Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft
Berlin-Dahlem

Development of laboratory methods for testing effects of chemicals and pesticides on Collembola and earthworms

Entwicklung von Labormethoden zur Prüfung der Wirkung von chemischen Stoffen und Pflanzenschutzmitteln auf Collembolen und Regenwürmer

by
Dr. Frank Riepert
and
Dr. Christine Kula

Biologische Bundesanstalt für Land- und Forstwirtschaft
Institut für Ökotoxikologie im Pflanzenschutz Berlin und
Abteilung für Pflanzenschutzmittel und Anwendungstechnik Braunschweig

Heft 320

Berlin 1996

Herausgegeben
von der Biologischen Bundesanstalt für Land- und Forstwirtschaft
Berlin-Dahlem

Parey Buchverlag Berlin
Kurfürstendamm 57, D-10707 Berlin

ISSN 0067-5849 ISBN 3-8263-3125-7

Die Deutsche Bibliothek – CIP-Einheitsaufnahme

Riepert, Frank: Development of laboratory methods for testing effects of chemicals and pesticides on Collembola and earthworms = Entwicklung von Labormethoden zur Prüfung der Wirkung von chemischen Stoffen und Pflanzenschutzmitteln auf Collembolen und Regenwürmer / by Frank Riepert and Christine Kula. Hrsg. von der Biologischen Bundesanstalt für Land- und Forstwirtschaft Berlin-Dahlem. – Berlin: Parey [in Komm.],1996.
(Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft Berlin-Dahlem; H. 320)
ISBN 3-8263-3125-7
NE: Kula, Christine:; Biologische Bundesanstalt für Land- und Forstwirtschaft <Berlin; Braunschweig>:
Mitteilungen aus der...

1996 Kommissionsverlag Parey Buchverlag Berlin, Kurfürstendamm 57, 10707 Berlin Printed in Germany by Arno Brynda, Berlin

Table of contents - Inhaltsverzeichnis

I. Preface

In all countries in the European region some type of control legislation exists concerning industrial and agricultural chemicals. The European Communities Directive for the notification of new chemicals (Council Directive on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances (67/548/EEC)) requires the manufacturer or importer of a new substance to submit 'a technical dossier supplying the information necessary for evaluating the foreseeable risks, whether immediate or delayed, which the substances may entail for man and the environment .....'. The information required under the 6th Amendment (Council Directive amending for the sixth time Directive 67/548/EEC) concerns the identity use pattern, fate and toxicity of a chemical. Base-set information includes parameters which are fundamental for risk evaluation. A stepwise testing scheme is employed, with a basic test package required of substances with production volumes of up to 1 ton per year, and respectively more intensive testing required of substances with production volumes of 100 tons per year (cumulative 500 tons produced) and 1000 tons (or cumulative 5000 tons produced). Ecotoxicological studies on the base level are restricted to the aquatic compartment. Tests in levels 1 and 2 comprise further studies on the ecotoxic effects including tests of terrestrial organisms (earthworms and higher plants), and the persistence of the substance. The testing necessary at level 2 should preferably be discussed between the manufacturer and the competent authority taking into consideration the information available from the levels below.

For approval of agricultural plant protection products (pesticides) the detailed requirements vary considerably between countries but are now harmonized at least for member countries of the EU by the Council Directive (91/414/EEC) concerning the placing of plant protection products on the market. Applications for approval must generally contain information on composition, intended use, efficacy, toxicity and possible side effects on non-target organisms.

The approval of an active ingredient to be taken up into Annex I (active substances permitted in EEC - accepted plant protection products) of Council Directive 91/414/EEC requires among others again ecotoxicological studies on the toxicity to earthworms and other non-target soil macroorganisms. Test data and documents submitted for notification of a substance or registration of a pesticide must be elaborated on the basis of internationally harmonized guidelines or other accepted scientific test methods. Only data obtained from tests which proved to be a reliable tool for measuring ecologically relevant effects can build a basis for a hazard assessment. In addidition, to be accepted internationally, test methods used in a hazard assessment programme should pass an international harmonization process. Very few test methods are available for testing effects of chemicals and pesticides on soil fauna. Both methods presented are part of the harmonization programm of the Technical Committee 190 'Soil Quality of the International Standards Organisation' and are provided for the OECD test guidelines programme.

This publication intends to provide testing laboratories, authorities and organisations involved in harmonization processes with more detailed information gained from experiences gathered in collaborative studies of national or international working groups and in experimental work at the Institute for Ecotoxicology in Plant Protection and the Biology Division of the Department for Plant Protection Products and Applications Techniques of the Federal Biological Research Centre for Agriculture and Forestry (Biologische Bundesanstalt für Land- und Forstwirtschaft).

Vorwort

Alle Länder Europas besitzen in irgendeiner Form eine Gesetzgebung zur Überprüfung von Chemikalien, die in der Industrie oder Landwirtschaft verwendet werden. Die Richtlinie der

Europäischen Gemeinschaften zum Anmeldeverfahren neuer Stoffe (Richtlinie des Rates zur Angleichung der Rechts- und Verwaltungsvorschriften für die Einstufung, Verpackung und Kennzeichnung gefährlicher Stoffe (67/548/EWG)) verlangt vom Hersteller oder Importeur u.a. die Vorlage „einer technischen Beschreibung mit Angabe der Einzelheiten, die die Beurteilung der vorhersehbaren sofortigen oder späteren Gefahren ermöglicht, die der Stoff für den Menschen und die Umwelt darstellen kann...". Die in der 6. Änderungsrichtlinie (Richtlinie des Rates zur 6. Änderung der Richtlinie 67/548/EWG) verlangten Angaben betreffen Stoffidentität, Verbleib und Toxuzität eines Stoffes. Die in der Grundstufe des Anmeldeverfahrens geforderten Angaben beinhalten die für die Risikoabschätzung unerläßlichen Prüfgrößen. In einem abgestuften Prüfverfahren wird für Stoffe mit einem Produktionsvolumen unterhalb einer Jahrestonne ein Basispaket an Prüfungen gefordert, gefolgt von eingehenderen Prüfungen bei Produktionsvolumen von 100 Jahrestonnen (500 Tonnen insgesamt seit Herstellungsbeginn) und bei 1000 Jahrestonnen (5000 Tonnen insgesamt seit Herstellungsbeginn). Ökotoxikologische Untersuchungen auf der Grundstufe beschränken sich auf den aquatischen Bereich. Prüfungen auf Stufe 1 und 2 umfassen zusätzliche Untersuchungen zu ökotoxischen Wirkungen einschließlich solcher an Organismen des terrestrischen Bereichs (Regenwurm und höhere Pflanzen) und zur Persistenz eines Stoffes. Die Prüfungen der Stufe 2 werden in Absprache mit dem Hersteller oder Einführer unter Berücksichtigung der Informationen aus den bereits durchgeführten Untersuchungen der darunterliegenden Stufen vereinbart.

Die genauen Anforderungen der Zulassungsverfahren für Pflanzenschutzmittel der einzelnen Staaten unterscheiden sich beträchtlich, sind aber nun wenigstens zwischen den Mitgliedsländern der Europäischen Union durch die Richtlinie 91/414/EWG des Rates über das Inverkehrbringen von Pflanzenschutzmitteln vereinheitlicht. Zulassungsanträge müssen im allgemeinen Informationen über die Zusammensetzung, Anwendungsgebiete, Wirksamkeit, Toxizität und möglichen Nebenwirkungen auf Nicht-Zielorganismen beinhalten. Der Antrag zur Aufnahme eines Wirkstoffes in Anhang I (für die Verwendung in Pflanzenschutzmitteln zulässige Wirkstoffe) der Ratsrichtlinie 91/414/EWG verlangt u.a. auch Untersuchungen zur Toxizität an Regenwürmern und anderen Bodenorganismen, die nicht Ziel der Bekämpfung sind.

Die bei der Anmeldung eines Stoffes oder der Zulassung eines Pflanzenschutzmittels vorgelegten Prüfdaten und Unterlagen müssen auf der Grundlage von international harmonisierten Richtlinien oder anderen wissenschaftlich anerkannten Testmethoden erarbeitet sein. Für die Risikoabschätzung können nur solche Daten Verwendung finden, die aus Prüfungen gewonnen wurden, die als zuverlässige Instrumente für die Messung ökologisch relevanter Wirkungen gelten. Außerdem sollten die zur Risikoabschätzung verwendeten Testmethoden einen internationalen Harmonisierungsprozeß durchlaufen haben, um international anerkannt zu werden. Es gibt nur sehr wenige Testmethoden zur Prüfung der Wirkungen von Chemikalien und Pflanzenschutzmitteln auf die Bodenfauna. Beide hier vorgestellten Methoden sind Teil des Harmonisierungsprogramms des Technischen Komitees 190 „Soil Quality" der International Standards Organisation (ISO) und sollen in das Richtlinienprogramm der OECD aufgenommen werden.

Diese Veröffentlichung soll dazu beitragen, Testlaboratorien, Behörden und Organisationen, die mit Harmonisierungsverfahren befaßt sind, mit detaillierteren Informationen zu versehen, die aus gemeinsamen Studien nationaler und internationaler Arbeitsgruppen und aus experimentellen Arbeiten des Instituts für Ökotoxikologie im Pflanzenschutz und der Fachgruppe für biologische Mittelprüfung der Abteilung für Pflanzenschutzmittel und Anwendungstechnik der Biologischen Bundesanstalt für Land- und Forstwirtschaft gewonnen wurden.

II.German Summaries - deutsche Zusammenfassungen

Entwicklung eines Standardtests mit *Folsomia candida* Willem, 1902 (Collembola: ISOTOMIDAE) als Testorganismus zur ökotoxikologischen Prüfung und Bewertung von Chemikalien nach dem Gesetz zum Schutz vor gefährlichen Stoffen

Die Eignung von Bodenorganismen als Indikatoren subletaler Wirkungen von Stoffen, deren toxische Eigenschaften unter standardisierten Laborbedingungen zu prüfen sind, hängt in großem Maße davon ab, ob die ausgewählte Art sowohl ökotoxikologischen als auch praktischen Ansprüchen gerecht wird. Hierzu zählt, ob die Art unter Laborbedingungen unter vertretbarem Aufwand zu züchten und zu halten ist, ob genügend Informationen darüber vorliegen, in welchem Ausmaß eine Art während ihres Lebenszyklus einer Stoffbelastung ausgesetzt ist und ob sie am Umsetzungsprozeß organischer Substanz im Boden beteiligt ist.

Auf Grund ihrer Lebensweise, Vebreitung und Funktion spielen Collembolen eine bedeutende Rolle in Bodenökosystemen. Darüberhinaus ist das überaus wichtige Kriterium ihrer Eignung als Testorganismus durch ausreichend verfügbare Erfahrungen in der Haltung und Zucht verschiedener Arten erfüllt.

Auf der Grundlage zweier Forschungsprojekte, ausgeführt beim Bundesgesundheitsamt in Berlin, zur Standardisierung von Zuchtverfahren für fünf Collembolenarten (*Hypogastrura bengtsoni, Onychiurus fimatus, Folsomia candida, Proistotoma minuta, Sinella coeca)* und zur Entwicklung eines standardisierten Testverfahrens zur Prüfung der akuten Toxizität von Umweltchemikalien an Springschwänzen unter besonderer Berücksichtigung von *Folsomia candida*, wurde von einer ad hoc AG unter Federführung des Institutes für Chemikalienprüfung der Biologischen Bundesanstalt für Land- und Forstwirtschaft ein Verfahrensvorschlag entwickelt, der insbesondere für Prüfungen zur Verfügung steht, die im Anmeldeverfahren nach dem Chemikaliengesetz erforderlich sein können.

Das Testprinzip liegt in der Bestimmung des Schwellenwertes einer Testsubstanz, bei dem unter festgelegten Bedingungen die Reproduktionsrate von Springschwänzen der Art *Folsomia candida* in einem künstlichen Bodensubstrat während einer 4-wöchigen Versuchsdauer im Vergleich mit einer unbehandelten Kontrolle signifikant verringert ist.

Die in 'Ringtests' gewonnenen Versuchsergebnisse, die die Konzeption der Prüfmethode bestimmt haben, werden dargestellt und liefern den Hintergrund für den Verfahrensvorschlag und seinen Anhang mit unverbindlichen Vorschlägen u.a. zur Haltung und Zucht der Versuchstiere und zur Auswertung der Versuchsgefäße.

Da u.a. auch in den Niederlanden und in Dänemark ähnliche Methoden existieren, und für standardisierte Methoden zur biologischen Charakterisierung von Böden weltweit ein großes Interesse besteht, ist es gelungen, den vorgestellten Verfahrensvorschlag bei der 'International Standards Organisation' (ISO) als Grundlage für die Erarbeitung einer Norm einzubringen.

Die Beschreibung des Verfahrensvorschlages entspricht daher formal dem Aufbau der ISO-Normen.

Entwicklung einer Methode zur Prüfung subletaler Auswirkungen von Pflanzenschutzmitteln auf die Regenwurmart *Eisenia fetida* - Vergleich von zwei Ringversuchen

Um die Auswirkungen von Pflanzenschutzmitteln auf die Reproduktion von Regenwürmern zu untersuchen, wurde eine Testmethode entwickelt. Als Testsubstrat diente wie im Akuttest (OECD-Richtlinie Nr. 207) ein künstliches Substrat (artificial soil). Als Testorganismus wurde die Art *Eisenia fetida* oder *Eisenia andrei* eingesetzt. Das zu untersuchende Pflanzenschutzmittel wurde so praxisnah wie möglich appliziert, das heißt zum Beispiel auf die Bodenoberfläche gesprüht. Testparameter waren die Anzahl der im Versuchszeitraum geschlüpften und überlebenden Jungtiere und die Gewichtsentwicklung und Mortalität der adulten Tiere im Versuchsverlauf.

Um diesen Versuchsansatz auf seine Verwendbarkeit zu überprüfen, wurden in den Jahren 1990/91 und 1991/92 zwei Ringversuche mit zwei verschiedenen Pflanzenschutzmitteln durchgeführt. Für den zweiten Ringversuch wurde die Methode in einigen Details abgeändert. Der wichtigste Unterschied bestand in der Expositionsdauer der adulten Tiere. Diese wurde von 6 Wochen im ersten Versuch auf 4 Wochen im zweiten Versuch reduziert. Außerdem wurde das Futter im zweiten Ringversuch nicht zentral von der koordinierenden Stelle verteilt.

Die Ergebnisse beider Ringversuche bestätigen die Eignung der Methode im Rahmen der Risikoabschätzung für Pflanzenschutzmittel. Es zeigte sich, daß eine Expositionszeit der adulten Tiere von 4 Wochen zu vergleichbaren Ergebnissen führt wie die 6-wöchige Exposition. Allerdings ergaben sich im zweiten Ringversuch dadurch Probleme, daß das von den Versuchsteilnehmern verwendete Futter nicht in allen Fällen geeignet war. Daher waren im zweiten Ringversuch einige Versuche aufgrund mangelnder Reproduktion nicht brauchbar.

Werden die Versuche mit zu geringer Reproduktion und ohne gerichtete Dosis-Wirkungsbeziehung von einer vergleichenden Betrachtung ausgeschlossen, erweist sich die Methode als sensibel und zuverlässig.

Das im Ringversuch eingesetzte Pflanzenschutzmittel mit dem Wirkstoff Benomyl wurde auch hinsichtlich seiner Eignung als toxischer Standard geprüft. Aufgrund der vorliegenden Ergebnisse zeigt sich eine gute Eignung des Wirkstoffs Benomyl und damit auch des verwandten Wirkstoffs Carbendazim als toxischer Standard für diesen Versuchstyp.

Die Erkenntnisse aus beiden Ringversuchen sind in die Erstellung einer BBA-Prüfrichtlinie (BBA, 1994) und eines ISO-Richtlinienentwurfs (ISO-DIS 11268-2) eingeflossen.

Frank Riepert, Berlin

III. A standard test method with *Folsomia candida* Willem, 1902 (Collembola: *Isotomidae*) as test organism for the ecotoxicological testing of new and/or existing chemicals following the hazard assessment concept of the act on protection against dangerous substances

Entwicklung eines Standardtests mit *Folsomia candida* Willem, 1902 (Collembola: *Isotomidae*) als Testorganismus zur ökotoxikologischen Prüfung und Bewertung von Chemikalien nach dem Gesetz zum Schutz vor gefährlichen Stoffen

III.1 Development of the test method
Entwicklung der Methode

CONTENTS

1. Introduction

The increased knowledge on soil pollution caused by the release of chemical substances and their metabolites has created a need for appropriate test methods which allow to determine the soil quality or adverse effects of chemicals.

Because of the great variety of soil organisms and a very complex system of interactions it is extremely difficult to determine the impact caused by the release of substances on life communities of the soil. As ecosystemical approaches are very timeconsuming and expensive, the legal test requirements are fulfilled by tests studying only the effects on single species, the test organisms being considered as representative to the community of soil organisms.

Microarthropods as e.g. springtails are said to have an important function regarding the maintainance of soil functions. Due to their short life cycles, high numbers of species and their high density, important requirements for using them as indicator organisms are fullfilled. An expert group initiated by the Institut für Chemikalienprüfung (Institute for Chemical Examination) of the Biologische Bundesanstalt für Land- und Forstwirtschaft (Federal Biological Research Centre for Agriculture and Forestry) startet in 1985 to elaborate a draft guideline for ecotoxicological testing of chemicals on Collembola. The test protocol was tested for several years in the laboratory and used by contract institutes for the testing of chemicals. At present, this method is involved in a harmonization process to become an international standard for the biological testing of soil quality.

