiolabelled instead of non-labelled substances is recommended for the presented test method, it is possible to analyse for total radioactivity only (i.e. parent and metabolites). However, if analytically feasible, quantification of parent compound and metabolites at steady state or at the end of the uptake phase can provide important information. The samples should then be cleaned so that the parent compound can be quantified separately. If single metabolites exceed 10% of total radioactivity in the analysed sample(s), the identification of these metabolites is recommended.

49. Due to low individual biomass, in contrast to earthworm samples it is not possible to determine the concentration of test item in each individual enchytraeid worm. Therefore, pooling of the individuals sampled from a given test vessel is acceptable. If pooling involves the reduction of the number of replicates, it does, hovever, restrict the statistical procedures which can be applied to the data. If a specific statistical procedure and power are required, then an adequate number of replicate test vessels to accommodate the desired pooling, procedure and power, should be included in the test.

50. It is recommended, that the BAF is expressed both as a function of total dry weight, and, when required (i.e. for highly lipophilic substances), as a function of the lipid content (see paragraph 16). Suitable methods should be used for determination of lipid content (e.g., Gardner et al. 1985, Randall et al. 1991). These methods use a chloroform/methanol extraction technique. However, to avoid the use of chlorinated solvents, a modification of the Bligh & Dyer method (Bligh & Dyer 1959) as described in De Boer et al. (1999) should be used. Since the various methods may not give identical values, it is important

to give details of the method used. When possible, i.e. if sufficient worm tissue is available, the lipid analysis should ideally be made on the same sample or extract as that produced for analysis for the test item, since the lipids often have to be removed from the extract before it can be analysed chromatographically (OECD 1996). Alternatively, control animals may be used to measure the lipid content, which can then be used to normalise BAF values. This latter approach reduces the contamination of equipment with the test item.

DATA AND REPORTING

Treatment of results

51. The uptake curve of the test item is obtained by plotting its concentration in/on the worms during the uptake phase against time on arithmetic scales. When the curve has reached a plateau, that is, become approximately parallel to the time axis, calculate the steady state BAF_{ss} from:

$$\frac{C_a \text{ at steady state or at end of uptake phase (mean)}}{C_s \text{ at steady state or at end of uptake phase (mean)}}$$

52. When no steady state is reached, the BAF, and the rate constants should be determined as described below.

53. Determine the accumulation factor (BAF_K) as the ratio k_s/k_e. The elimination rate constant (k_e) is usually determined from the elimination curve (i.e. a plot of the concentration of the test item in the worms during the elimination phase). The uptake rate constant k_s is then calculated given k_e and a value of C_a which is derived from the uptake curve. See Annex 2 for a description of these methods. The preferred method for obtaining BAF_K and the rate constants, k_s, and k_e, is to use non-linear parameter estimation methods on a computer. If the elimination is obviously not first-order, then more complex models should be employed (see Annex 2).

Test report

54. The test report must include the following information:

Test item:

- purity, physical nature and, physicochemical properties e.g. log K_{ow}, water solubility;
- substance identification data; source of the test item, identity and concentration of any solvent used;
- if radiolabelled, the precise position of the labelled atoms, the specific radioactivity, and the percentage of radioactivity associated with impurities.

Test species:

- scientific name, strain, source, any pre-treatment, acclimation, age, size-range, etc..

Test conditions:

- test procedure used;
- type and characteristics of illumination used and photoperiod(s);
- test design (e.g. number and size of test vessels, soil mass and height of soil layer, number of replicates, number of worms per replicate, number of test concentrations, duration of uptake and elimination phases, sampling frequency);
- rationale for the choice of test vessel material;
- method of test item preparation and application as well as reasons for choosing a specific method;
- the nominal test concentrations, the means of the measured values and their standard deviations in the test vessels, and the method by which these values were obtained;
- source of the constituents of the artificial soil or - if natural media are used - origin of the soil, description of any pre-treatment, results of the controls (survival, biomass development, reproduction), soil characteristics (pH, total organic carbon content, particle size distribution (percent sand, silt, and clay), WHC_{max}, percent water content at start and at end of the test, and any other measurements made);
- detailed information on the treatment of soil and worm samples, including details of preparation, storage, spiking procedures, extraction, and analytical procedures (and precision) for the test item and lipid content (if measured), and recoveries of the test item.