2. Requirements for the test

The complex of 'ecotoxicological' tests in relation to the assessment of potential environmental hazards of chemicals has been discussed by several authors (e.g. IGLISCH, 1985, 1986; SCHLOSSER, 1985; BECKER, 1986; LANGE, 1986; RUDOLPH & BOJE, 1986; NUSCH 1986; PETER and FRANKE, 1993). The topics reviewed range from the definition of 'ecotoxicology' in relation to 'toxicology' over the requirements for ecotoxicological test systems to questions concerning the evaluation of data and the precautionary measures to be taken to minimize potential hazards to the natural balance.

For IGLISCH (1986) the objective of the previous national and international harmonization process within the chemicals legislation and associated research projects is focused on the ecochemical behaviour of the chemical. The choice of the test organisms showed that they were used just for this purpose neglecting biocoenotic aspects. In consequence the authentic legal objective of environmental protection was not taken into account sufficiently.

Describing the contents of urgent improvements of the hazard assessment concept, in particular in the field of terrestrial ecotoxicology, the author did not recommend an updating of existing acute test methods or the adoption of new ones but insisted on replacing them by subacute tests. The following steps on the way to an improved concept are proposed:

- Search for representative soil organisms
- Elaboration of conditions for maintaining sufficient numbers in culture
- Development of subacute tests
- Validation of the test methods

The suitability of soil organisms used in the laboratory as indicator organisms for potentially harmful effects of chemicals is characterized - besides the more practical aspect of being easy to culture - by the fact to what extent, their life cycle takes place within the upper soil layer

with respect to possible chemical exposure, and to what extent they participate at the decomposition process in the soil (IGLISCH, 1989).

The function and significance of an animal species for the community of soil organisms or even the ecosystem is therefore a subject repeatedly discussed when the suitability of a test organism has to be defined.

The question is whether the response of a single species to a chemical stress factor may be considered as representative for any organisation level within the ecosystem.

Are there 'model organisms' as for example a collembola species or population, which may be considered as representative of enchytraeae, larvae of dipterae or other soil organisms with similar functions, and can mechanisms of response be found for assessing long-term hazards ? According to the present knowledge the answer is no.

However the political agreement aiming at a prospective prevention of any risks compels to extrapolations between different ecosystematical levels without being scientifically proven or verified. An example for this is given by the development of a model designed for deducing ecotoxicological threshold levels applicable to a whole ecosystem. OKKERMAN et al., 1993, tried to validate recent extrapolation models. With some restrictions they concluded that toxicity data gained from single-species tests are a good basis just to determine threshold levels for aquatic ecosystems.

3. Collembola as test system

3.1 Properties meeting the requirements for a test organism

Due to their mode of life, distribution and function there is no doubt about the biological significance of Collembola for the soil (FOLSOM, 1933; SCHALLER, 1950 and 1970; SPAHR 1981; DUNGER, 1983, HERGARTEN, 1984; IGLISCH, 1985; van STRAALEN, 1989; KLIRONOMOS et al., 1992). Their sensitivity is direct or indirect effects of pesticides and industrial chemicals has been described in serveral publications (ULBER, 1979; FRAMPTON, 1988; CROMMENTUIJU, 1994). Moreover, the experience gained in mass breeding and rearing of Collembola is a decisive factor with regard to their suitability as test organisms.

3.2 Basis for the development of a test with Collembola

Studies carried out to develop such a test method require a sufficient number of homogeneous animals. As the availability of animals living in the field is influenced by seasonal or climatic factors only a species being cultured in the laboratory could meet this requirement.

The objective of a thesis promoted by the Umweltbundesamt (Federal Environmental Agency) and carried out by SPAHR (1981a, 1981b and 1983) within a research project on soil arthropods as test organisms in ecotoxicology at the Institut für Wasser- Boden- und Lufthygiene (Institute for Hygiene of Water, Soil and Atmosphere)of the Bundesgesundheitsamt (Federal Health Office) was to develop a standardized breeding method. For five Collembola species *Hypogastrura bengtsoni, Onychiurus fimatus, Folsomia candida, Proistotoma minuta* and *Sinella coeca* simple methods for mass breeding were described.

Additionally, on the basis of the results from this study and a method described by THOMPSON & GORE (1972) a 'Standardized test method for studying the acute toxicity of environmental chemicals on springtails (Collembola), with particular consideration of Folsomia candida' was developed within the research project 'Development of test models for studying the toxicity of environmental chemicals on terrestrial animals under field and laboratory

conditions'. The principle of this method was to determine the acute toxicity of a substance on six weeks old Collembola after a test period of 24 hours on a substrate consisting of refined sea sand to which different doses of the test substance were added. To test this method 8 chemicals and 2 plant protection products were used.

For routine testing not only *Folsomia candida* but also *Proisotoma minuta* and *Sinella coeca* proved to be suitable organisms because they are easily reared and their reproducibility was high, whereas the reproduction capacity of *Hypogastrura bengtssoni* and *Onychiurus fimatus* was too low. Beyond the development of this test, fundamentals for the identification of sublethal effects describing the effects on reproduction were prepared and test parameters (e.g. feeding capacity, oviposition, hatching rate) proposed.

An additional approach regarding the choice of suitable test parameters for the identification of sublethal effects was made by the research project 'Development of methods for testing the toxicity of chemicals on indifferent arthropod species' funded by the Federal Minister of Food, Agriculture and Forestry and carried out at the Institute of Applied Zoology of the University Bonn (DIELMANN, 1984; TALBOT, 1987). The species were selected according to the life forms occurring in the different soil layers - *Folsomia candida* as an euedaphic to hemiedaphic species, *Tomocerus flavescens* as an epiedaphic species and *Onychiurus armatus* preferably living in deeper layers (edaphon) - and to their biology and taxonomic position. The parameters studied were moulting interval, time of oviposition, impairment of the locomotion system and the water budget, embryonic development and hatch rate of eggs. Representatives of the chemical industry also reported about first experiences made with bioassays using Collembola as test organisms (personal information). On the basis of the above mentioned studies on culturing techniques for Collembola (SPAHR) and on the acute test (WOLF-ROSKOSCH), HEIMBACH (verbal report) modified the method of the acute test, in particular regarding the substrate used, and tested it successfully. Instead of the quartz sand an artificial soil substrate was used as described in the internationally (EC, OECD) harmonized earthworm test.

3.3 Concept for the experimental design of a sublethal test method on Collembola

As a result of the discussion about an adequate test with soil arthropods for the identification of sublethal effects the following items were agreed on:

- Collembola are considered to be an adequate representative for testing the effects of a substance on soil arthropods.
- Within a range of concentrations of a test substance the test parameters selected should be adequate to show sublethal effects of a test substance mixed into the soil substrate.
- Reproduction rate and population development are recognized to be suitable parameters.
- The length of the test period should comprise a life-cycle to assess any effects on reproduction.

On this basis a sub-working group was established to elaborate a draft proposal of a guideline. In addition, the development of the test was supported by studies carried out at the Institut für Bodenzoologie der Freien Universität Berlin (Institute of Soil Zoology of the Free University of Berlin) the results being published by JANCKE 1989.

3.3.1 Test species

With respect to the potential of the taxon Collembola to adapt to the soil layers inhabited it seemed appropriate to keep species available which are representative of the differently

exposed habitats. This would allow to select the appropriate species from a range of available collembola species dependent on the contamination conditions.

WOLTERS (1986) formulated the requirements to be fulfilled by Collembola when used as test organisms as follows:

- The species should be multivoltine in order to have sufficient test animals available at any season and to guarantee an assessment of the reproduction behaviour independent of time;
- great numbers should be easy to culture under conditions close to nature;
- the laboratory breeding stock should remain genetically stable over a long period of time.

However, there is hardly a Collembola species to meet all these requirements. In particular, the last requirement mentioned is to be considered with the greatest scepsis. Even breeding stocks kept under conditions close to nature e.g. of *Onychiurus fimatus* and *O. furcifer* are susceptible to instability regarding their ratio and reproduction success. For that reason, a permanent examination of the cultures and a regular renewal by field populations seems to be appropriate. As a result of the studies carried out by SPAHR (1981), continued by WOLF-ROSKOSCH (1983), breeding experience existed with the following species: *Hypogastrura bengtssoni, Folsomia candida, Proisotoma minuta , Sinella coeca* and *Onychiurus fimatus.*

Three of these species, i.e. *Folsomia candida, Proisotoma minuta and Sinella coeca,* are particularly suitable for routine testing because their rearing is easy and their life-cycle is short. The two other species had a relatively low reproduction capacity.

As on the one hand there was no information from relevant literature which would allow for the conclusion that the response to chemicals would be the same for different Collembola species and on the other hand there was no evidence whether the differences observed were specific to the species, substrate, habitat or behaviour, experience in testing was to be gained for practical reasons only with one species. Possible slight variations regarding the sensitivity of such a 'laboratory species' in comparison with field populations had to be accepted because it could not be guaranteed, when field populations are used, that a sufficient number of animals would be always available for tests, regardless of the fact that difficulties with the taxonomic identification had to be expected.

Under these aspects the following three species were considered as being adequate:

Isotoma tigrina, Proisotoma minuta and *Folsomia candida.*

As most of the culturing experience was gained with *Folsomia candida* and as the two other species did not belong to a different soil layer and no advantages were to be expected as for the extrapolation to field conditions, the working group finally selected *Folsomia candida* as test organism.

3.3.2 Test parameters

Parameters measuring directly the function or productivity of the test organism were left out of consideration for describing sublethal chemical effects. It was generally agreed that such effects may be determined indirectly by means of the reproduction parameters, as for example the period required to complete a life-cycle (from hatching to oviposition), the embryonic development and the number of offspring.

Methodical experience existed in the working groupfor testing the period of embryonic development and the population development starting at different stages.

The number of offspring (F_1) which can be determined quantitatively was selected as parameter for the determination of sublethal chemical effects.

3.3.3 Test substrate

The choice of the test substrate is dependent on the length of the test period and the test criteria. Besides the methodical aspect regarding the influence of the test substrate on the practicability of the numerical assessment of the test criteria it must be considered that the impact of a chemical to the test organism is not an absolute figure but always correlated to the medium. Due to the requirement of the Chemicals Act to assess the environmental risk of potentially hazardous substances, and the objectives stipulated by ordinance, a substrate simulating natural soil properties but reproducible in the laboratory was assumed to be preferable to substrates consisting of an inert matrix. Taking into account the advantages and disadvantages of artificial soil in comparison to the substrates gypsum and sea sand used for breeding purposes and in short term tests, the artificial soil as described for the earthworm test (OECD, EC) was prefered. Due to its components the adsorption properties are similar to an arable soil (with high organic matter content) and the artificial soil substrate has the advantage of being standardized and reproducible.

A further advantage of this substrate is the fact that the springtails are able to penetrate the substrate. In the case of the euedaphic species *Folsomia candida* being used as test organism this test design is likely to meet better the conditions of living and exposure in the natural biotope habitat. On the other hand, the fact that the collembola may easily penetrate the substrate makes the verification of the test criteria as for example oviposition and hatching more difficult during the test period and at the end of the test.

3.3.4 Test period

The implementation of the objectives of the test in a standardized and reproducible test procedure requires a time schedule providing under the test conditions a good estimate of the periods of the various developmental stages.

As the main objective of the test is studying the effect of a substance on the reproduction rate, the period of exposure should begin with the period of the sexual maturity of the adults and end with the hatching from the eggs. Thus, two periods have to be determined:

1. The period of time of the embryonic and larval development in order to fix the beginning of exposure with a test substance.
2. The period of time from the subadult stage (e.g. the fifth larval stage) to the hatch of the F_1-generation in order to determine the exposure period and to fix the end of the test.

The results obtained from observations concerning the periods of time necessary to reach specific developmental stages relevant for the test are summarized in Table 1. For comparison, Table 2 shows results taken from literature by JANCKE (1989).

Table 1: Periods needed by F. candida under the test conditions to reach defined developmental stages

Type of Development	Period (d) Range	Observations N
Embryonic development	7 - 12	7
Larval development	11 - 18	5
Hatch - Oviposition	14 - 30	8

Table 2: For Comparison, periods compiled from different authors (from JANCKE, 1989; modified to SNIDER, 1973)

	Green 1964	Marshall & Kevan 1962	Milne 1960	Snider 1973	Marshall & Kevan 1962
Temperature (^{0}C)	25	24	24	21	20
Embryonic development (days)	7	7 - 10	7 - 9	9 - 11	10 - 15
Days between hatching and maturity	13 - 18	11 - 30	20 - 24	13 - 29	
Days between Oviposition	6 - 8	11 - 13		5,5 - 12	14 - 16

When planning the test schedule it was proposed to fix the beginning of a test by the completion of the fifth larval stage in order to assure that the animals at the beginning of oviposition are exposed for a sufficient time to the test chemical.

However, as in view of the wide range of the developmental periods the exact determination of this sub-adult stage turned out to be difficult, it was decided at first to start the test with recently hatched instars. Because of the vulnerability of these animals the age of the test organisms at the beginning of the test was extended to 10 days. At this age, the larval development is not yet finished and there is enough time left for exposure induced effects until the beginning of oviposition.

The reproducibility of the test results requires a minimum of synchronisation of the development. Therefore hatching was restricted to an interval of 48 hours. (Techniques to obtain synchronized cultures are described in Annex A.1.6 of part II.) The test should end when the F_1 generation has hatched from the eggs laid by the parental springtails. According to the given developmental periods this was to be expected 3-4 weeks after the 10 days old animals had been placed on the substrate.

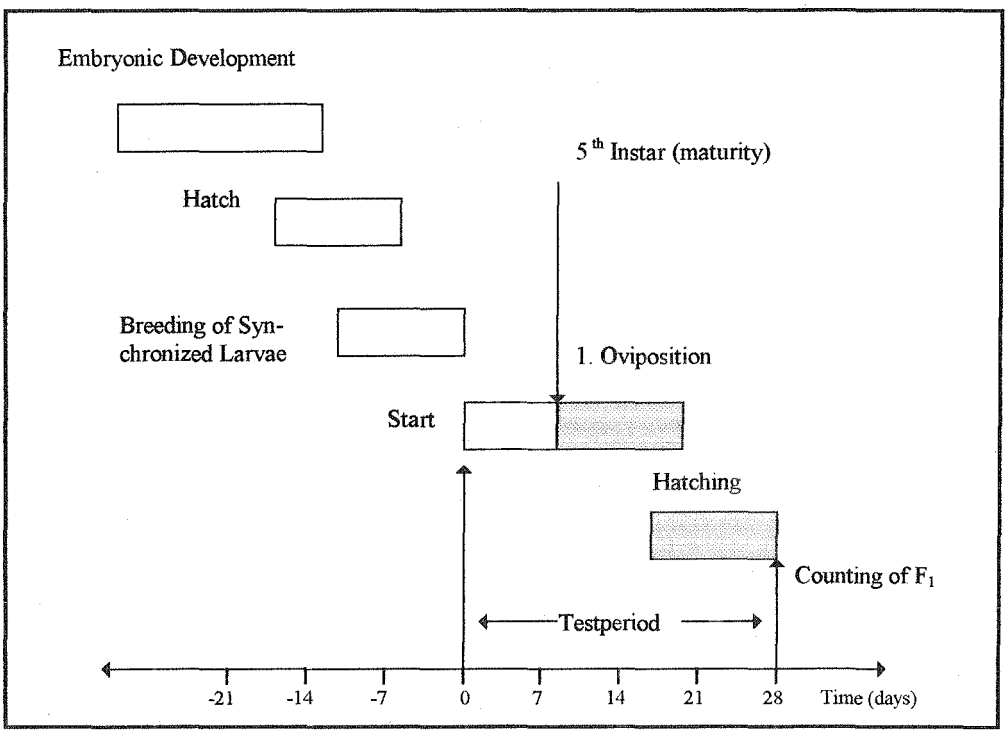


Figure 1: Sequence of different steps following the preparation and performance of the test.

The step sequence starts with the embryonic development of an induced oviposition by transferring springtails to fresh breeding containers and ends with the counting of the juveniles (F_1 - generation) in each of the test containers. The time at which the test organisms are transferred to the test containers (start of the test) is given the value 0 on the horizontal axis. Preceeding periods (test preparation) have negative values.

3.3.5 Food supply during the test

To decide whether feeding of the springtails during the test period is required or not, 10 springtails at the age of 10 to 12 days were allowed to feed upon granulated dry yeast spread on a small glass plate placed on the substrate others were kept without any food supply. After a period of 5 and 6 weeks respectively the number of living adults and juveniles was determined.

The containers without any food supply showed an increased mortality (15-30%) of the adults and no reproduction at all, whereas in the containers supplied with food the average mortality of the adults was smaller than 10 % and the mean number of juveniles was 200 (after 5 weeks) and 500 respectively (after 6 weeks).

In order to optimize the reproduction success another test was carried out to find the adequate quantity required during the test period.

Depending on the type of application different quantities of granulated dry yeast were added to 30 g wet substrate in the test containers.

Ten springtails at the age of 10-14 days were transferred to the test containers. After a period of 4 weeks the mortality of the adults and the number of juveniles was determined in each of the classes and replicates.