Results:

- mortality of the control worms and the worms in each test vessel and any observed abnormal behaviour (e.g., soil avoidance, lack of reproduction in a bioaccumulation test with enchytraeids);

- the dry weight to wet weight ratio of the soil and the test organisms (useful for normalisation);

- the wet weights of the worms at each sampling occasion; for earthworms, the wet weights at start of the test, and at each sampling occasion before and after gut purging;

- the lipid content of the test organisms (if determined on testing occasion);

- curves, showing the uptake and elimination kinetics of the test item in the worms, and the time to steady state;

- C_a and C_s (with standard deviation and range, if appropriate) for all sampling times (C_a expressed in g kg^{-1} wet and dry weight of whole body, C_s expressed in g kg^{-1} wet and dry weight). If a biota-soil accumulation factor (BSAF; see Annex 1 for definition) is required (e.g. for comparison of results from two or more tests performed with animals of differing lipid content), C_a may additionally be expressed as g kg^{-1} lipid content of the organism, and C_s may be expressed as g kg^{-1} organic carbon (OC) of the soil;

- BAF (expressed in kg soil kg^{-1} worm), soil uptake rate constant k_s (expressed in g soil kg^{-1} of worm d^{-1}), and elimination rate constant k_e (expressed in d^{-1}); BSAF (expressed in kg soil OC kg^{-1} worm lipid content) may be reported additionally;

- if measured: percentages of parent compound, metabolites, and bound residues (i.e. the percentage of test item that can not be extracted with common extraction methods) detected in soil and test animals;

- methods used for statistical analyses of the data.

Evaluation of results:

- compliance of the results with the validity criteria as listed in paragraph 17;

- unexpected or unusual results, e.g. incomplete elimination of the test item from the test animals.

ANNEX 1

DEFINITIONS AND UNITS

Bioaccumulation is the increase in concentration of the test item in or on an organism relative to the concentration of the test item in the surrounding medium. Bioaccumulation results from both bioconcentration and biomagnification processes (see below).

Bioconcentration is the increase in concentration of the test item in or on an organism, resulting exclusively from uptake via the body surface, relative to the concentration of the test item in the surrounding medium.

Biomagnification is the increase in concentration of the test item in or on an organism, resulting mainly from uptake from contaminated food or prey, relative to the concentration of the test item in the food or prey. Biomagnification can lead to a transfer or accumulation of the test item within food webs.

The elimination of a test item is the loss of this substance from the test organism tissue by active or passive processes, that occurs independently of presence or absence of the test item in the surrounding medium.

The bioaccumulation factor (BAF) at any time during the uptake phase of this bioaccumulation test is the concentration of test item in/on the test organism (C_a in g kg^{-1} dry weight) divided by the concentration of the substance in the surrounding medium (C_s as g kg^{-1} of dry weight of soil). In order to refer to the units of C_a and C_s, the BAF has the units of kg soil kg^{-1} worm.

The steady state bioaccumulation factor (BAF_{ss}) is the BAF at steady state and does not change significantly over a prolonged period of time, the concentration of the test item in the surrounding medium (C_s as g kg^{-1} of dry weight of soil) being constant during this period of time.

The biota-soil accumulation factor (BSAF) is the lipid-normalised concentration of the test item in/on the test organism divided by the organic carbon-normalised concentration of the test item in the soil at steady state. C_a is then expressed as g kg^{-1} lipid content of the organism, and C_s as g kg^{-1} organic content of the soil; the BSAF has the units of kg OC kg^{-1} lipid.