Table 3: Feeding test with 10 springtails per container using different quantities of granulated dry yeast and two types of application

yeast mixed into the substrate			yeast spread on the substrate		
Quantity (mg/30g)	**no. of adults**	**no. of juveniles**	**Quantity (mg/30 g)**	**no. of adults**	**no. of juveniles**
5	10	0	0,5	10	150
	7	0		9	150
10	9	0	1,0	10	150
	10	0		8	190
15	10	0	1,5	9	320
	9	0		10	320
20	9	0	2,0	10	280
	10	0		10	350
25	9	0	2,5	10	480
	9	0		10	350
30	8	0	3,0	10	160
	10	0		9	350

Good reproduction rates were obtained when 15 to 25 mg granulated yeast were spread on the substrate surface. Based on this result it was proposed to feed the springtails at the beginning of the test with about 2 mg of dry granulated yeast per test container and to replace food during the test if necessary.

3.3.6 Experimental design of a first approach

Test principle:	Subadult collembola are exposed to sublethal concentrations of a chemical mixed into an artificial soil substrate over a period of time including oviposition, hatching and first instar stages.
Test organism:	*Folsomia candida* (Willem).
Test parameter:	Reproduction rate, duration of embryonic development.
Test substrate:	Artificial soil substrate in the same composition as used for the toxicity test on earthworms. The test substrate consists of the wet basic soil substrate, the test substance and deionized water.

Test period:	The test starts with 10 days old sub-adult springtails and ends after an exposure period of 3 to 5 weeks.
Test conditions:	For practical reasons the preliminary test conditions choosen were those of the earthworm test, i.e. the test containers are kept in a climatic chamber at 20 ± 1 ^{0}C and 70-90 % relative humidity.
	As test containers glass containers of 100 ml capacity were used able to be closed by appropriate glass lids or plastic covers. Each container was filled with 30 g test substrate.
	The springtails were allowed to feed upon 2 mg granulated dry yeast spread on the soil substrate.

3.4. Validation of the experimental design

In a series of ring tests with participation of laboratories of the industry and the University of Göttingen priority was given to optimizing start and end of the test period within the life cycle of *Folsomia candida* and the examination of the test parameters in view of relevance and practicability.

3.4.1 Test substances

The test substances used were the chemicals chloroacetamide, potassium dichromate, the plant protection products Dursban flüssig (480/l Chlorpyrifos), Pentachlorophenol (Na-salt) and the surfactant Tetrapropylenebenzenesulphonate (TPBS). Except for Dursban flüssig, which was selected as effective reference substance because of its insecticide effects, the other substances were chosen because guidance data were available from the so-called 25 substances programme (LANGE, 1985) funded by the Umweltbundesamt (Federal Environmental Agency) to validate the test inventory of the Chemicals Act.

3.4.2 Fixing the date of the test evaluation

As not only the reproduction rate (number of juveniles per test container) but also the period of embryonic development were chosen as test parameters, 3 evaluation dates (3, 4 and 5 weeks after placing the adults on the test substrate) were appointed. Not only the number of replicates was three fold, but also the juveniles of the 2nd oviposition had developed after 5 weeks, which increased the counting effort (up to 1000 animals/container) considerably. With respect to the laborious and timeconsuming procedure the evaluation was reduced to two dates, i.e. after 3 and 4 weeks.

The influence of the evaluation date (3 or 4 weeks after placing the sub-adults on the test substrate) is demonstrated by results of tests using Pentachlorophenol as test substance. These test results are appropriate because a relative great number of tests had been conducted by various laboratories and in most cases good concentration-response relations were obtained.

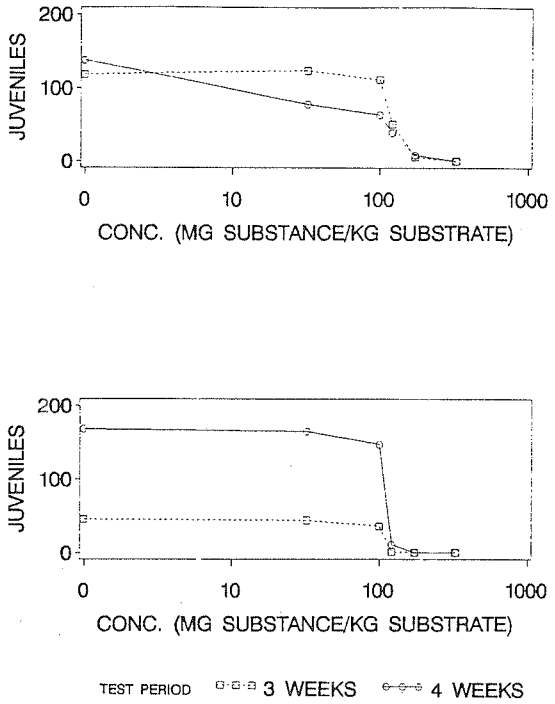


Figure 2: Two representative examples of concentration - response relationships obtained from tests using Na-PCP demonstrating an ambiguous effect of the length of the test period.

In 3 of 7 cases a significant difference between the juveniles counted at different evaluation dates was observed (left Figure). When the test was evaluated after 3 weeks, the number of animals was significantly lower in all concentrations as well as in the control and only slight effects could be observed. In the other 4 cases (right figure) the differences between the counts after the different test periods were not significant.

The advantage of a later evaluation date becomes obvious when the frequency distribution of the variation coefficients (standard deviation in % of the mean) to different size classes is considered (Fig. 3).

After a test period of 3 weeks the variation coefficient in most cases exceeds 40%, while after a period of 4 weeks it is smaller than 40 %. These data confirm that according to the periods of time indicated in Table 1 and 2 for the larval and embryonic development, the hatch of the F_1 - generation may be expected with sufficient confidence not earlier than after a period of 4 weeks.

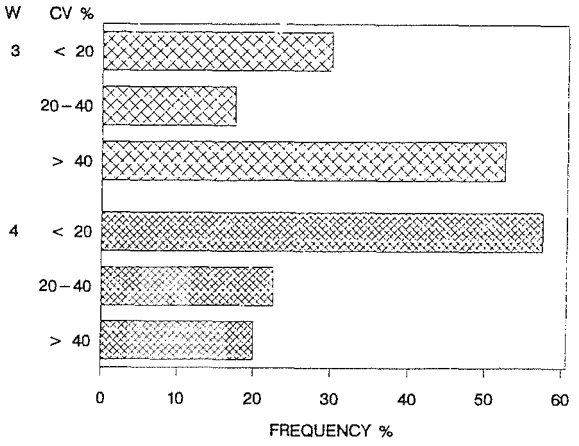


Figure 3: Distribution of coefficients of variation (CV) of reproduction rates obtained from tests using Na-PCP depending on the length of the test period (3or 4 weeks (W)).

3.4.3 Determination of effects

In order to make comparisons between treatments and the untreated control it is necessary to extract adult and juvenile Collembola from the substrate. The easiest way to do this is to flood the substrate. Because of their nonwettability Collembola drift to the water surface where they can be counted. Different counting techniques were used during the ring test. A possible procedure which has proven to be practicable and sufficient by precise is described in Annex B of part II. The prerequisite of all procedures is that they permit an efficient and consistent recovery of the number of juveniles per test unit. Any technique used for counting of the juveniles therefore has to be validated before it is applied.

After the living adults and juveniles were counted, 3 and 4 weeks respectively after placing the parental springtails on the substrate, the fourfold repeated treatments were compared with the controls.

For comparing the mean value (significance level $\alpha = 0,05$) the statistical analysis was based on the 'General Linear Models' procedure and the multiple T-test according to RYAN-EINOT-GABRIEL-WELSCH (EINOT & GABRIEL, 1975; WELSCH, 1977) using the SAS^R - statistic programme.

For the test parameters mortality of parental animals and number of juveniles per test container (reproduction rate) the LOEC (Lowest Observed Effect Concentration) was determined. Its definition is the following: The lowest tested concentration of the test substance at which the substance is observed to have a significant effect when compared with the control. All test concentrations above the LOEC must have a harmful effect equal to or greater than those observed at the LOEC.

Table 4: Ranges of threshold levels (LOEC) determined in N tests with *Folsomia candida* for the test parameters mortality and reproduction after a 4 weeks period (a). The ranges of the minimal and maximal concentrations applied in these tests are given seperately (b). Test substances were Chloroacetamid (ClAcetA), Dursban flüssig (Dursban), Potassium Dichromate (K_2 Cr_2 O_7), Pentachlorphenol (Na-PCP) and Tetrapropylenebenzenesulfonate (TPBS).

a)

		LOEC		
		N	MIN	MAX
Substance	**Testparameter**			
ClAcetA	Mort.	1.00	56.00	56.00
	Repr.	1.00	32.00	32.00
Dursban	Mort.	9.00	0.10	100
	Repr.	10.00	0.02	56.00
$K_2Cr_2O_7$	Mort.	5.00	562.00	1000.00
	Repr.	7.00	178.00	1000.00
Na-PCP	Mort.	10.00	56.00	562.00
	Repr.	13.00	32.00	316.00
TPBS	Mort.	5.00	562.00	1000.00
	Repr.	9.00	178.00	1000.00

b)

	min. Conc.		max. Conc.	
Substance	**MIN**	**MAX**	**MIN**	**MAX**
ClAcetA	5.00	5.00	56.00	56.00
Dursban	0.02	32.00	0.18	200.00
$K_2Cr_20_7$	0.01	1000.00	100.00	10 000.00
Na-PCP	10.00	178.00	100.00	1000.00
TPBS	10.00	200.00	562.00	1000.00

When determining the threshold values for the two test parameters mortality of parental animals and reproduction rate, i.e. the number of juveniles per test container (Table 4), the sensitivity of the test parameters should be assessed and compared also with other similar test systems. Threshold levels of tests with three soil organisms, *Enchytraeus albidus*, *Eisenia fetida* and *Folsomia candida* using the same artificial soil substrate are compiled in Table 5. As for financial reasons the tests were not carried out according to GLP conditions an evaluation as for the reproducibility of this method can only be made with certain restrictions.

Table 5: LOEC/NOEC or LC 50 in mg/kg dry weight in test systems using artificial soil as substrate

Testorganism	*Enchytraeus albidus*		*Folsomia candida*		*Eisenia fetida*	
Substance	**LOEC Mort. (LC 50)**	**LOEC Reprod. (NOEC)**	**LOEC Mort. (LC 50)**	**LOEC Reprod. (NOEC)**	**LOEC Mort. (LC 50)**	**LOEC Reprod. (NOEC)**
TPBS	1000[2] (18[3])	316[2] (10[3])	1000[2] 562 - > 1000[4]	178 - 316[2] 178 - 1000[4]	(> 1000[1]) (1000[3])	
Na-PCP	>100[2] (122[3])	100[2] (90[3])	316 - 562[2] 56 - 562[4]	178 - 316[2] 32 - 316	(28 - 190[3]) 100[2] (190[1]) (81 - 172[5])	56[2]
Dursban	> 0,56[2]		(0,3[6]) 0,02 - 0,56[4] 0,18 - 0,32[2]	0,02 - 0,18[4]	1077[7] 560[2]	(484[7]) 100[2]
$K_2Cr_2O_7$	56[2] (713[3])	100[2] (1000[3])	3000 - 10 000[4] 2000 - 3000[2]	1000 - 3000[4] 1500 - 2000[2]	> 1000[1] (1700[3])	
ClAcetA	(4[3])	(3[3])	32 - 56[4]	32[4]	(22 - 40[3])	

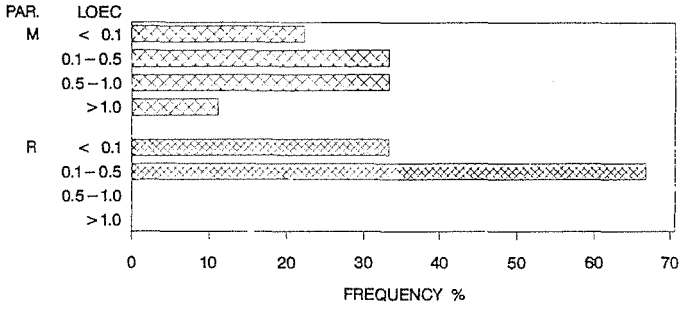
[1] UBA (1985)
[2] RIEPERT (unpublished)
[3] RÖMBKE (1989)
[4] Collembola-WG
[5] van GESTEL
[6] THOMPSON (1972)
[7] WEI-CHUN (1993)

3.4.4 Sensitivity of test parameters

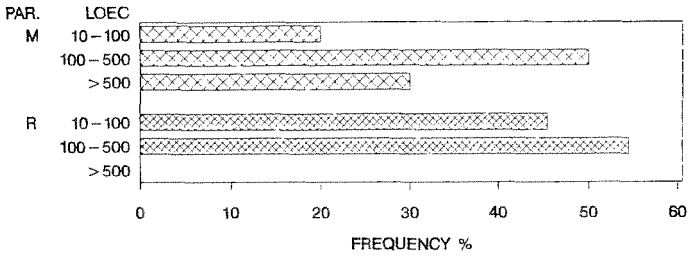
When the ranges of the LOEC levels of the mortality of the parental animals and of the reproduction rate are compared obviously the reproduction is a parameter sufficiently sensitive in this test system.

This becomes even more obvious when e.g. for the three substances Dursban, $K_2CR_2O_7$ and Na-PCP being tested several times a frequency distribution of the LOEC values on level classes is performed for each type of the test parameters (Fig. 4).

Dursban flüssig



Pentachlorphenol (Sodium Salt)



Tetrapropylenbenzenesulfonate (TPBS)

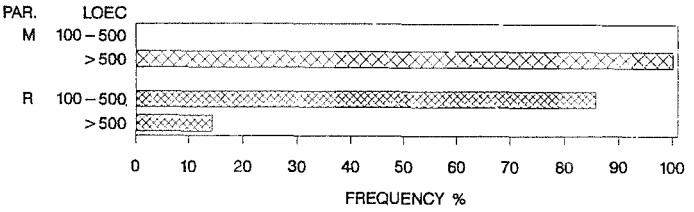


Fig. 4: Frequency distribution of LOEC levels for the test parameters mortality (M) and reproduction (R) determined in tests using Dursban flüssig (Dursban), Pentachlorophenol (Na-PCP) and Tetrapropylenebenzenesulfonate (TPBS) as test substances.

Concerning the moderately toxic fungicide Na-PCP and the slightly toxic surfactant TPBS the majority of the LOEC values for reproduction can be classified into a lower concentration class compared to the LOEC values for mortality. As expected, this is not the case for Dursban flüssig. It can be supposed that the low reproduction rate is the direct consequence of the rapid mortality due to acute toxic effects.

3.4.5 Impact of the components of the substrate on the level of the effect parameters

The artificial soil substrate having been used already in the OECD/EU earthworm test was selected as test substrate among other reasons because of the fact that this substrate was already standardized internationally thus making a comparison of the effects of substances between the various test organisms (*lumbricidae, enchytraeidae, collembola*) possible. However, despite of its obvious advantages, the composition of the substrate was exposed to criticism even during the development and harmonization process of the earthworm guideline. In comparison with 'normal' arable soil, its high portion of organic matter, due to its peat content of 10 %, seems to be not very appropriate for simulating the soil properties which influence substantially the bioavailability and thus the possible toxicity of a substance. Van GESTEL (1992) reviewed with reference to earthworm toxicity tests the present knowledge concerning this problem.

The results obtained from tests carried out with the substances $K_2 Cr_2 O_7$, Na-PCP and TPBS added to substrates of which the peat portion was reduced to 5 and 2% respectively show that the toxicity depends on the chemical behaviour of the test substance and the portion of peat in the substrate.

Table 6: Mean effect levels (LOEC) of the test substances potassium dichromate, ($K_2 Cr_2 O_7$) Pentachlorophenol (Na-Salt) and Tetrapropylenebenzenesulfonate (TPBS) at different portions of peat in the test substrate

COMPARISON BETWEEN DIFFERENT PEAT CONTENTS		LOEC MEAN % PEAT IN THE SUBSTRAT		
SUBSTANCE	**TEST PARAMETER**	**2**	**5**	**10**
$K_2Cr_2O_7$	Mortality	562	n.d.	5155
	Reproduction	178	562	3300
Na-PCP	Mortality	n.d.	n.d.	301
	Reproduction	100	100	131
TPBS	Mortality	n.d.	n.d.	912
	Reproduction	562	562	347

3.5 Quality criteria

3.5.1 Mortality of parental animals in the control containers

The first quality criterion to be fulfilled in the proposed test procedure is that the mortality of the adults should not exceed 10 % at the end of the test.

Fig. 6a shows by the example of 12 tests carried out by 4 laboratories the frequency distribution of the mortality of parental animals depending on the date of evaluation after 3 or 4 weeks. In 40% of the cases, either at the 3 or 4 weeks evaluation date, the tolerable limit of 10 % mortality was exceeded.

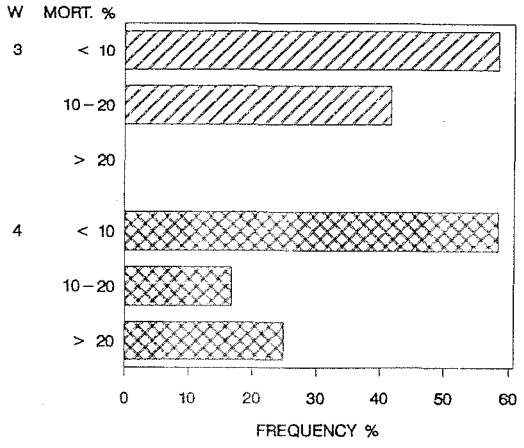


Fig. 5a: Percent frequency distribution of the parental mortality in the control containers varied by the length of the test period (3 or 4 weeks)

3.5.2 Minimum reproduction in the control containers

The other quality criterion selected requires a mean reproduction rate per test container of at least 100 juveniles in the control. As shown by Figure 6b this criterion was not met by 50 percent of the cases after a 3 weeks period whereas after a 4 weeks period the percentage was smaller than 10 %.

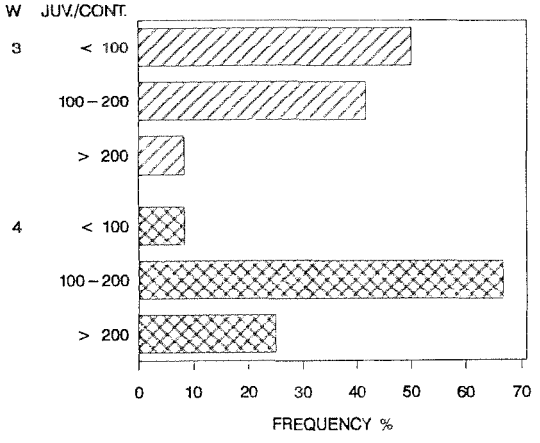


Figure 5b: Percent frequency distribution of reproduction rates (Juv./Cont.) in the control containers varied by the length of the test period (3 or 4 weeks)

To confirm these results 20 springtails were kept individually under the same test conditions in 20 ml glass cylinders. A mean reproduction rate of 39 offspring per adult springtail with a minimum of 0 and a maximum of 60 was found. 90 % had more than 10 and 60 % had more than 40 offspring (Figure 6c).

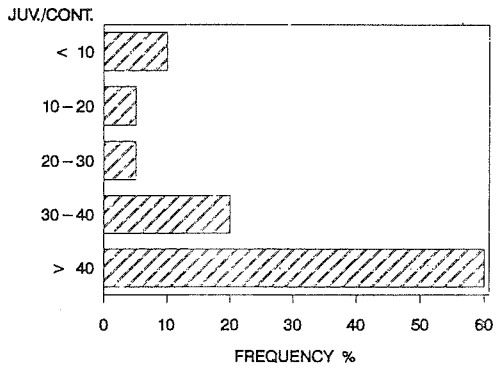


Figure 5c: Percent frequency distribution of reproduction rates (Juv./Cont.) of 20 adult springtails kept individually in 20 ml glass cylinders.

4. Discussion

The studies contributed by the different research projects (4.1) and the ad hoc working group with respect to the characterisation of the biology and appropriate culturing conditions of different Collembola species as well as the definition of adequate test conditions for an acute and a reproduction test primarely result from the need for providing practicable and generally accepted methods for the implementation of the Chemicals Act.

These experiences may be applied as well for testing side effects of plant protection products as the purpose of the Plant Protection Act is - similar to the Chemicals Act - to take precautionary measures against unjustifiable effects on the natural balance by providing appropriate test procedures. The principal problems associated with ecotoxicological testing and the difficulties in transforming test results into precautionary measures have been sufficiently discussed (for example RUDOLPH, 1983; RUDOLPH & BOJE, 1986).

On the initiative of the OECD, Van STRAALEN and Van GESTEL (1993) formulated criteria for selecting test organisms belonging to the taxonomic group of the arthropods. The proposed criteria are assigned to three types with taxonomic, ecological and exposure related relevance.

As for practical reasons the test inventory is always limited to a relatively small number of tests, the two first mentioned selection criteria aim at the representation of the taxonomic and functional variety; the objective of the third one is the way, the selected test organism is exposed within its biotope to chemical impact. These criteria are completed by F. PEDERSEN and L. SAMSØE- PETERSEN (1993) by aspects of sensitivity. In addition, the strategy of reproduction and the dispersal capacity should be considered and not only representative organisms of slightly affected sites but also organisms subjected to high exposure probability should be included in order to compensate possible phenomena of adaptation.

Collembola satisfy undoubtedly the above mentioned criteria with respect to their biotope which according to the species includes the litter layer or the humus containing soil horizons, by their distribution on ecosystems, both close to nature and subjected to anthropogenic influence, and by their capacity of being primary and secondary decomposers.

As for their representativeness for a soil biocoenosis the Collembola which belong to the primary wingless insects (*Apterygota)* are distributed in approximately 3500 species over all climatic zones, being often cosmopolite (DUNGER, 1983).

Their different life forms express the adaptation to the soil layers inhabited. Their significance for the soil biology at a population density of up to 4500 animals per litre soil is due to the fact that they contribute to the decomposition of plant detritus and animal excrements. They are supposed to have an indirect influence on the mineralization process as the microbial coloni-

zation is facilitated by the surface enlargement (SPAHR, 1981, EIJSACKERS, 1994, Van GESTEL & Van STRAALEN, 1994).

In an experiment made by Van der DRIFT & JANSEN (1977) a stimulation of fungi respiration, i.e. of fungi activity, was observed. KLIRONOMOS et al. (1992) examined the effect of Collembolan grazing on the microfungal succession on spruce litter in a microcosm system. The experiments demonstrated selective feeding as primary saprophytes were preferred to secondary saprophytes. The absense of the grazing springtails slowed down the rate at which the primary saprophytes were replaced by the secondary ones. They concluded that these data support the hypothesis that fungal succession observed in the field on decaying litter may result from preferential grazing by micro arthropods.

For the test presented here the eu- to hemidaphic species *Folsomia candida* was chosen from a number of species because at that time most of the experience in culturing had been gained for this species. The required data concerning taxonomy, distribution, anatomy and physiology, life cycle and reproduction biology were summarized by JANCKE (1989).

This decision made for pragmatic reasons was exposed to criticism in the working group with respect to the relevance for agricultural ecosystems in view of the exposure and function of this species. In order to give more consideration to the aspect of representativity it was proposed to complete the test system by an epiedaphic species as soon as the biological requirements would be fulfilled. There is of course no doubt about the differences in sensivity between the species. Nevertheless no agreement could be reached with respect to the determination of an indicator species meeting ideally all requirements of a test system. The importance of the aspect of easy culturing in practice was demonstrated by the multiple problems which arose at the beginning for making available a sufficient number of test organisms, though detailed information on breeding and rearing conditions was given by the mentioned research studies. Altogether it can be said however, that the procedures and conditions for culturing proposed by GOTO (1960), TÖRNE (1964) and SPAHR (1981) have proved to be applied successfully. Concerning the breeding substrate usually composed of a mixture of plaster of Paris and activated charcoal THOMSON & GORE (1972) and BOOTH (1983) explained that the type of these constituents and their ratio may have a decisive influence on the breeding result.

The recommendations given in the Annex of part II are the result of experiences gained by several ring tests. However they need repeated verification under the specific conditions of a test. The culturing conditions are discussed in detail in the Thesis of JANCKE (1989).

4.1 Test Substrate

The medium applied in this test corresponds completely to the artificial soil substrate of the 'Earthworms, acute Toxicity Test' (OECD 1981).

The advantage of this substrate is to be sufficiently standardized, to be used internationally and if moistured adequately to enable the hemiedaphic species *Folsomia candida* to penetrate into the substrate. A disadvantage frequently complained about is the rather high organic matter content compared to that in arable soil, which is due to the 10 percent peat proportion in the substrate resulting in a high adsorption capacity to non-polar substances. In some cases the toxic effects of a substance might be underestimated if the model situation of the test is extrapolated to field conditions.

The influence of soil characteristics like pH and organic-matter content on the bioavailability and toxicity of a substance to the earthworm *Eisenia fetida andrei* was demonstrated for several chlorinated phenols by Van GESTEL & WEI-CHUN MA (1988) and Van GESTEL & Van DIS (1988). Differences between soils almost completely disappeared when LC_{50} values in

mg/kg dry soil were recalculated towards values in mg/l pore water using sorption coefficients (Van GESTEL and MA (1988, 1990).

For ionizable compounds, (e.g. Pentachlorophenol) not only the organic-matter content but also the soil pH influences sorption. Having found that sorption was correlated with lipophilicity after correction for dissociation a chance is seen by these authors to extrapolate toxicity data using sorption data calculated by means of QSARs.

A reduction of the peat proportion from 10 to 5 and 2 percent respectively during the development of the experimental design of the test method resulted in a significant lower toxic level for potassium dichromate and a reduced toxic level for pentachlorophenol (Na-salt) having however no effect on the toxic level of the surfactant Tetrapropylenebenzenesulfonate (TPBS). Therefore it can be assumed that also for Collembola the availability of a test compound influences to a great extent the manifestation of toxic effects.

The main reason for maintaining the composition of the test substrate was to be able to compare different test systems for their sensitivity towards a chemical impact.

For purposes of environmental risk assessment the normalization of the concentration effect relationship by taking into consideration the bioavailable amount of a substance due to the chemical/physical, soil/substrate properties (PEDERSEN & SAMSOE-PETERSEN, 1993) could be a useful approach to overcome problems arising from the extrapolation of toxiic levels found under the test conditions.

In course of the harmonization process in the EEC of the Earthworm, Acute Toxicity Tests, (OECD 1981) and the discussions around the substrate to be used, HEIMBACH, 1984, demonstrated that a good correlation was found between the LC_{50} levels of 19 pesticides of different groups and types of action and other chemicals using the artificial soil substrate and another substrate called artisol.

This is surprising as the artisol substrate consisting of water, silica and glass balls contains no organic-matter and therefore the adsorption behaviour should be different. Both substrates enable worms an uptake of the chemicals through skin and gut.

No correlation was found with filter paper as contact medium where worms could not feed on.

Even if the reasons for the good correlations between the artificial soil and the artisol substrate have some speculative character, it may be concluded that the effects observed are not only due to the properties of the substance and the substrate and their interactions but also to the type of exposure of the test organism.

Comparing two natural soils with the artificial soil substrate Van GESTEL, 1991 found differences between the LC_{50} values in toxicity tests with the earthworm *Eisenia fetida* which were attributed to different pH-values and amounts of organic matter. These differences due to different soil or substrate characteristics nevertheless were smaller than those between LC_{50} values of tests carried out by different laboratories. According to the experience gathered with the Collembola test the conclusions drawn by van GESTEL that the artificial soil substrate is suitable for standardized earthworm testing can be transferred to the collembola test as well.

4.2 Test parameter

The main objective of the test method proposed is the reproductive success of the collembola species *Folsomia candida* determined as number of counted juveniles per test container. An estimate of the mortality rate of the parental springtails is part of the test evaluation but is not equivalent to the LC_{50} being assessed as a parameter for acute toxicity. The range of concen-

trations selected in an experimental design for acute toxicity testing should include concentrations resulting in a low and high mortality rate as well.

Mortality assessed in this test does not comprise effect levels of a higher mortality rate. Threshold levels like LOEC or LC10 are subject to a higher statistical error than the LC50, as levels of beginning toxicity are more difficult to separate from other effects causing mortality. Nevertheless even if mortality is determined as a by-product, it is needed for the estimation of the fitness of the test organism and to predict effects at the population level (LÖKKE & Van GESTEL, 1993).

The reproductive success expressed as quantity of juveniles per test container only holds as definition for reproduction rate, i.e. mean number of offspring per adult springtail, if no parental mortality has occurred during the test period. Due to the specific toxic properties of the test chemicals mortality of adults was not correlated in any case with a significant reduction of offspring. Strong correlation is found using substances with high acute toxicity, e.g. an insecticide, resulting in a high mortality rate of the adults before oviposition has begun. Parental mortality without effects on reproduction compared to the control, may be due to subchronic effects of the test substance or to compensation by individuals with a higher fitness. Density related factors should not play a major role for a population of ten springtails living in a test container with a volume of 100 ml. The test being not designed as a life cycle test gives no information on the probability for the survival of the offspring until they reach maturity.

An experimental design trying to assess effects on the embryonic development, manifested by a delayed hatching, by sequential evaluation dates was not successful. As the number of test units increases very much due to the extraction technique destroying the testing unit, this approach turned out to be too laborious.

Other parameters used in sublethal tests like growth or feeding activity were not taken into account as the experimental expense was not acceptable bearing in mind the uncertainty of the ecological interpretation of the parameters.

4.3 Quality criteria

The selection of the quality criteria and the definition of tolerable deviations depends on both, the precision desired when estimating a test parameter and the expense which reasonably should be achieved considering good laboratory practice.

By experience with numerous bioassays using different test organisms of the soil meso- and macrofauna it has been shown that these criteria cannot be combined in any case.

Factors affecting the fitness of the test organisms apart from the effects expected by a test substance may include genetical deviations of the laboratory strain, mechanical injuries by handling the animals and substrate induced effects.

In 40 percent of the cases mortality in the control containers exceeded the tolerance fixed at 10 % on average per test container making the test invalid. In these tests and others carried out later, it was observed that a mean average mortality of 20 percent in the control containers had no influence on the number of juveniles found at the end of the test compared with counts of control containers without parental mortality.

After having carried out numerous tests, growing experience in handling the test organisms resulted in a mortality in the control containers which remained in most cases under the limit of 10 percent. As the main effect parameter reproduction is not affected by low parental mortality in course of the test period, which may happen after oviposition has occurred, it is proposed to fix the tolerance limit at 20 percent even if a loss in information about substance related effects

on parental mortality has to be accepted. The other quality criteria requiring a mean reproduction rate of at least 100 offspring per test container was met in more than 90 percent of all cases.

A similar distribution of reproduction rates was found in tests where springtails were kept singular under the same conditions in 20 ml glass cylinders. 10 percent had 10 or less offspring and 80 percent more than 30 offspring.

A quantity of 10 percent having a low oviposition rate or laying unfertile eggs seems to be a normal rate at least under laboratory conditions. In addition recurrent periods of low oviposition were observed in the breeding containers throughout the year. Studies carried out by several authors with respect to the reproduction behaviour of *Folsomia candida* give a rather heterogeneous picture (JANCKE 1984).

According to a review of SNIDER, 1973, two of four authors report of similar reproduction rates found at higher temperature and under different rearing conditions. As a possible reason for the enormous range of 10 to > 500 offspring per adult different genetical strains are supposed apart from different rearing conditions (HUTSON 1978 b).

4.4 Reference substance

To ensure the quality of a test system it is usually recommended in ecotoxicological test guidelines or standards to perform tests with a reference substance regularly, e.g. once or twice a year. If available a range of effect levels based in most cases on results from ring tests is given as reference. Substances to be used as reference substance should have a slope of the concentration-response relationship which is not too steep with respect to the parameter under investigation to enable a valid determination of a concentration affecting the test organism to a defined percentage. Normally this is the EC 50 as for statistical reasons the confidence limits are smallest in this area of the relationship.

The parameter used in this study is the LOEC which is defined as the lowest tested concentration of the test substance at which the substance is observed to have a significant effect when compared with the control. All test concentrations above the LOEC must have a harmful effect equal to, or greater than those observed at the LOEC. This type of effect level determined with a statistical procedure (ANOVA) depends on the spacing of the concentrations applied in the test and the variability between the replicates of the control and the treatments. Therefore LOEC levels determined by different laboratories for the same test substance may differ much more than EC 50 values.

Of the test substances used for the methodical development only Na-PCP and TPBS might have been appropriate to form a reference level. The other substances applied were not effective ($K_2 Cr_2 O_7$), the effect on reproduction was due to the high acute toxicity to the parental collembola (Dursban), or a sufficient number of results is missing (Chloroacetamid). As both substances, Na-PCP and TPBS, are not available anymore because of regulation (Na-PCP) or have been replaced by better degradable compounds (TPBS) no additional tests were carried out to limit the range of the LOEC level.

For purposes of recommending a reference effect level, substances should be selected to their toxic properties showing a good concentration effect relationship if an adequate spacing of the test concentrations is used.

With respect to results gained from an ISO ring test in 1995 using the herbizide Betanal plus (a.i. Phenmedipham) and the insecticide E 605 forte (a.i. Parathion) as test substances a

meeting of participating experts recommended Betanal plus as reference substance. An approach based on the determination of a concentration resulting in a defined percentage of an adverse effect (EC_x) by a regression method might be preferable.

Problems associated with determining the NEC (No Effect Concentration) as measure of chronic toxicology were discussed on a workshop held in September 1994 in The Hague, the Netherlands (F. Noppert et al., 1994). As both, NEC/NOEC and LOEC, are approaches for the same goal but come from different sides the conclusions drawn by the working group should hold for the LOEC as well. One of the most important recommendations of the workshop is, that the NOEC should (in due time) be replaced by some other measure for (almost) no effect. It is indicated that research is needed to choose between an EC_x value and a parametric NEC estimate. If an EC_x value is chosen the preferred value of x is 5 or 10 percent.

5. Summary

The suitability of soil organisms being used as indicators of potential sublethal effects of chemical substances under standardized laboratory test conditions depends to a large extent on how the selected test species meets all the requirements of ecology and practicability. Among other minor aspects this requires information about practical aspects like rearing and breeding under laboratory conditions, to which extent a species in its life cycle is exposed to a substance and to the functional role of the selected species e.g. its contribution to the breakdown of organic matter in soil.

Due to their life cycle, distribution and function Collembola are playing a major role in soil ecosystems. Furthermore the availability of sufficient experience in breeding and rearing of several species covers one of the most important requirements a test system should meet.

Based on two investigations carried out at the Bundesgesundheitsamt (Federal Health Institute) Berlin, to standardize methods for breeding of five Collembola species (*Hypogastrura bengtsoni, Onychiurus fimatus, Folsomia candida, Proistotoma minuta, Sinella coeca)* and to develop a standardized test method for testing the acute toxicity of environmental pollutants to springtails with special respect to *Folsomia candida*, a draft proposal for a test required within the notification of new chemicals under the Chemicals Act was developed by a working group chaired by the Institut für Chemikalienprüfung (Institute for chemical Examination) of the Biologische Bundesanstalt für Land- und Forstwirtschaft (Federal Biological Research Center for Agriculture and Forestry). The principle of the test is described by the assessment of that level of a test substance at which under defined conditions the reproduction rate of springtails of the species *Folsomia candida* WILLEM, 1902 (Collembola: Isotomidae) kept in an artificial soil substrate is significantly reduced after a 4 weeks exposure period compared to an untreated control.