A plateau or steady state is defined as the equilibrium between the uptake and elimination processes that occur simultaneously during the exposure phase. The steady state is reached in the plot of BAF against time when the curve becomes parallel to the time axis and three successive analyses of BAF made on samples taken at intervals of at least two days are within 20% of each other, and there are no statistically significant differences among the three sampling periods. For test items which are taken up slowly, more appropriate intervals would be seven days (OECD 1996).

Bioaccumulation factors calculated directly from the ratio of the soil uptake constant and the elimination rate constant (k_s and k_e, respectively - see below) are termed kinetic bioaccumulation factor (BAF_K).

The organic carbon-water partitioning coefficient (K_{oc}) is the ratio of a substance's concentration in/on the organic carbon fraction of a soil and the substance´s concentration in water at equilibrium.

The octanol-water partitioning coefficient (K_{ow}) is the ratio of a substance's solubility in n-octanol and water at equilibrium, also sometimes expressed as P_{ow}. The logarithm of K_{ow} (log K_{ow}) is used as an indication of a substance´s potential for bioaccumulation by aquatic organisms.

The uptake or exposure phase is the time during which the test organisms are exposed to the test item.

The soil uptake rate constant (k_s) is the numerical value defining the rate of increase in the concentration of the test item in/on the test organism resulting from uptake from the soil phase. k_s is expressed in g soil kg^{-1} of worm d^{-1}.

The elimination phase is the time, following the transfer of the test organisms from a contaminated medium to a medium free of the test item, during which the elimination (or the net loss) of the substance from the test organisms is studied.

The elimination rate constant (k_e) is the numerical value defining the rate of reduction in the concentration of the test item in/on the test organism, following the transfer of the test organisms from a medium containing the test item to a substance-free medium; k_e is expressed in d^{-1}.

ANNEX 2

Calculation of uptake and elimination parameters

The main endpoint of a bioaccumulation test is the bioaccumulation factor, BAF. The measured BAF can be calculated by dividing the concentration in the test organism, C_a, by the concentration in the soil, C_s, at steady state. If the steady state is not reached during the uptake phase, the BAF is calculated in the same manner for the end of the uptake phase (day 21). However, it should be noted if the BAF is based on steady state concentrations or not.

The preferred means for obtaining the kinetic bioaccumulation factor (BAF_K), the soil uptake rate constant (k_s) and the elimination rate constant (k_e) is to use non-linear parameter estimation methods on a computer, e.g., based on the models described by Spacie & Hamelink (1982)(64). Given a set of sequential time concentration data and the model equations

$$C_a = \frac{k_s}{k_e} * C_s(1-e^{-k_e t}) \qquad 0 < t < t_c \quad \text{[equation 1]}$$

or

$$C_a = \frac{k_s}{k_e} * C_s(e^{-k_e(t-tc)} - e^{-k_e t}) \qquad t > t_c \quad \text{[equation 2]}$$

where C_a = concentration of substance in worms [g kg^{-1} wet or dry weight]
k_s = uptake rate constant in tissue [g soil kg^{-1} of worm d^{-1}]
C_s = concentration of substance in soil [g kg^{-1} of wet or dry weight]
k_e = elimination rate constant [d^{-1}]
t_c = time at the end of the uptake phase

these computer programs calculate values for BAF_K, k_s and k_e.

When the background concentration in the non-exposed worms e.g. on day 0 differs significantly from zero (this may e.g. be the case for metals), this background concentration ($C_{a,0}$) has to be included in these equations, to make them read:

$$C_a = C_{a,0} + \frac{k_s}{k_e} * C_s(1-e^{-k_e t}) \qquad 0 < t < t_c \quad \text{[equation 3]}$$

and

$$C_a = C_{a,0} + \frac{k_s}{k_e} * C_s(e^{-k_e (t-tc)} - e^{-k_e t}) \qquad t > t_c \quad \text{[equation 4]}$$