The results gained in ring tests to elaborate the test design are presented giving background information for the draft proposal of the method and its informative annex which includes techniques for culturing of the test organisms and for counting the offspring at the end of the test. The editorial design of the draft proposal corresponds to the ISO format for international standards.

The worldwide interest in biological methods for testing soil quality and the existence of similar test methods in the Netherlands and Denmark enabled to establish this method as a work item in the ISO Technical Committee 190, Soil Quality, as a basis within the harmonization process of a new standard.

6. Bibliography

BOOTH, R.G., 1983: Effects of plaster - charcoal substrate variation on the growth and fecundity of *Folsomia candida (Collembola, Isotomidae)*. Pedobiologia 25(3), 187-195.

CROMMANTUIJU, T., 1994: Sensitivity of soil arthropods to toxicants. Vrije Universiteit te Amsterdam, Academisch Proefschrift, 140 p.

DIELMANN, H., 1984: Testmethoden zur Überprüfung der Auswirkung sublethaler Dosen auf Collembolen. In: Entwicklung von Testmethoden zur Prüfung der Toxizität von Chemikalien an indifferenten Arthropoden-Arten. Forschungsauftrag 82 HS 008 BML.

DRIFT, J. van der, JANSEN, E., 1977: Grazing of springtails on hyphal mats and its influence on fungal growth and respiration. Ecol. Bull. (Stockholm) 25, 203-209.

DUNGER, W., 1983: Tiere im Boden. Neue Brehm Bücherei, A. Ziemsen Verlag, Wittenberg Lutherstadt, 280 p.

EIJSACKERS, H., 1994: Ecotoxicology of soil organisms: seeking the way in a pitch dark labyrinth, in Ecotoxicology of Soil Organisms, DONKER, M.H., EIJSACKERS, H. and HEIMBACH, F. Eds. LEWIS Publishers, Boca Raton, 1994, chap. 1.

FOLSOM, J.W., 1933: The economic importance of Collembola. J. econ. Ent. 26, 934-939.

Van GESTEL, C.A.M. & WEI-CHUN MA, 1988: Toxicity and bioaccumulation of chlorphenols in earthworms in relation to bioavailability in soil. Ecotoxicology and Environmental Safety 15, 289-297.

FRAMPTON, G.K., 1988: The effects of some commonly - used foliar fungicides on Collembola in winter barley: laboratory and field studies. Ann. Appl. Biol. 113, 1-14.

Van GESTEL, C.A.M., 1991: Earthworms in ecotoxicology. Diss. Univ. von Utrecht, 197 p.

Van GESTEL, C.A.M., 1992: The influence of soil characteristics on the toxicity of chemicals for earthworms: a review. Ecotoxicology of Earthworms, Intercept, Andover, pp. 44-54.

Van GESTEL, C.A.M. & Van STRAALEN, N.M., 1994: Ecotoxicological test systems for terrestrial invertebrates, in Ecotoxicology of Soil Organisms, DONKER, M.H., EIJSACKERS, H. and HEIMBACH, F., Eds. Lewis Publishers, Boca Raton, 1994, Chap. 14.

GOTO, H.E., 1960: Simple techniques for the rearing of collembola. Ent. mont. mag. 96, 138-140.

GREEN, C.D., 1964: The life history and fecundity of *Folsomia candida* (Willem) var distincta (Bagnall) collembola: *Isotomidae*. Proc. R. ent. Soc. Lond. (A) 39, 125-128.

HEIMBACH, F., 1984: Correlations between three methods for determining the toxicity of chemicals to earthworms. Pestic. Sci. 15, 605-611.

HERGARTEN, W., 1985: Die Collembolenfauna verschieden bewirtschafteter Flächen am Niederrhein. Decheniana (Bonn) 138, 135-148.

HUTSON, B.R., 1978: Influence of pH, temperature and salinity on the fecundity and longevity of collembola. Pedobiologia 18(3), 163-179.

IGLISCH, J., 1985: Bodenorganismen für die Bewertung von Chemikalien. Zeitschrift für angewandte Zoologie 72(4), 395-431.

IGLISCH, I., 1986: Hinweise zur Entwicklung von Testverfahren zum Nachweis subakuter Wirkungen von Chemikalien. Zeitschrift für angewandte Zoologie 73(2), 199-218.

JANCKE, G., 1989: Modellversuche zur subakuten und subletalen Wirkung von Herbiziden auf Collembolen im Hinblick auf ein Testsystem für Umweltchemikalien. Zool. Beitr. (N.F.) 32, 261-299.

KLIRONOMOS, J.N., WIDDEN, P., DESLANDES, I., 1992: Feeding preferences of the collembolan Folsomia candida in relation to microfungal succession on decaying litter. Soil biology and biochemistry 24(7), 685-692.

LANGE, A.W., 1985: Überprüfung der Durchführbarkeit von Prüfungsvorschriften und der Aussagekraft der Stufe 1 und 2 ChemG - 25 - Stoffe - Programm - Zusammenfassung der Diskussionsergebnisse des Abschlußseminars des F u. E - Vorhabens 106 0411 am 17./18.1.1985, Umweltbundesamt, 52 p.

LØKKE, H., Van GESTEL, C.A.M., 1993, (Editors): Development, improvement and standardization of test systems for assessing sublethal effects of chemicals on fauna in the soil ecosystem. Report from a workshop held in Silkeborg, Denmark, Jan. 18-19, EC Environmental Research Programme, Contract no. EV5V-CT92-0218.
MARSHALL, V.G. & KEVAN, D.K. Mc E., 1962: Preliminary observations on the biology of *Folsomia candida* Willem, 1902 (Collembola: *Isotomidae).* Can. Ent. 94, 575-586.
MILNE, S., 1962: Phenology of a natural population of soil collembola. Pedobiologia 2, 41-52.
NOPPERT, F., N. van der HOEVEN & A. LEOPOLD, 1994: How to measure no effect, towards a new measure of chronic toxicity in ecotoxicology. Workshop Report The Hague The Netherlands September 9th 1994 - Publications of The Netherlands Working Group on Statistics and Ecotoxicology.
NUSCH, E.A., 1986: Möglichkeiten und Grenzen der Aussagekraft ökotoxikologischer Tests. Vom Wasser 67, 213-220.
OECD, Paris 1981: Guidelines for testing chemicals-method 207, earthworm, acute toxicity tests.
OKKERMAN, P.C. et al., 1993: Validation of some extrapolation methods with toxicity data derived from multiple species experiments. Ecotoxicology and environmental safety 25, 341-359.
PEDERSEN, F., SAMSOE-PETERSEN, L., 1993: Discussion paper regarding guidance for terrestrial effects assessment, 1. Draft, Water Quality Institute Horsholm, Denmark, p. 47.
PETER, H., FRANKE, C., 1992: Ökotoxikologische Prüfungen nach dem Chemikaliengesetz (ChemG) - Durchführung und Bewertung. Z. Umweltchem. Ökotox. 4 (6), 333-338.
RÖMBKE, J., 1989: Entwicklung eines Reproduktionstests an Bodenorganismen - Enchytraeen. Abschlußbericht FE-Vorhaben 10603051/01, 90 p.
RÖMBKE, J., 1989a: Enchytraeus albidus (*Enchytraeidae, Oligochaeta)* as a test organism in terrestrial laboratory systems. Arch. Toxicol. Suppl. 13, 403-405.
RUDOLPH, P., 1983: Erkenntnis- und wissenschaftstheoretische Überlegungen bei der Bestimmung der Umweltgefährlichkeit nach dem Chemikaliengesetz durch ökotoxikologische Testverfahren. UBA - Texte 27, Umweltbundesamt .
RUDOLPH, P., BOJE, R., 1986: Ökotoxikologie - Grundlagen für die ökotoxikologische Bewertung von Umweltchemikalien nach dem Chemikaliengesetz. Handbuch des Umweltschutzes, ECOMED.
SCHLOSSER, H.J., BECKER, H., 1986: Ökotoxikologische Bewertung von Chemikalien. Gesunde Pflanzen, 38. Jahrg., Heft 6, 299-303.
SNIDER, R.M., 1973: Laboratory observations on the biology of *Folsomia candida* (Willem) (Collembola: *Isotomidae).* Rev. Ecol. Biol. Sol. 10, 103-124.
SPAHR, H.J., 1981: Experimentell-ökologische Untersuchungen über die Haltungsbedingungen von Collembolen in praxisorientierten Massenzuchten. Diplomarbeit, Inst. f. Tierphysiologie und angewandte Zoologie, FU- Berlin.
SPAHR, H.J., 1981: Die bodenbiologische Bedeutung von Collembolen und ihre Eignung als Testorganismen für die Ökotoxikologie. Anzeiger für Schädlingskunde, Pflanzenschutz, Umweltschutz 54, 27-29.
SPAHR, H.J., 1983: Experimentell-ökologische Untersuchungen über die Haltungsbedingungen von Collembolen in praxisorientierten Massenzuchten. Experimental ecological studies of the conditions for the keeping of collembolans under practical conditions of mass breeding. Zeitschrift für Angewandte Zoologie (Germany, F.R.) V. 70(4), 473-505.
Van STRAALEN, N.M. & Van GESTEL, C.A.M., 1993: Ecotoxicological test methods using terrestrial arthropods. Report nr. D93002, Department of Ecology and Ecotoxicology, Vrije Universiteit Amsterdam, 63 p.

TALBOT, G., 1987: Untersuchungen zur Entwicklung eines standardisierten Mortalitäts- und Reproduktionstests für Pflanzenschutzmittel bei Collembolenarten. Diplomarbeit, Bonn.
THOMPSON, A.R., GORE, F.L., 1972: Toxicity of twenty-nine insecticides to *Folsomia candida:* laboratory studies. J. econ. Entomol. 65, 1255-1260.
TÖRNE, E.V., 1964: Über die Anzucht und Haltung individuenreicher Collembolenpopulationen. Pedobiologia Bd. IV, 256-264.
ULBER, B.; 1979: Einfluß von Zuckerrüben-Herbiziden auf Mortalität und Verhalten von Onychiurus fimatus GISIN (Collembola, Onychiuridae). Z. Angew. Entomol. 87, 143-153.
WEI-CHUN, MA & JOS BOLT, 1993: Differences in Toxicity of the Insecticide Chlorpyrifos to Six Species of Earthworms *(Oligochaeta, Lumbricidae)* in Standardized Soil Tests. Bull. Environ. Contam. Toxicol. 50, 864-870.
WOHLGEMUTH, D., 1989: Einfluß von Bodenparametern auf die Cadmiumtoxizität, ermittelt durch die Reproduktionsrate von Collembolen (Folsomia candida). Dipl. FB Biologie, FU Berlin, 106.
WOLF-ROSKOSCH, F., 1983: Standardisiertes Testverfahren zur Prüfung der akuten Toxizität von Umweltchemikalien an Springschwänzen, unter besonderer Berücksichtigung von Folsomia candida. UBA-Texte 3, 83-110.

III.2 DRAFT METHOD

EFFECTS OF SOIL POLLUTANTS ON COLLEMBOLA (*FOLSOMIA CANDIDA*):

METHOD FOR THE DETERMINATION OF EFFECTS ON REPRODUCTION

ISO COMMITTEE DRAFT 11267, VERSION 3/96

CONTENTS

1 Scope

This International Standard describes a method for determining the effects on reproduction of *Folsomia candida* by dermal and alimentary uptake of a substance using a defined artificial soil substrate treated with a defined amount of that substance. The method is not applicable to volatile substances i.e. substances for which H (Henry's constant) or the air/water partition coefficient is greater than 1, or for which the vapour pressure exceeds 0,0133 Pa at 25 °C.

NOTE 1 - The stability of the test substance cannot be assured over the test period. No allowance is made in the test method described for the degradation of the test substance.

2 Normative references

The followings standards contain provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 10390 - 1994 Soil quality - Determination of pH

ISO 11465 - 1993 Soil quality - Determination of dry matter and water content on a mass basis - Gravimetric method

ISO 11274 - 199 Soil quality - Determination of the water retention characteristic - Laboratory methods[1)]

ISO 11268-1-1993 Soil quality - Effects of pollutants on earthworms (*Eisenia fetida*) Part 1: Determination of acute oxicity using artificial soil substrate

3 Definitions

LOEC (lowest observed effect concentration):

The lowest tested concentration of the test substance at which the substance is observed to have a significant effect when compared with the control. All test concentrations above the LOEC must have a harmful effect equal to, or greater than that observed at the LOEC.

NOEC (no observed effect concentration): The test concentration immediately below the LOEC.

EC 50: The concentration estimated to reduce the reproduction rate at the end of the test to 50 per cent compared to the control.

Reproduction rate: Mean number of offspring per test vessel after 28 days.

4 Principle

10 to 12 days old springtails (*Folsomia candida*) are placed in a defined artificial substrate containing the test substance at different concentrations. They are incubated until offspring (F_1) emerge from eggs laid by mature adults. Normally in the control offspring emerges after 28 days. The number of offspring is determined.

[1)] In course of preparation

5 Reagents

5.1 Biological material

In this test 10 - 12 days old juvenile springtails of the species *Folsomia candida* (Willem) are used. (Synchronisation of breeding see Annex A.1).

5.2 Test substrate

A defined artificial soil is used as a basic soil substrate (ISO-Standard 11268-1).

The test substrate consists of the wet basic soil substrate, the test substance and deionized water.

5.2.1 Soil Substrate

	In percent of dry mass
Spaghnum peat, air dried for about 24 hours, (about 10 - 12 % water content) as finely ground as practicable and with no visible plant remains	10 %
Kaolinite clay (air dry) containing not less than 30 % kaolinite	20 %
Industrial quartz sand (air dry),	68-69 %

(predominantly fine sand with more than 50 % of particle size 0,05 - 0,2 mm) dependent on $CaCO_3$ needed.

Add sufficient (0,5 to 1 %) Calcium carbonat ($CaCO_3$), pulverized recognized analytical grade to bring the pH (measured in 1 mol/l KCl solution) to 6,0 ± 0,5 at the start of the test.

NOTE - the amount of calcium carbonate required will depend on the components of the soil substrate and should be determined by measurements on sub-samples (see Annex C.2) immediately before the test.

The dry constituents are blended in the correct proportions and mixed thoroughly in a large-scale laboratory or in a household mixer. A part of the totally needed volume of deionized water is added while mixing is continued. This is the basic soil substrate. The overall water content of the test and control substrate respectively has to be fitted in a way to give the substrate a crumbly structure to enable springtails to penetrate substrate cavities. Normally such a structure is achieved by a water content corresponding to 40 - 60 % of total water holding capacity. The water holding capacity is determined in accordance with ISO 11274 or with the method given in annex C.1. Water content and pH are determined in a mixed sample immediately before the start of the test and at the end of the test in an additional container of the control and each concentration tested.

NOTE - Allowance should be made for any water or quartz sand that are to be used for introducing the test substance into the soil.

5.2.2 Control substrate

The control substrate consists of the basic substrate and deionized water. If the preparation of the test requires the use of an auxiliary agent, an additional control treated like the test substrate without the test substance is needed.

5.3 Reference substance

As a reference substance the herbicide Betanal plus (a.i. 160 g/l Phenmedipham) is recommended. Effects on reproduction (α = 0,05) shall be observed at concentrations of between 100 and 200 mg of Betanal plus (corresponding to 16 and 32 mg Phenmedipham) per kg dry mass of the substrate.

To ensure the quality of the test system it is recommended to perform tests with the reference substance regularly, e.g. once or twice a year.

6 Apparatus

Standard laboratory equipment, and:

6.1 Glass containers able to be closed tightly of about 100 ml capacity and with a diameter of about 5 cm.

6.2 Apparatus capable of measuring pH and water content of the substrate.

6.3 Exhaustor for transfer of springtails (see Annex A).

7 Test environment

7.1 Enclosure, capable of being controlled to a temperature of 20 °C ± 2 °C.

7.2 Light source, capable of delivering a constant illuminance of 400 lx to 800 lx at a controlled light dark cycle of 16 : 8 h or constant light.

8 Procedure

8.1 Preparation of the test

8.1.1 Test concentrations

At least 5 concentrations in a geometric series at a factor not exceeding (e.g. $\sqrt[4]{10} \sim 1.8$) should be selected to give an estimation of the LOEC/NOEC of the reproduction rate.

The concentrations of the test substance shall be expressed as mass of substance per dry mass of soil substrate (mg/kg) (see 5.2).

If no other relevant toxicity data are available the concentrations selected to provide the LOEC/NOEC are based on the results of the preliminary test (see 8.2).

8.1.2 Introduction of the test substance

8.1.2.1 Water soluble substances

Immediately before starting the test, dissolve the quantity of the test substance in the water required for the replicates of one concentration (or that portion of it necessary to wet the soil substrate in order to meet the requirements of 5.2.1) and mix it thoroughly with the basic soil substrate before introducing it into the test containers.

Continue as described in 8.1.3.

8.1.2.2 Substances insoluble in water but soluble in organic solvents

Dissolve the quantity of test substance required to obtain the desired concentration in a volatile solvent (such as acetone or hexane) and mix it with a portion of the quartz sand required. After having evaporated the solvent by placing the container under a fume hood, add the remainder of the basic substrate and the water and mix it thoroughly before introducing it into the test containers. Continue as described in 8.1.3.

NOTE - Ultrasonic dispersion, organic solvents, emulsifiers or dispersants may be used to disperse substance with low aqueous solubility. When such auxiliary substances are used, all test concentrations and an additional control should contain the same minimum amount of auxiliary substance.

WARNING - Apropriate precautions should be taken when dealing with solvent vapour to avoid danger from inhalation or explosion, and to avoid damage to extraction equipment, pumps etc.

8.1.2.3 Substances insoluble in water or organic solvents

For a substance insoluble in a volatile solvent, prepare a mixture of 10 g of finely ground industrial quartz sand (see 5.2) and the quantity of the test substance required to obtain the desired concentration. Add the remainder of the basic substrate and the water and mix it thoroughly before introducing it into the test containers.

Continue as described in 8.1.3.

8.1.3 Introduction of the test organisms

Ten juvenile 10 - 12 days old springtails are placed into each test container of each concentration and control.

Springtails are tapped or sucked from the breeding containers to transfer them to the test containers.

Before they are transferred to the test containers, it is suitable to check the test organisms for damages. This is necessary to reduce control mortality and to avoid systematic trial errors, respectively. This can easily be done using an exhaustor (see Annex A.2).

8.1.4 Control container

Prepare control containers in the same way as the test containers without the test substance. If the preparation of the test requires the use of auxiliary substances (see 8.1.2.2) use additional control containers. Treat these containers in the same way as those without the test substance.

8.2 Preliminary test (optional)

If it is necessary to determine the range of concentrations for use in the final test, perform a preliminary acute test on mortality for four concentrations of the test substance and a control (for example, 0, 1, 10, 100 and 1000 mg/kg) on 10 10 to 12 days old springtails per concentration and per container.

Prepare the test containers as indicated in 8.1.2 Place the test containers in the test environment described in clause 7.

At the beginning of the test about 2 mg of granulated dry yeast is fed per test container. Open the test container shortly twice a week for aeration.

After 14 d, count live springtails in each container. For each container and each concentration, calculate the percent mortality. Note the symptoms observed on the animals.

NOTE - Due to fast degradation of dead springtails missing springtails are supposed to have died during the test period.

8.3 Final test

The concentrations selected to provide the LOEC/NOEC (EC 50 if possible) are based on the results of the preliminary test or other toxicity data, suitable to derive a dose-response for reproduction.

Substances do not need to be tested at concentrations higher than 1000 mg/kg dry mass of test substrate.

After mixing the test substance (see 8.1.2) into the test substrate for one concentration, each test container (replicate) is filled with 30 g wet weight of the test substrate.

To insure easy immigration of springtails, the substrate filled into the test container should not be pressed.

For each concentration and control 5 replicates have to be provided.

To check pH and humidity of the test substrate it is recommended to provide and additional container for each concentration and the control.

At the beginning of the test about 2 mg of granulated dry yeast is fed per test container. Check food consumption during the test period and add yeast if necessary.

Determine the water content and the pH in the presence of 1 mol/l KCl of the artificial soil at the beginning of the test (when acid or basic substances are tested, do not adjust the pH) and at the end of the test.

Open the test containers shortly twice a week for aeration. After a two weeks period water content is checked by reweighing the additional test containers. Compensate water if loss of water exceeds 2 % of the initial water content.

8.4 Determination

4 weeks after introducing the parental springtails on the test and control substrate, the number of organisms has to be determined. For this purpose the test substrate is poured into a 500 to 600 ml container and watered. After gentle stirring of the suspension with a spatula springtails will drift to the water surface. Adults and juveniles, if present, are counted by a suitable procedure (see Annex B) and numbers are reported.

9 Calculation and expression of results

9.1 Calculation

The data should be presented in tabular form, indicating for each concentration the mean number of adults and juveniles. For each concentration a statistical analysis of the homogeneity and normality of replicate results should be made. If this is proved, an appropriate statistical analysis should be used to test whether differences between the control and the test concentrations are significant (P=0.05). If these requirements are not fulfilled, it is recommended to use non-parametric methods, e.g. the U-test by Mann & Whitney.

NOTE - When the data are adequate the EC 50 value and the confidence limits (P=0.05) should be determined using standard methods (Trimmed Spearman-Karber or equivalent method).

9.2 Expression of results

Indicate in mg/kg dry mass soil

- the lowest concentration tested with significant difference versus control(s) (LOEC),
- the test concentration immediately below the LOEC (NOEC) and optionally,
- the concentration at which the reproduction rate is reduced by 50 % compared to the control (EC 50).

All observations have to be described and interpreted for assessment of the effects of the test substance.

10 Validity of the test

10.1 The mortality of the adults in the control(s) should not exceed 20 % at the end of the test.

10.2 The reproduction rate should reach a minimum of 100 instars per control vessel.

10.3 The coefficient of variation of reproduction in the control should not exceed 30 %.

11 Test report

The test report shall refer to this standard and, in addition to the results expressed as in section 9.2, shall provide the following indications:

- Detailed description of the test substance and information on physical and chemical properties if helpful for the interpretation of the test result
- Complete description of the biological material employed (species, age, breeding conditions, supplier)
- method of preparation of the test substrate with indication of the auxiliary substances used for a low-/non-water-soluble substance
- results obtained with the reference substance, if performed detailed conditions of the test environment
- Table giving the per cent mortality of the adults obtained for each concentration and for the control(s)
- number of dead or missing adults and number of offspring per test container at the end of the test
- the lowest concentration causing significant effects (LOEC)
- the highest concentration causing no observed effects (NOEC) EC 50 for the inhibition of reproduction and the method used for calibration
- description of obvious or pathological symptoms or distinct changes in behaviour observed in the test organisms per test container
- water content and pH of artificial soil at start and at end of the test for the control and each concentration
- all operating details not specified in the standard, and any occurrences liable to have affected the results.

ANNEXES

A (informative) Techniques for rearing and breeding

A.1 Conditions for rearing and breeding

A.1.1 Breeding substrate

Plaster of paris: plaster for stucco (pH 6.4)

Activated charcoal: pulverized chemical activated charcoal, pH 6-7.

Plaster of paris and activated charcoal are mixed in a ratio of 8:1 (weight/weight). Depending on the type of plaster, 60-100 g water are added to 100 g of this mixture. This substrate provides a highly moistured substrate, the charcoal adsorbing waste gases and excretes. The dark backround allows easy observations.

Measuring pH: As the contact water on the water saturated substrate surface is essential for springtails, pH can easily be determined by using pH indicators, placed on the wet substrate surface.

A.1.2 Breeding containers

Commercial plastic containers of about 400 ml volume may be used. The containers are filled to a depth of about 1 cm with the breeding substrate. After drying, deionized water is added near to saturation point. Moisture content can be maintained automatically by using a wick, implanted in the substrate and sucking water from a water bath beneath or by supplying evaporated water with a pipette until the substrate is saturated but without water standing on the substrate surface.

The breeding containers are tightly closed by suitable covers. They have to be aerated periodically (e.g. in combination with feeding) by lifting the cover for a short time.

The covers may also be perforated for aeration by a needle.

(**Warning**: Predacious mites may penetrate)

A.1.3 Climatic conditions

For keeping and breeding a climatic chamber with a controlled temperature of 20-22 °C and 70-80 % r H with constant lighting at 400 to 800 lux (or light - dark cycle 16:8 h) is most suitable.

A.1.4 Food

For breeding and for the test granulated dry yeast is used as food supply. Feeding the breeding containers once or twice a week is recommended. To avoid spoiling by fungi, food should be applied rather short or removed if it is apparently spoiled by extreme fungal growth. Other suitable food may be applied as well.

A.1.5 Transfer

After a period of about 8 weeks the springtails are transferred to fresh breeding containers by tapping or blowing. The transfer to fresh containers usually induces oviposition.

A.1.6 Test organisms of a standard age

To obtain 10-12 days old juvenile springtails for the test, egg clusters are transferred from breeding containers with a fine spatula or hair brush to a freshly prepared breeding substrate. After 48 h the egg clusters are removed and the instars meanwhile hatched from eggs are fed.

The egg clusters are easily removed if they are placed on small pieces of breeding substrate or cover glasses laid on the breeding substrate. After another period of 10 days, the animals provided can be used for the test.

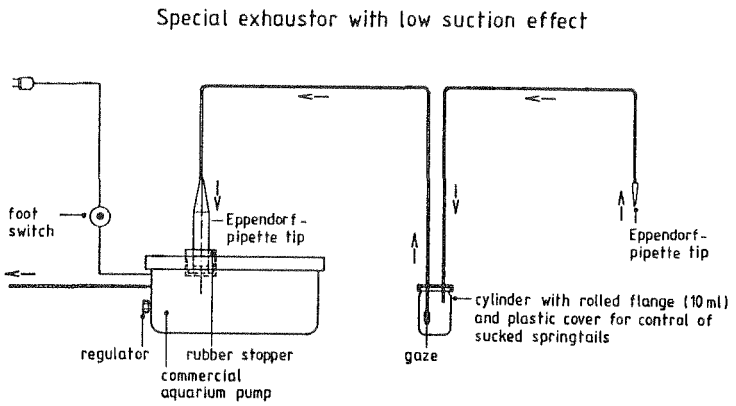
As an alternative, 10-12 days old juvenile springtails may be obtained by placing a number of adult springtails in (small) containers with a bottom of plaster of Paris and let them lay eggs for 2 days. After this period, the adults are removed. Twelve days after the first juveniles have emerged from the eggs, the juveniles can be used for the test. To ensure a successful synchronization, it is advisable to check the containers for egg production before removing the adults. In some cases, the adults do not immediately start laying eggs and only few eggs are produced after 2 days. In that case it is advisable to keep the adults for one day more in the containers.

For both methods, it should be recommended to avoid overcrowding in the containers, as this may lead to a reduced growth. As a consequence, the 10-12 days old animals used for the test may be too small and not yet able to produce a sufficient number of eggs to meet the requirement of having at least 100 juveniles per test container in the control.

A.2 Transfer of springtails to the test containers

The springtails are easily transferred from the breeding substrate to the test substrate by an exhaustor. An example is shown in the Figure below.

Figure of apparatus for transfer of springtails



The collembola are sucked individually through a pipette tip to a small covered container to control damage of springtails. (Be sure that the suction performance of the pump is low, otherwise springtails may be damaged.) After removing the cap, springtails provided for one test container are transferred on to the substrate surface of the test containers.

A hand-made exhauster may be used as well. Such an exhauster can be made of a small glass tube and a piece of flexible plastic tube. For this purpose e.g. a Pasteur pipette might be used, the small end of which has been removed; the resulting hole in the tube should be kept in a flame to smoothen the sharp edges. The other end of the tube is covered with a piece of very fine-mesh gaze, and a piece of flexible plastic tube is fixed to it. With such an exhauster, the juveniles can be sucked up by mouth and transferred to the test containers.

B (informative) Techniques for counting juvenile springtails at the end of the test

For evaluating the effect on reproduction, the juveniles, swimming on the water surface of the watered substrate have to be counted, and not estimated. Especially when reproduction is high, the use of technical devices to facilitate counting is recommended.

If the swimming juveniles are distributed evenly over the water surface, a counting grid may be used and a sample taken at random is counted.

Aggregations of instars on the water surface sometimes are prevented by adding a drop of oil for sewing-machines. The accuracy of the estimation method should be known. The average error should not exceed 10 %.

Another practical method uses a culture counter to count the animals on a projected picture (slide) of the water surface. Normal photographic equipment (single lens reflex camera, macro lens or other device for close-up photography) is adequate.

Film speed has to be adjusted according to light intensity (e.g. flash or cold light source) and the desired shutter speed. If a cold light source is used, the minimum film speed value is 400 ASA. A useful relation between a sufficient projection format on a screen and the beaker volume is met with volumes of 50-100 ml (lens used: Zeiss S-Planar 1:2.8 f=60 mm, angular field 39° , min. focus 0.24 m). To improve the contrast between white springtails and surrounding water surface, water may be coloured dark with ink.

NOTE - To avoid errors in determining mortality of the parental springtails, the number of liveadults floating on the water surface should be counted by using a binocular microscope.

C (informative) Determination of the water holding capacity of the artificial soil

The following method has been found to be appropriate for the purposes of this test used alternatively to ISO standard 11274.

C.1 Apparatus

C.1.1 Glass tube, approximately 20 mm to 50 mm diameter and at least 100 mm in lenght.

C.1.2 Water bath at room temperature.

C.1.3 Filter paper.

C.1.4 Drying oven set to 105 ± 5 °C.

C.1.5 Balance, capable of weighing with an accuracy of ± 0.1 g.

C.2 Method

Plug the bottom of the tube with a filter paper, and after filling with the artificial soil substrate to a depth of 5 cm to 7 cm, place the tube on a rack in a water bath. Gradually submerge the tube until the water level is above the top of the soil. Leave the substrate sample in the water for about three hours.

As not all water absorbed by the substrate capillary can be retained, the tube containing the sample should be placed for a period of two hours on very wet finely ground quartz sand for draining. The same quartz sand as that used for the soil substrate is satisfactory.

Weigh the sample, dry it to constant mass at 105 °C and re-weigh it.

C.3 Calculation of the water capacity (WC)

$$WC \text{ (in \% of dry weight)} = \frac{S - T - D}{D}$$

S = water saturated substrate + mass of tube + mass of filter paper

T = tare (mass of tube + mass of filter paper)

D = dry mass of substrate

D Guidance to adjust the pH of the artificial soil

To estimate how much $CaCO_3$ is needed to obtain the desired pH (KCl) of 6.0 ± 0.5 artificial soil is prepared by mixing peat, sand and kaolin and water as described. Small portions are taken and mixed with different amounts of $CaCO_3$ e.g. corresponding with concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 % dry weight. From these portions , the pH is determined as described in ISO standard 10390 and the results ar plotted in a graph as pH versus amount $CaCO_3$. From this graph, the amount of $CaCO_3$ necessary to obtain a pH of 6.0 ± 0.5 can be estimated.

E Suppliers

Quartz sand:	F 36	Quarzwerke Frechen GmbH Kaskadenweg 40 50226 Frechen
Kaolinite:	1. Type 1777	Grubenkontor Wunsiedel Ziegler & Co. 95632 Wunsiedel
	2. Lohrheimer Kaolin W	Erbslöh & Co. Geisenheimer Kaolinenwerke 65366 Geisenheim

F Bibliography

SPAHR (1981): Anz. Schädlingskde., Pflanzenschutz, Umweltschutz 54, 27 - 29.

WOLF-ROSKOSCH (1983): Chemikaliengesetz Heft 3, Texte 27/38, Umweltbundesamt, 83 - 109.

OECD (1984): Guideline for testing of chemicals 'Earthworms, Acute Toxicity Tests', No. 207.

EG (1988): Amtsblatt der Europäischen Gemeinschaften, Nr. L 133/3, Teil C, "Toxizität für Regenwürmer: Prüfung in künstlichem Boden".

JANCKE (1989): Zool. Beiträge, neue Folge, 32, 261 - 299.

HAMILTON, H.A., RUSSO, R.C. & THURSTON, R.V. (1977, 1978): Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. Environ. Sci. Technol. 11, 714-719. Correction Environ. Sci. Technol. 12, 417.

III.3 List of Experts who cooperated in the development of the test method

Participants of national ring tests (1985-1989)

R. Fischer
Hoechst Roussel Agri Vet Company
c/o NOR-AM Chemical Company
Rt. 2. Country Rd 1324
Pikeville, North Carolina 27863
U.S.A.