In cases where a significant decrease of the test substance concentration in the soil is observed over time during the uptake phase, the following models can be used (e.g. Widianarko & van Straalen 1996, Sousa et al. 2000):

$$C_s = C_0(e^{-k_0 t}) \qquad \text{[equation 5]}$$

where C_s = concentration of substance in the soil [g kg^{-1} wet or dry weight]
k_0 = degradation rate constant in soil [d^{-1}]
C_0 = initial concentration of substance in soil [g kg^{-1} of wet or dry weight]

$$C_a = \frac{k_s}{k_e - k_0} * (e^{-k_0 t} - e^{-k_e t}) \qquad 0 < t < t_c \quad \text{[equation 6]}$$

$$C_a = \frac{k_s}{k_e - k_0} * (e^{-k_0 tc} - e^{-k_e tc}) * e^{-k(t-tc)} \qquad t > t_c \qquad \text{[equation 7]}$$

where C_a = concentration of substance in worms [g kg^{-1} wet or dry weight]
k_s = uptake rate constant in tissue [g soil kg^{-1} of worm d^{-1}]
k_0 = degradation rate constant in soil [d^{-1}]
k_e = elimination rate constant [d^{-1}]
t_c = time at the end of the uptake phase

When steady state is reached during the uptake phase (i.e. $t = \infty$), equation 1

$$C_a = \frac{k_s}{k_e} * C_s(1-e^{-k_e t}) \qquad 0 < t < t_c \qquad \text{[equation 1]}$$

may be reduced to:

$$C_a = \frac{k_s}{k_e} * C_s$$

or

$$C_a/C_s = k_s/k_e = BAF \qquad \text{[equation 8]}$$

Then $k_s/k_e * C_s$ is an approach to the concentration of the test item in the worm tissue at steady state ($C_{a,ss}$).

The elimination kinetics can be modelled using the data from the elimination phase and applying the following model equation and a computer-based non-linear parameter estimation method. If the data points plotted against time indicate a constant exponential decline of the test item concentration in the animals, a one-compartment model (equation 9) can be used to describe the time course of elimination.

$$C_a(t) = C_{a,ss} * e^{-k_e t} \qquad \text{[equation 9]}$$

Elimination processes sometimes appear to be biphasic, showing a rapid decline of C_a during the early phases, that changes to a slower loss of test items in the later phases of the elimination (e.g., Spacie & Hamelink 1982 (64), Franke et al. 1994 (26)). The two phases can be interpreted by the assumption, that there are two different compartments in the organism, from which the test item is lost with different velocities. In these cases specific literature should be studied (e.g., Van Gestel & Ma 1990, Jager et al. 2000, Jager et al. 2003, Vijver et al.2005).

Using the model equations above, the kinetic parameters (k_s and k_e) may also be calculated in one run by applying the first order kinetics model to all data from both the uptake and elimination phase together. For a description of a method that may allow for such a combined calculation of uptake and elimination rate constants, references Janssen et al. (1991)(39), Van Brummelen & Van Straalen (1996)(68) and Sterenborg et al. (2003)(66) may be consulted.

$$C_a = \frac{k_s}{k_e}\left[\left(\frac{k_s}{k_e} * C_s(1-e^{-k_e t})*(m=1)\right) + \left(\frac{k_s}{k_e} * C_s(e^{-k_e(t-tc)} - e^{-k_e t})*(m=2)\right)\right]$$

m = 1 for uptake phase and 2 for elimination phase

Nevertheless, these model equations should be used with caution, especially when changes in the test chemical´s bioavailability, or (bio)degradation occur during the test (see e.g. Widianarko & van Straalen 1996).

ANNEX 3

EXAMPLEs OF Activity SCHEDULEs FOR SOIL Bioaccumulation tests

Earthworm test

a) Uptake phase with 8 sampling dates used for calculation of kinetics

Day	Activities
-6	Conditioning of the prepared soil for 48 h;
-4	Spiking of the soil fraction with the test item solution; evaporating of any solvent; mixing of the soil constituents; distributing the soil to the test vessels; equilibration at test conditions for 4 days (3 weeks for metal-spiked soil);
-3 to -1	Separation of the test organisms from the culture for acclimation; preparation and moisturising of the soil constituents;
0	Measuring temperature, and soil pH; removing soil samples from treated vessels and solvent controls for determination of test item concentration; weighing and randomised distribution of the worms to the test vessels; retaining of sufficient subsamples of worms for determination of analytical background values, wet and dry weight, and lipid content; weighing of all test vessels to control soil moisture; controlling air supply, if closed test system is used;
1	Controlling air supply, recording worm behaviour and temperature; taking soil and worm samples for determination of test item concentration;
2	Same as day 1;
3	Controlling air supply, worm behaviour and temperature;
4	Same as day 1;
5 - 6	Same as day 3;
7	Same as day 1; control soil moisture by re-weighing the test vessels and compensate evaporated water;
8 - 9	Same as day 3;
10	Same as day 1;

11 - 13	Same as day 3;
14	Same as day 1; control soil moisture by re-weighing the test vessels and compensate evaporated water;
15 - 16	Same as day 3;
17	Same as day 1;
18 - 20	Same as day 3;
21	Same as day 1; measuring temperature and soil pH; control soil moisture by re-weighing the test vessels; end of uptake phase; transfer worms from remaining exposed replicates to vessels containing clean soil for elimination phase (no gut-purging); sampling of soil and worms from solvent controls.
	Pre-exposure activities (equilibration phase) should be scheduled taking into account the properties of the test substance.
	Activities described for day 3 should be performed daily (at least on workdays).

b) Elimination phase

Day	Activities
-6	Preparation and moisturising of the soil constituents; conditioning of the prepared soil for 48 h;
-4	Mixing of the soil constituents; distributing the soil to the test vessels; incubation at test conditions for 4 days;
0 (end of uptake phase)	Measuring temperature and soil pH; weighing and randomised distribution of the worms to the test vessels; transfer worms from remaining exposed replicates to vessels containing clean soil; taking soil and worm samples after 4 - 6 h for determination of test item concentration;
1	Controlling air supply, recording worm behaviour and temperature; taking soil and worm samples for determination of test item concentration;
2	Same as day 1;
3	Controlling air supply, worm behaviour and temperature;
4	Same as day 1;
5 - 6	Same as day 3;
7	Same as day 1; control soil moisture by re-weighing the test vessels and compensate evaporated water;

8 - 9	Same as day 3;
10	Same as day 1;
11 - 13	Same as day 3;
14	Same as day 1; control soil moisture by re-weighing the test vessels and compensate evaporated water;
15 - 16	Same as day 3;
17	Same as day 1;
18 - 20	Same as day 3;
21	Same as day 1; measuring temperature and soil pH; control soil moisture by re-weighing the test vessels; sampling of soil and worms from solvent controls.

Preparation of the soil prior to start of elimination phase should be done in the same manner as before the uptake phase.

Activities described for day 3 should be performed daily (at least on workdays).

Enchytraeid test

a) Uptake phase with 8 sampling dates used for calculation of kinetics

Day	Activities
-6	Conditioning of the prepared soil for 48 h;
-4	Spiking of the soil fraction with the test item solution; evaporating of any solvent; mixing of the soil constituents; distributing the soil to the test vessels; equilibration at test conditions for 4 days (3 weeks for metal-spiked soil);
-3 to -1	Separation of the test organisms from the culture for acclimation; preparation and moisturising of the soil constituents;
0	Measuring temperature, and soil pH; removing soil samples from treated vessels and solvent controls for determination of test item concentration; weighing and randomised distribution of the worms to the test vessels; retaining of sufficient subsamples of worms for determination of analytical background values, wet and dry weight, and lipid content; weighing of all test vessels to control soil moisture; controlling air supply, if closed test system is used;
1	Controlling air supply, recording worm behaviour and temperature; taking soil and worm samples for determination of test item concentration;
2	Same as day 1;
3	Controlling air supply, worm behaviour and temperature;
4	Same as day 1;
5 - 6	Same as day 3;
7	Same as day 1; control soil moisture by re-weighing the test vessels and compensate evaporated water;
9	Same as day 1;
10	Same as day 3;
11	Same as day 1;
12 - 13	Same as day 3;
14	Same as day 1; measuring temperature and soil pH; control soil moisture by re-weighing the test vessels; end of uptake phase; transfer worms from remaining exposed replicates to vessels containing clean soil for elimination phase (no gut-purging); sampling of soil and worms from solvent controls.
	Pre-exposure activities (equilibration phase) should be scheduled taking into account the properties of the test substance.