P. Gode
Henkel KGaA
Ökologie
Henkelstraße 67
40589 Düsseldorf

F. Heimbach
Bayer AG
Pflanzenschutzzentrum
Pf.F(CE-OE-Geb. 6620
51368 Leverkusen-Bayerwerk

D. Heimann-Detlefsen
An der Renne 45
31139 Hildesheim

H.J. Jatzek
BASF AG
DUU/WL-A 520
Carl-Bosch-Str. 38
67067 Ludwigshafen

F. Riepert
Biologische Bundesanstalt
für Land. und Forstwirtschaft
Institut für Ökotoxikologie
im Pflanzenschutz
Königin-Luise-Str. 19
14195 Berlin

J. Römbke
ECT Ökotoxikologie GmbH
Böttgerstr. 2-14
65439 Flörsheim am Main

P. Rudolph
Ministerium für Umwelt, Naturschutz
und Raumordnung Brandenburg
Abt. Immissionsschutz
Albert-Einstein-Str. 42/46
14473 Potsdam

M. Schleuter
Bundesanstalt für Gewässerkunde
Kaiserin-Augusta-Anlagen 15
56068 Koblenz

B. Ulber
Institut für Pflanzenpathologie
und Pflanzenschutz der
Georg-August-Universität
Grisebachstr. 6
37077 Göttingen

G. Weigmann
Freie Universität
Institut für Tierphysiologie
und angewandte Zoologie
Tietzenweg 85 - 87
12203 Berlin

V. Wolters
Justus-Liebig-Universität
Institut für allgemeine
und spezielle Zoologie
Stephanstr. 24
35390 Gießen

C.A.M. van Gestel
Department of Ecology and Ecotoxicology
Vrije Universiteit Amsterdam
De Boelelaan 1087
NL 1081 HV Amsterdam

Participants of the first ISO Ring Test 1993

Dr. K. van Gestel
Vrije Universiteit Amsterdam
Faculteit Biologie
de Boelelaan
NL-1081 HV Amsterdam
The Netherlands

Dr. Nelly van der Hoeven
TNO Institute
of Environmental Sciences
Schoemakerstraat 97
P.O. Box 6011
NL-2600 JA Delft
The Netherlands

Dr. H.J. Jatzek
BASF AG
DUU/WL-A520
Carl-Bosch-Str. 38
D-67067 Ludwigshafen
Germany

Frau B. Lang
BioChem GmbH Karlsruhe
Labor Cunnersdorf
D-04451 Cunnersdorf
Germany

Dr. R. Petto
IBACON
Institut für Biologische Analytik
und Consulting
Industriestr. 1
D-64380 Roßdorf
Germany

Mrs.
Estelle Bjornestad
Water Quality Institute
Head Office - Science Park Horsholm
11, Agern Alle
Forskningscentret
DK-2970 Horsholm
Denmark

Dr. F. Riepert
Biologische Bundesanstalt
für Land- und Forstwirtschaft
Institut für Ökotoxikologie
im Pflanzenschutz
Königin-Luise-Str. 19
D-14195 Berlin
Germany

Dr. Schmuck
Bayer-AG
Pflanzenschutzzentrum
Pf-F/CE-OE-Geb.6620
D-51368 Leverkusen-Bayerwerk
Germany

Leon R.M. de Poorter
Aqua Sense
Postbus 41 125
NL-1009 EL Amsterdam
The Netherlands

Participants of the second ISO Ring Test 1995

Jos Brils
Aqua Sense
Postbus 95 125
NL-1090 HC Amsterdam
The Netherlands

Dr. K. van Gestel
Vrije Universiteit Amsterdam
Faculteit Biologie
de Boelelaan
NL-1081 HV Amsterdam
The Netherlands

Dr. F. Heimbach
Bayer AG
Pflanzenschutzzentrum
PF-F/CE-OE-Geb. 6620
D-51368 Leverkusen-Bayerwerk
Germany

Dr. H.J. Jatzek
BASF AG
DUU/WL-A520
Carl-Bosch-Str. 38
D-67067 Ludwigshafen
Germany

Frau B. Lang
BioChem GmbH Karlsruhe
Labor Cunnersdorf
D-04451 Cunnersdorf
Germany

Dr. R. Heusel
Hoechst Schering AgrEvo GmbH
Umweltforschung G36
D-65926 Frankfurt/M.
Germany

Mr. R. Knoben
Witteveen & Bos
Postbus 233
NL-7400 AE Deventer
The Netherlands

Dr. Ralf Petto
IBACON
Institut für Biologische Analytik
und Consulting
Industriestr. 1
D-64380 Roßdorf
Germany

Dr. F. Riepert
Biologische Bundesanstalt
Institut für Ökotoxikologie
im Pflanzenschutz
Königin-Luise-Str. 19
14195 BERLIN
Germany

Frau Dr. Süß
Biologische Bundesanstalt
Institut für Ökotoxikologie
im Pflanzenschutz
Stahnsdorfer Damm 81
D-14532 Kleinmachnow
Germany

Christine Kula, Braunschweig

IV. Development of a test method on sublethal effects of pesticides on the earthworm species *Eisenia fetida/Eisenia andrei* - comparison of two ringtests

Entwicklung einer Methode zur Prüfung subletaler Auswirkungen von Pflanzenschutzmitteln auf die Regenwurmart *Eisenia fetida/Eisenia andrei* - Vergleich von zwei Ringversuchen

IV.1 Evaluation of ringtests
Auswertung von Ringversuchen

CONTENTS

1. Introduction

The evaluation of side-effects of pesticides on soil organisms is mainly based on testing of earthworms. Earthworms have been chosen because of their contributions to soil fertility and often high biomass in soils. Besides this, earthworms are a relatively well known systematic group within soil fauna with a limited number of species. Knowledge on biology and ecology of this group is better than in many other soil organisms.

In 1984 a laboratory test on acute toxicity was published (OECD, 1984). Since indigenous species of the European region are difficult to culture in the laboratory, *Eisenia fetida* is used for standard testing in the laboratory. The species, originating from the mediterranean region and occurring in substrates with high organic matter content, has a short generation time and a high reproductive potential.

Mortality is the main endpoint in the acute toxicity test according to OECD-guideline no. 207. Body weight change of the test animals also has to be recorded. It can be used as a test parameter indicating sublethal effects. As the animals are not fed during the test and the test duration is only 14 days it is not possible to gain information on sublethal effects on an appropriate time scale.

To overcome these disadvantages a test on reproduction toxicity was developed in the Federal Biological Research Centre for Agriculture and Forestry and tested in two ringtests. The aim of the investigation was to establish a test on sublethal effects within the authorization procedure of pesticides. Because of the problems with indigenous earthworm species mentioned above, *Eisenia fetida* was chosen to serve as test species.

Eight laboratories participated in a first ringtest in 1990/91 (KOKTA, 1992). In 1991/92 a second ringtest was initiated with participation of 14 laboratories. The aim of the second ringtest was to improve repeatability and to test the system using an appropriate standardisation. On the basis of these results an ISO-Standard (ISO-DIS 11268-2) is in preparation.

2. Test design

The test design of the second ringtest was closely related to the method used in 1990/91 (KOKTA, 1992). The main similarities and dissimilarities are listed in table 1.

Table 1: Test design in the two ringtests on sublethal effects on earthworms

	FIRST RINGTEST 1990/91	SECOND RINGTEST 1991/92
Test organisms	*Eisenia fetida* or *Eisenia andrei*	*Eisenia fetida* or *Eisenia andrei*
Test substrate	artificial soil	artificial soil
Food mixed into substrate before introducing earthworms	without additional food	with additional food (e.g. 10 g dried cattle manure/kg dry weight substrate)
Test boxes	bellaplast boxes	appropriate boxes
Application	on soil surface	on soil surface
Exposure of adults	6 weeks	4 weeks
Hatching and growth of juveniles	4 weeks	4 weeks
Overall test duration	10 weeks	8 weeks
Food given during experiment	cattle manure (same charge)	appropriate food
Feeding	when required	once a week
Number of replicates	3-4	4

In the second ringtest exposure of adult earthworms was shortened from six to four weeks. Thus the overall test duration was eight weeks instead of 10 weeks. Furthermore the test boxes and the food were not distributed from one charge as in the first ringtest. The two pesticides tested (active ingredients benomyl and parathion) were applied with appropriate laboratory spraying equipment. No residue analysis was performed in parallel to the tests, but the amount of spraying dilution was checked by weighing empty test boxes before and after application.

In the first ringtest the food given was commercially available cattle manure from the same charge, normally used as fertilizer. In the second ringtest there were no instructions on the food to be used. However an advice was given that the food should be tested on its „feeding quality", because toxic effects can appear due to high ammonia content, especially in commercially available cattle manure. In the second ringtest most laboratories used commercially available cattle manure as food. Nevertheless this food was not tested on its usefulness by all participants carefully, resulting in experiments with nearly no reproduction. In some tests freshly collected cattle manure was used after having been dried, finely ground and moistened. Oat meal brans were used in one test.

3. Endpoints

Mortality

Mortality of adults is evaluated after four weeks of exposure by handsorting the surviving adults from the substrate. In very few cases the adults managed to escape from the test boxes. When it was clear from which test box they originated, these adults were summed up with the dead ones because they did not contribute to fertility .

Number of juveniles

The reproductional success is evaluated by counting the number of juveniles which hatched and grew up for some time in the contaminated substrate. By allowing the juveniles to grow up to a certain extent, exposure of the juveniles in the contaminated substrate is given. Additionally the juveniles can only be counted directly or expelled with a water bath if a certain size is reached.

The number of juveniles was not adjusted to dead or missing adults. So the real number of juveniles per adult might be higher than indicated in the tables. One problem of adjusting to the number of surviving adults is that it is not known when these animals died. In some cases, higher mortality of adults seems to be compensated by a higher reproduction of the surviving adults.

Concerning exposure it has to be kept in mind that by sorting out the adults after 4 weeks of exposure, the substrate is mixed. Therefore the pesticide residues are not longer concentrated in the upper soil layer, but are distributed homogenously in the substrate during the second part of the test when the juveniles hatch and grow up.

Body weight of adults

Body weight change is evaluated by weighing adult animals at test start and in the end. When a high number of animals died within the test, the determination of body weight related to surviving adults might lead to misinterpretation because the surviving animals could be the most fittest. The lower the number of survivors the bigger might be the mistake.

According to VAN GESTEL et al (1992), cocoon production appears to be negatively correlated with body weight. For this reason both parameters should be evaluated in a study.

Feeding activity and behaviour

The animals are fed on the soil surface. Feeding activity gives an insight into the behaviour of test animals in the first 4 weeks of the test. For means of standardization a weekly feeding interval with a defined amount of food is preferred and has prooved to be sufficient, but it is also possible to feed ad libitum.

Behaviour of test animals is an important factor which to some extent should be evaluated in the test. Surface crawling and feeding activity can give information on a possible repellent effect. Hyperactivity should be taken care of because it might lead to the escape of the animals from the test boxes.

Morphological changes

As in the acute toxicity test, adults and juveniles should be examined for morphological changes. In practice, these effects predominate in the acute test where higher concentrations are tested.

4. Test substances

The pesticides DuPont Benomyl (active ingredient 500 g/kg benomyl) and E 605 forte (active ingredient 500 g/l parathion) were used in both ringtests. Both products originated from one charge each and were distributed to all participants except one case (test no. 16 with benomyl, second ringtest) where another charge was used.

The fungicide DuPont Benomyl was applied with a single application rate of 250 g product/ha and a 5-fold rate. Benomyl is a benzimidazole-fungicide mainly used in orchards and cereal fields. The insecticide E 605 forte was applied with a single application rate of 210 ml product/ha and a 5-fold rate. Parathion is a non-systemic organophosphorous insecticide used against insects in different crops.

No reference compound was used in these tests. The fungicide benomyl was also tested on its usefulness to serve as a reference compound in further tests.

5. Results

5.1 First ringtest

For comparison of both ringtests the results of the first test (KOKTA, 1992) are repeated in brief. Eight laboratories participated in this test (n = 8).

The application of benomyl resulted in significant effects on juvenile numbers in 2 tests in the 1-fold rate (see Table A, annex). In the 5-fold rate significant effects on juvenile numbers occurred in 7 tests. Significant effects on weight were observed in 2 tests in the 1-fold rate and in 5 studies in the 5-fold rate.

The application of parathion (see Table B, annex) in the 1-fold rate resulted in significant effects on the numbers of juveniles in 2 tests and in significant effects on body weight in 1 test. In the 5-fold rate in 5 studies significant effects on the numbers of juveniles and in 3 studies significant effects on weight occurred.

Tables 2 and 3 list the percent change of juvenile numbers compared to control. With benomyl (table 2) there was a reduction of more than 20 % in the 1-fold rate in 5 studies. This effect was significant in 2 studies. A reduction of more than 30 % in the 1-fold rate was observed in 5 studies, with a significant effect in 2 studies. In the 5-fold rate there was a reduction of more than 20 % in all studies. This effect was significant in 7 studies. There was an effect of more than 30 and 40 % in 7 studies.

Table 2: Juvenile numbers in % of control numbers (control = 100 %) in the first ringtest with benomyl (raw data see table A, annex)

STUDY NUMBER (first ringtest)	1-FOLD (125g ai/ha)	5-FOLD (625 g ai/ha)
1	43.6	0.8 *
2	107.4	56.1 *
3	69.4	47.2 *
4	106.6	5.3 *
5	141.9	72.7
6 (after 4 weeks)	65.9	31.7 *
7	45.7 *	4.2 *
8 #	57.8 *	14.2 *

#In this laboratory the 1.5-fold resp. 7.5-fold rate was tested

* sign. $p \leq 0.05$, Williams-test

With parathion (table 3) there was a reduction of more than 20 % in the 1-fold rate in 4 studies. This effect was significant in 2 studies. In the 5-fold rate there was a reduction of more than 20 % in 6 experiments. The effect was significant in 5 studies. A reduction of 30 % was observed in 5 studies. This effect was significant in all cases.

Table 3: Juvenile numbers in % of control numbers (control = 100 %) in the first ringtest with parathion (raw data see table B, annex)

STUDY NUMBER (first ringtest)	1-FOLD (210 g ai/ha)	5-FOLD (1050 g ai/ha)
1	47.0	28.7 *
2	63.9 *	61.1 *
3	141.3	84.4
4	88.5	60.1 *
5	76.8	105.3
6 (after 4 weeks)	133.0	76.1
7	62.9 *	39.3 *
8 #	132.6	64.3 *

#In this laboratory the 1.5-fold resp. 7.5-fold rate was tested

* sign. $p \leq 0.05$, Williams-test

Tables 4 and 5 show that there were few cases with a significant reaction of either number of juveniles or body weight in the 1-fold rate in benomyl and parathion. In the 5-fold rate there was a dose-related effect in nearly all experiments. In most cases number of juveniles alone and number of juveniles combined with body weight were the parameters indicating an effect.

Table 4: Parameters showing a significant reduction with benomyl in the first ringtest (j = number of juveniles, b = body weight)

STUDY NUMBER (first ringtest)	1-FOLD (125 g ai/ha)	5-FOLD (625 g ai/ha)
1	-	j, b
2	-	j
3	-	j
4	-	j
5	-	b
6	b	j, b
7	j	j, b
8 #	j, b	j, b

in this laboratory the 1.5-fold resp. 7.5-fold rate was tested

Table 5: Parameters showing a significant reduction with parathion in the first ringtest (j = number of juveniles, b = body weight)

STUDY NUMBER (first ringtest)	1-FOLD (105 g ai/ha)	5-FOLD (525 g ai/ha)
1	-	j
2	j	j
3	-	b
4	-	j
5	-	-
6	b	b
7	j	j, b
8 #	-	j, b

in this laboratory the 1.5-fold resp. 7.5-fold rate was tested

5.2 Second ringtest

There were 14 laboratories which participated in the second ringtest. The list of participants is given in chapter IV.3. In some cases laboratories conducted more than one test, so that more than 14 study numbers can occur in the tables listing the results.

5.2.1 Results with Benomyl

Concerning mortality of adults, 13 tests (n=16) showed no mortality in the control (Table C, annex). In 2 studies (tests no. 10 and 13) a mortality of more than 10 % in the control and both treatments occurred, without any dose-response-relationship. In test no. 1 there was a significant effect on mortality in the 5-fold dose, indicating an acute pesticide effect.

The number of juveniles found was in the same order of magnitude in both ringtests, although the exposure of the adults was reduced to 4 weeks in the second ringtest. In 11 studies a quality criterion of at least 30 juveniles as a mean for the control boxes was reached (Table C, annex). The tests no. 1 and 2 nearly reached this value. By including both, 13 experiments had about 30 juveniles or more in the controls.

There were two studies with nearly no reproduction (tests no. 13 and 15), which is defined as less than 10 juveniles/test box. In both studies the food used was commercially available cattle manure. In test no. 8 there was a mean juvenile number of 18 in the control. This experiment

does not meet the standard criterion of 30 juveniles per control box. However in this study a low variation in juvenile numbers between test boxes was observed, therefore the reduction of the number of juveniles in the treated boxes was significant.

When all studies including those with a low number of juveniles are taken into account, there was a significant effect on the number of juveniles in 11 tests with the 1-fold rate, and in 32 experiments with the 5-fold rate.

Compared to test start, control animals lost body weight in 5 studies (Table C, annex). In 4 studies weight gain was less than 10 % of the initial weight. In 7 studies weight increased during the study by more than 10 %. The quality criterion for weight in the current reproduction toxicity test guideline (BBA, 1994) states that adults should not loose more than 20 % of their initial weight. This criterion is fulfilled in all studies of this ringtest. There was a significant effect on body weight in 2 studies in the 1-fold rate and in 7 studies in the 5-fold rate.

Some experiments were not used for further comparison when interpreting the results for different reasons. Studies with nearly no juveniles, no dose-response relationship concerning control and both treatments, and less than 4 replicates, are put in parenthesis in the tables and excluded from some general comparisons. In test no. 16 the benomyl used was elder than 2 years. It is assumed that for this reason no effects were observed.

Table 6 lists the percent change of the number of juveniles compared to control. Only 11 studies out of 16 are compared. The others are excluded for the reasons mentioned above. In the 1-fold rate there are only 10 numbers for comparison, because in study number 5 some replicates of the 1-fold rate were not valid. A reduction of more than 20 % was observed in 6 studies in the 1-fold rate; this effect was significant in 5 of these studies. A significant reduction of more than 30 % was observed in 4 studies in the 1-fold rate. In the 5-fold rate there was a reduction of more than 20 % in all studies and of more than 50 % in 9 studies. The effect was significant in 10 experiments.

Table 6: Juvenile numbers in % of control numbers (control = 100 %) in the second ringtest with benomyl (raw data see table C, annex)

STUDY NUMBER (Second ringtest)	1-FOLD (125 g ai/ha)	5-FOLD (625 g ai/ha)
1	163.1	4.3 *
2	103.5	57.6
3	63.8 *	12.5 *
4	65.5 *	38.2 *
5	-	55.1 *
6	86.5	1.0 *
7	20.3 *	0 *
8	85.4	13.5 *
9	3.4	38.6 *
10	38.7 *	35.9 *
11	71.9 *	20.6 *

* sign. $p \leq 0{,}05$, Williams-test

In Table 7 the parameters showing a significant reduction compared to the control are listed for the different studies. In most studies juvenile number was the most sensitive parameter. A significant reduction in body weight was only observed in the 5-fold rate and in no case body weight alone showed an effect. Only in one study also mortality was significantly affected.