	Activities described for day 3 should be performed daily (at least on workdays).

b) Elimination phase

Day	Activities
-6	Preparation and moisturising of the soil constituents; conditioning of the prepared soil for 48 h;
-4	Mixing of the soil constituents; distributing the soil to the test vessels; incubation at test conditions for 4 days;
0 (end of uptake phase)	Measuring temperature and soil pH; weighing and randomised distribution of the worms to the test vessels; transfer worms from remaining exposed replicates to vessels containing clean soil; taking soil and worm samples after 4 - 6 h for determination of test item concentration;
1	Controlling air supply, recording worm behaviour and temperature; taking soil and worm samples for determination of test item concentration;
2	Same as day 1;
3	Controlling air supply, worm behaviour and temperature;
4	Same as day 1;
5 - 6	Same as day 3;
7	Same as day 1; control soil moisture by re-weighing the test vessels and compensate evaporated water;
8 - 9	Same as day 3;
10	Same as day 1;
11 - 13	Same as day 3;
14	Same as day 1; measuring temperature and soil pH; control soil moisture by re-weighing the test vessels; sampling of soil and worms from solvent controls.
	Preparation of the soil prior to start of elimination phase should be done in the same manner as before the uptake phase.
	Activities described for day 3 should be performed daily (at least on workdays).

Annex 4

ARTIFICIAL SOIL - PREPARATION AND STORAGE RECOMMENDATIONS

Since natural soils from a particular source may not be available throughout the year, and indigenous organisms as well as the presence of micropollutants can influence the test, an artificial soil is recommended for use in this test. Several test species can survive, grow, and reproduce in this soil, and maximum standardisation as well as intra- and interlaboratory comparability of test and culture conditions are provided.

Soil constituents

Peat:	10%	Sphagnum-peat, in accordance with the OECD Guideline 207 (OECD 1984);
Quartz sand:	70%	Industrial quartz sand (air dried); grain size: more than 50% of the particles should be in the range of 50-200 µm, but all particles should be ≤ 2 mm;
Kaolinite clay:	20%	Kaolinite content ≥ 30 %;
Calcium carbonate:	≤ 1%	$CaCO_3$, pulverised, chemically pure.

As an option, the organic carbon content of the artificial soil may be reduced, e.g. by lowering the peat content to 4-5% of dry soil and increasing the sand content accordingly. By such a reduction in organic carbon content, the possibilities of adsorption of test chemical to the soil (organic carbon) may be decreased, and the availability of the test chemical to the worms may increase (Van Gestel 1992). It has been demonstrated that *Enchytraeus albidus* and *Eisenia fetida* can comply with the validity criteria on reproduction when tested in field soils with lower organic carbon content (e.g. 2.7%) (Hund-Rinke et al., 2000 (33), Roembke et al., 2000 (57)), and there is experience that this can also be achieved in artificial soil with 5% peat.

Preparation

The dry constituents of the soil are mixed thoroughly (e.g. in a large-scale laboratory mixer). This should be done about one week before starting the test. The mixed dry should be moistened with deionised water at least 48 h before application of the test item in order to equilibrate/stabilise the acidity. For the determination

of pH a mixture of soil and 1 M KCl solution in a 1:5 ratio is used. If the pH value is not within the required range (6.0 ± 0.5), a sufficient amount of $CaCO_3$ is added to the soil, or a new batch of soil is prepared.

The maximum water holding capacity (WHC) of the artificial soil is determined according to ISO 11268-2 (ISO 1998). At least two days before starting the test, the dry artificial soil is moistened by adding enough deionised or reconstituted water to obtain approximately half of the final water content. The final water content should be 40% to 60% of the maximum WHC. At the start of the test, the pre-moistened soil is divided into as many batches as the number of test concentrations and controls used for the test, and the moisture content is adjusted to 40 - 60% of WHC_{max} by using the solution of the test item and/or by adding deionised or reconstituted water (see paragraphs 22 - 25). The moisture content is determined at the beginning and at the end of the test (at 105 °C). It should be optimal for the species' requirements (the moisture content can also be checked as follows: when the soil is gently squeezed in the hand, small drops of water should appear between the fingers).

Storage

The dry constituents of the artificial soil may be stored at room temperature until use. The prepared, pre-moistened soil may be stored in a cool place for up to 3 days prior to spiking; care should be taken to minimise evaporation of water. Soil spiked with the test item should be used immediately unless there is information indicating that the particular soil can be stored without affecting the toxicity and bioavailability of the test item. Samples of spiked soil may then be stored under the conditions recommended for the particular test item until analysis.

ANNEX 5

SPECIES OF TERRESTRIAL OLIGOCHAETES RECOMMENDED FOR TESTING BIOACCUMULATION FROM SOIL

Earthworms:

The recommended test species is *Eisenia fetida* (Savigny 1826), belonging to the family Lumbricidae. Since 1972 it is divided into two subspecies (*Eisenia fetida* and *Eisenia andrei*; Bouche 1972). According to Jaenike (1982) they are true, separate species. *Eisenia fetida* is easily recognised by its bright intersegmental yellow stripes whereas *Eisenia andrei* has a uniform, dark red colour. Originating probably from the region of the Black Sea, they are distributed world-wide today, especially in anthropogenically modified habitats like compost heaps. Both can be used for ecotoxicological as well as bioaccumulation tests.

Eisenia fetida and *Eisenia andrei* are commercially available, e.g. as fish bait. In comparison to other lumbricid earthworms, they have a short life-cycle, reaching maturity within ca. 2 – 3 months (at room temperature). Their temperature optimum is approximately at 20 - 24°C. They prefer relatively moist substrates with a nearly neutral pH and a high content of organic material. Since these species are widely used in standardised ecotoxicological tests for about 20 years, their culturing is well established (OECD 1984; Venter & Reinecke 1988).

Both species can be bred in a wide range of animal wastes. The breeding medium recommended by ISO (1998) is a 50:50 mixture of horse or cattle manure and peat. The medium should have a pH value of about 6 to 7 (regulated with calcium carbonate), a low ionic conductivity (less than 6 mS/cm or less than 0.5 % salt concentration) and should not be contaminated excessively with ammonia or animal urine. Also, a commercial gardening soil free of additives, or artificial soil according to OECD (1984), or a 50:50 mixture of both can be used. The substrate should be moist but not too wet. Breeding boxes of 10 litre to 50 litre volume are suitable.

To obtain worms of standard age and mass, it is best to start the culture with cocoons. Therefore, adult worms are added to a breeding box containing fresh substrate to produce cocoons. Practical experience has shown that a population density of approximately 100 adult worms per kg substrate (ww) leads to good reproduction rates. After 28 days, the adult worms are removed. The earthworms hatched from the cocoons are used for testing when mature after at least 2 months but less than 12 months.

Worms of the species described above can be considered healthy if they move through the substrate, do not try to leave the substrate, and reproduce continuously. Very slow motioning or a yellow posterior end (in the case of *E. fetida*) indicate substrate exhaustion. In this case, fresh substrate and/or a lower number of animals per box is recommended.

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Enchytraeids:

The recommended test species is *Enchytraeus albidus* Henle 1837 (white potworm). *Enchytraeus albidus* is one of the biggest (up to 15 mm) species of the annelid oligochaete family Enchytraeidae and it is world-wide distributed (e.g. Bell 1958). *Enchytraeus albidus* is found in marine, limnic and terrestrial habitats, mainly in decaying organic matter (seaweed, compost) and rarely in meadows (Kasprzak 1982). This broad ecological tolerance and some morphological variations indicate that there might be different races for this species.

Enchytraeus albidus is commercially available, sold as food for fish. It should be checked whether the culture is contaminated by other, usually smaller species (Römbke & Moser 1999). If contamination occurs, all worms should be washed with water in a Petri dish. Large adult specimens of *Enchytraeus albidus* are then selected (by using stereomicroscope) to start a new culture. All other worms are discarded. Its life cycle is short as maturity is reached between 33 days (at 18 °C) and 74 days (at 12 °C). Only cultures which have been kept in the laboratory for at least 5 weeks (one generation) without problems should be used for a test.

Other species of the *Enchytraeus* genus are also suitable, especially *E. luxuriosus*. This species is a true soil inhabitant, which has been newly described by Schmelz & Collado (1999). If other species of *Enchytraeus* are used, they must be clearly identified and the rationale for the selection of the species should be reported.

Enchytraeus crypticus (Westheide & Graefe 1992) is a species belonging to the same group like *E. luxuriosus*. It has not been found to exist with certainty in the field, having only been described from earthworm cultures and compost heaps (Römbke 2003). Its original ecological requirements are therefore not known. However, recent laboratory studies in various field soils have confirmed that this species has a broad tolerance towards soil properties like pH and texture (Jänsch et al. 2005). In recent years, this species has often been used in ecotoxicological studies because of the simplicity of its breeding and testing (e.g. Kuperman et al. 2003). However, it is small (3 – 12 mm; 7 mm on average (Westheide & Müller 1996)) and this makes handling more difficult compared with *E. albidus*. When using this species instead of *E. albidus*, the size of the test vessel can but needs not to be smaller. In addition, it has to be considered that this species reproduces very rapidly having a generation time of less than 20 days at 20 ± 2°C (Achazi et al. 1999) and even quicker at higher temperatures.

Enchytraeids of the species *Enchytraeus albidus* (as well as other *Enchytraeus* species) can be bred in large plastic boxes (e.g. 30 x 60 x 10 cm) filled with a mixture of artificial soil and commercially available, uncontaminated garden soil free of additives. Compost material must be avoided since it could contain toxic substances like heavy metals. Fauna should be removed from the breeding soil before use by 3 times deep-freezing. Pure artificial soil can also be used but the reproduction rate could be slower compared to that obtained with mixed substrates. The substrate should have a pH of 6.0 ± 0.5. The culture is kept in an incubator at a temperature of 15 ± 2 °C without light. In any case, a temperature higher than 23 °C must be avoided. The artificial/natural soil moisture should be moist but not wet. When the soil is gently pressed by hand, only small drops of water should appear. In any case, anoxic conditions must be avoided (e.g. if a lid is used, the number of lid holes must be high enough to provide sufficient exchange of air). The breeding soil has to be aerated by carefully mixing it once per week.

The worms should be fed at least once per week ad libitum with rolled oats which are placed into a cavity on the soil surface and covered with soil (Scheffczyk, 2008, pers. comm.). If food from the last feeding date remains in the container, the amount of food given must be adjusted accordingly. If fungi grow on the remaining food, it should be replaced by a new quantity of rolled oats. In order to stimulate reproduction, the rolled oats may be supplemented with commercially available, vitamin amended protein powder every two weeks. After three months, the animals are transferred to a freshly prepared culture or breeding substrate. The rolled oats, which have to be stored in sealed vessels, should be autoclaved or heated before use in order to avoid infections by flour mites (e.g. *Glyzyphagus sp.*, Astigmata, Acarina) or predacious mites (e.g. *Hypoaspis*

(Cosmolaelaps) miles, Gamasida, Acarina). After disinfecting, the food is ground up so that it can easily be strewn on the soil surface. Another possible food source is baker's yeast or the fish food TetraMin®.

In general, the culturing conditions are sufficient if worms do not try to leave the substrate, move quickly through the soil, exhibit a shiny outer surface without soil particles clinging to it, are more or less whitish coloured, and if worms of different ages are visible. Actually, worms can be considered healthy if the they reproduce continuously.

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