Table 7: Parameters showing a significant reduction with benomyl (j = number of juveniles, b = body weight, m = mortality)

STUDY NUMBER (Second ringtest)	1-FOLD (125 g ai/ha)	5-FOLD (625 g ai/ha)
1	-	j, b, m
2	-	(j)
3	j	j, b
4	j	j
5	-	j, b
6	-	j, b
7	j	j, b
8	-	j, b
9	-	j
10	j	j
11	j	j

(j) reduction of juvenile number more than 40 %, but not significant

5.2.2 Results with Parathion

In 2 studies (n = 15) there was a mortality of 15 % in the control (see Table D, annex). This level of mortality also occurred in both studies in the pesticide-treated boxes.
The number of juveniles exceeded 30 in 11 experiments. In two studies the number was nearly 30, so that 13 studies reached about the standard of a mean number of 30 juveniles in control boxes. In 2 tests there was nearly no reproduction. There was a significant effect on the number of juveniles in 7 studies in the 1-fold rate and in 9 studies in the 5-fold rate.

In body weight there was a decrease in weight in the control boxes in 5 studies. In 2 of these tests the reduction in body weight was more than 10 % of the initial weight and in one case reduction was more than 20 %. This test (no. 15) was also not satisfactory concerning the number of juveniles and mortality in the control, so that there are different reasons to reject this test.

There was a significant decrease in body weight in 3 experiments in the 1-fold rate and in 5 experiments in the 5-fold rate. In tests no. 1 and 11 there was no clear dose-response-relationship concerning body weight, because there was an decrease in weight in the 1-fold rate whereas there was an increase in the 5-fold rate.

In Table no. 8 the percent change of juvenile numbers compared to control is listed, excluding the studies with only three replicates (no. 13) and without or with low reproduction (no. 14 and 15).

Table 8: Juvenile numbers in % of control numbers (control = 100 %) in the second ringtest with parathion (raw data see table D, annex)

STUDY NUMBER (Second ringtest)	1-FOLD (105 g ai/ha)	5-FOLD (525 g ai/ha)
1	125.9	92.4
2	107.8	78.8
3	69.6 *	37.7 *
4	35.9 *	34.1 *
5	103.1	83.6 *
6	101.6	95.5
7	98.1	47.6 *
8	110.8	75.6
9	24.2 *	7.0 *
10	82.0 *	66.1 *
11	53.0 *	77.2 *
12	95.7	84.1

* sign. $p \leq 0,05$, Williams-test

A reduction of more than 30 % in juvenile numbers in the 1-fold rate was found in 4 studies. The effect was significant in all cases. In the 5-fold rate there was a reduction of more than 20 % in 8 studies. This effect was significant in 6 studies. In 4 studies the reduction was more than 30 % and significant in all cases.

In Table 9 the parameters showing a significant reduction compared to the control are listed for the different studies. With parathion in the studies no. 1 and 2 body weight was the most sensible parameter. In these studies body weight was the only parameter showing a pesticide-related effect. In the other studies juvenile number or juvenile number and body weight showed a significant reduction. No dose-related effects on mortality were observed.

Table 9: Parameters showing a significant reduction with parathion (j = number of juveniles, b = body weight)

STUDY NUMBER (Second ringtest)	1-FOLD (105 g ai/ha)	5-FOLD (525 g ai/ha)
1	b	b
2	b	b
3	j	j, b
4	j	j
5	-	j, b
6	-	-
7	-	j
8	-	-
9	j	j
10	j	j
11	j, b	j, b
12	-	-

6. Comparison and discussion of both ringtests

6.1 Validity of results of control boxes

For decision on the quality and reproducibility of test results it is helpful to have criteria indicating that the test system generates reliable data. These criteria include results on a reference substance as well as a check of the control data. In the following the data of the control boxes are checked according to the quality criteria given in the BBA-guideline (BBA, 1994).

Mortality

Mortality in the control should not exceed 10 %. In the first ringtest in one control mortality exceeded 10 %. In the second ringtest in two studies mortality exceeded the limit of 10 %. In one of the cases (study number 13 in Table C, annex) also reproduction was not sufficient and body weight decreased by more than 20 % in the control. This test therefore should be rejected for several reasons. The other test with a control mortality of more than 10 % was test no. 10 in Table C. In this case mortality did not fulfill the quality criteria, whereas the number of juveniles and body weight were within the standard. This test was used for further comparison.

Number of juveniles

The BBA-guideline (BBA, 1994) on reproduction of earthworms prescribes a minimum reproduction of 30 juveniles/control box. It is difficult to compare both ringtests concerning this parameter because the adult exposure period differed between both tests.

Taking into consideration that often the same control was used for the tests with benomyl and parathion, in the first ringtest there were 11 different controls (Table E, annex). In one control there were less than 30 juveniles/test box. All the other controls reached the minimum of 30, although the exposure period was shorter than in the second ringtest.

The second ringtest comprised 23 different controls. In 17 controls there were more than 30 juveniles in the control boxes. In test no. 8 with benomyl (Table C, annex) the number of juveniles was 18 in the control. This does not reach the minimum number, but this test showed a low variation between test boxes and therefore a significant reduction was observed. This example shows that in some cases also tests with a low juvenile number might yield reliable results. On the other hand a certain reproduction must be given in a reproduction toxicity test. The number of 30 juveniles means about 3 juveniles/adult in 4 weeks. According to literature data on fertility, about 1 to 4 cocoons/worm/week are produced in artificial soil in most experiments (van Gestel, 1992). The number of juveniles per cocoon is about 1 to 4, sometimes even more. Therefore it should be possible to fulfill the criterion of 30 juveniles/test box, which means only about one cocoon/adult in the entire testing period.

Coefficient of variation of juvenile numbers

In the following comparison the studies with less than 30 juveniles in the control were excluded. For the first ringtest 10 different controls from both products remain for comparison (Table E, annex). The BBA-guideline says that the coefficient of variation of juvenile numbers should not exceed 50 %. In one case (test no.5) the coefficient of variation was 71.1 and so higher than 50 %, but this study was not typical and something might have been extraordinary because of a very high weight gain in the control and in the treatments. Using a limit value of 20 % for the coefficient of variation, 40 % of the studies do not fulfill this criterion. A limit value of 30 % reduces this number to 30 %.

For the second ringtest, 20 different control values were suitable for comparison (Table F, annex). A coefficient of variation of more than 50 % was reached in only one control for parathion (study number 8 in Table D, annex). In this study other quality criteria like the juvenile number were sufficient. The decrease in juvenile numbers of 25 % found in the 5-fold rate in this experiment was not statistically significant.

Setting the limit for the maximum coefficient of variation to 20 %, 13 controls were below this value. This means that 7 studies did not fulfill this criterion. Setting 30 % as an upper limit, 3 studies did not fulfill the criterion. In the case of study number 8 with benomyl there was a low juvenile number, so the study should also be rejected for this reason. Only in study no. 12 with parathion there are no additional criteria indicating that there might be a lack of reliability, despite the fact that there is a slight reduction in juvenile numbers which might indicate an effect. This study was used for further comparison.

To conclude, it can be stated that a coefficient of variation of less than 30 % might be a better indicator of reliable data than a coefficient of variation of less than 50 %.

Body weight

Test animals should not loose too much weight which might be an indicator for starvation or desiccation. The limit value in the BBA-guideline for a maximum decrease of weight is 20 % of the initial weight. Decrease observed in the first ringtest was less than 20 % in all experiments. Test no. 5 with a high number of juveniles showed a weight decrease of 14 %.

For the second ringtest the 20 % limit was also not exceeded and there are no hints from all the tests that this criterion should be changed. A high weight gain in the control like in studies number 6,7 and 8 with parathion might lead to a lower reproduction (VAN GESTEL et al, 1992). A high weight gain indicates that the animals are still in a growth phase instead of the reproductive phase. To develop a quality criterion from this fact seems not to be necessary, but a high weight gain should be considered when interpreting the results.

Food

In the second ringtest the food was not distributed from one source. In the first ringtest the food was tested on its 'feeding quality' by conducting a short pre-test before sending the food to the participants. In the second ringtest a quality check of the food should have been performed but was obviously not done in all laboratories. The current guideline (BBA, 1994) therefore gives advice on the problem of suitable food. This advice should be followed carefully.

6.2 Validity of test results

The results of both ringtests are in the same order of magnitude and indicate a suitable testing method. The second ringtest was done with a lower degree of standardization. This helped to point out critical points and to develop practicable validity criteria which are now laid down in the BBA-guideline and in the ISO-draft.

6.3 Benomyl as a reference substance

The results of both ringtest indicate that the active ingredient benomyl can be used as a reference substance in future, although there have been a few tests which did not show an effect. As the active ingredients benomyl and carbendazim are chemically related, carbendazim is a good choice for a reference substance too.

6.4 General problems and conclusions

Both ringtests showed comparable results and demonstrate that the method is reproducible. The 5-fold rate of benomyl and parathion showed an effect on either reproduction or body weight in both ringtests. In the 1-fold rate there was an effect in about on third up to half of the tests. These results were nearly identical for both ringtests.

Due to the close connection between reproduction and body weight both parameters must be investigated in the test and should be regarded equal concerning their biological significance. There are no hints from both ringtests on any factors which could have determined whether reproduction and/or body weight showed an effect. Taking all results from tables 4, 5, 7 and 8 together there was an effect on reproduction alone in 27 cases, on body weight alone in 9 cases and on reproduction and bodyweight simultaneously in 17 cases. This demonstrates that in most cases reproduction is the most sensitive parameter. The same data indicate that a simultaneous reaction of reproduction and body weight appears more often in the 5-fold rate than 1-fold rate.

Effects of Benomyl

Even with benomyl, which is relatively well known for effects on earthworms, not all experiments showed an effect. As in risk assessment normally there is only one test of each step, there might be problems identifying products or active ingredients which are a risk for earthworms. The validity criteria given solve most of the problem like in tests no. 5 in the first ringtest and in tests no. 1, 2 and 8 in the second ringtest. Additionally it seems to be necessary to test at least two application rates because there are tests like no. 5 in the first ringtest and no. 1 and 14 in the second ringtest with no clear dose-response relationship and where another rate tested indicates that there is an effect. To conclude it can be stated that testing more concentrations helps to get a clear answer to the questions whether there is a risk.

Effects of Parathion

In parathion there are some tests like no. 3, 4 and 5 in the first ringtest and test no. 6 in the second ringtest which do not show a dose-related effect. There are no additional hints by validity criteria which could explain this. It seems also from other experience that with parathion the effect cannot be predicted as clear as for example with benomyl.

Technical problems with the test

To avoid problems the test substance should not be too old. In one case benomyl from the previous ringtest was used and in this test no effects were found.

The problem of low juvenile numbers in some studies can be solved by improving food quality and testing the food before use.

Care has to be taken of the problem that adults might escape from the test boxes during the study, e.g. when the test substance has a repellent effect. In these ringtests this occurred in very few cases and therefore can be neglected here.

Another problem is that still unhatched cocoons are found after the four weeks of juvenile development period. As the amount of unhatched cocoons is reported to be low and is assumed to appear in the control as well as in the treatments no elongation of the second test phase seems necessary.

The preincubation period could also cause problems for the general condition of the worms when it is too short. This might be the reason for high variability of juvenile numbers in some cases. With the experience from both ringtests, the preincubation period can be extended up to seven days (ISO-draft 11268-2).

Concerning the connection between mortality and body weight development of the surviving worms it is assumed that a trigger will help to solve the problem. A suggestion is that when more than 15 % of test animals died during the experiment in the single test boxes, one should be careful relating the weight to the number of surviving animals. The effect might be much bigger than appearing from these values. Another possibility is to focus on the overall biomass and not on the mean body weight. With both pesticides tested, this problem did not play a major role. When higher mortality occurred, also significant effects on the weight of individuals were observed.

Evaluation of exposure

By feeding the animals on the soil surface during the test and having applied the test substance onto the soil surface, the earthworms had to work through a pesticide-treated soil layer to reach the food source. As there was a small amount of food mixed into the soil before test start in the second ringtest, the animals were not forced to enter the contaminated soil layer immediately. This procedure might reduce exposure to a realistic amount, better comparable to the field situation than in the first ringtest with no food mixed into the soil.

The plant cover of the soil at the time of application will reduce the amount of pesticide reaching the soil. This fact is not taken into account in the test design because it is difficult to standardize. It should be considered in risk assessment looking at the results of the test. The same is true for the results of the 5-fold rate. It is necessary and even better to test more than 2 rates for achieving reliable test results. For the risk assessment under field conditions it should be kept in mind that in this test the only safety factor for comparing sensitivity of species is testing the full rate without taking into account the plant cover of the soil and testing the 5-fold rate. Whereas some information is available on comparisons of acute toxic effects in different earthworm species (MA & BODT, 1993, KULA, 1994), it is difficult to compare effects on reproduction between species, because in indigenous species it is hard to stimulate reproduction in the laboratory.

The evaluation of juvenile numbers at the end of the test is one possibility of measuring reproduction. Another possibility is to evaluate cocoon production and hatching rate of the cocoons separately. It is not possible to determine both endpoints in the same artificial soil substrate because the number of cocoons produced is best determined by washing them out from the substrate. By doing this, the substrate is destroyed. Hatching rate is evaluated by incubating the cocoons on moist filter paper or in uncontaminated fresh artificial soil substrate. As the cocoons are a good shelter for the eggs, the exposure of juvenile earthworms to a contaminated substrate is assumed to be a sensitive period concerning pesticide exposure. It is well known that juvenile earthworms mainly live in the upper soil layer (LEE, 1985), so that the test will ensure a realistic exposure situation comparable to the field.

7. Summary

For testing side-effects of pesticides on reproduction of earthworms, a laboratory test was developed. An artificial test substrate ('artificial soil') according to OECD-guideline no. 207 was used. The species *Eisenia fetida* or *Eisenia andrei* served as test organism. The test substance was applied as close to field conditions as possible, for example on the soil surface. The number of juveniles produced in the test period, body weight change of surviving adults and mortality of adults were used as test parameters.

In 1990/1991 and 1991/1992 two ringtests with two different pesticides were conducted. In the second ringtest the method was changed in some details. The main difference was the shortening of the exposure period from 6 weeks in the first ringtest to 4 weeks in the second ringtest. Another difference was that in the first ringtest the food was distributed from one charge whereas in the second ringtest every participant selected the food on his own.

The results of both ringtests indicate the suitability of the method. The exposure period of 4 weeks proved to be sufficient. There were problems in the second ringtest by the fact that the food used was not suitable in all cases. For this reason some tests could not be evaluated due to missing reproduction.

The method is sensible and reproducible when studies without sufficient reproduction are excluded according to the validity criteria given.

The pesticide with the active ingredient benomyl was also tested with the intention that it could serve as a toxic standard in further testing. The results show that products with the active ingredient benomyl or carbendazim are a good choice for a toxic standard.

The results from both tests have been used within the development of a BBA test guideline and an ISO-draft guideline on testing reproduction of earthworms in the laboratory.

8. Bibliography

BBA, 1994: Auswirkungen von Pflanzenschutzmitteln auf die Reproduktion und das Wachstum von *Eisenia fetida/Eisenia andrei*. Richtlinien für die Prüfung von Pflanzenschutzmitteln im Zulassungsverfahren, Teil VI, 2-2, Januar 1994.

KOKTA, C. 1992: A laboratory test on sublethal effects of pesticides on earthworms. In: Becker, H.; Edwards, P. Greig-Smith, P.; Heimbach, F. (eds.) Ecotoxicology of earthworms, Intercept, Andover.

KULA, H. (1994): Species-specific sensitivity differences of earthworms to pesticides in laboratory tests. In: Donker, M.H., Eijsackers, H. & Heimbach, F. (eds.): Ecotoxicology of soil organisms. Lewis Publishers, Boca Raton. 241-250.

LEE, K.E. (1985): Earthworms - Their ecology and relationships with soils and land use. Academic Press, Sydney.

MA, W.C., BODT, J. (1993): Differences in toxicity of the insecticide chlorpyriphos to 6 species of earthworms (Oligochaeta, Lumbricidae) in standardized soil tests. Bull. Environ. Contam. Toxicol. 60, 864-870.

OECD, 1984: Guideline for testing of chemicals. No. 207. Earthworm acute toxicity tests. Adopted 4 April 1984.

Van GESTEL, C.A.M., DIRVEN-VAN BREEMEN, E.M., BAERSELMAN, R. (1992) Influence of environmental conditions on the growth and reproduction of the earthworm *Eisenia andrei* in an artificial soil substrate. Pedobiologia 36, 109-120.

9. Annex (tables)

Table A: Mortality of adults, number of juveniles and weight of surviving adults in the first ringtest with benomyl (tested product: DuPont Benomyl)

1-fold application rate: 250 g product/ha (= 125 g ai/ha)
5-fold application rate: 1250 g product/ha (= 625 g ai/ha)

MORTALITY OF ADULTS (IN %)

STUDY NUMBER (first ringtest)	CONTROL	1-FOLD RATE	5-FOLD RATE
1	0	0	3.0 ± 6.0
2	15.0 ± 19.0	5.0 ± 10	0
3	0	3.0 ± 6.0	0
4	0	0	3.1 ± 6.3
5	0	0	0
6 (after 4 weeks)	7.0 ± 6.0	13.0 ± 15.0	10.0 ± 10.0
7	0	3.0 ± 6.0	27.0 ± 38.0
8 #	0	0	0

NUMBER OF JUVENILES/TEST BOX

1	38.3 ± 16.3	16.7 ± 18.8	0.3 ± 0.5 *
2	88.3 ± 17.2	94.8 ± 13.8	49.5 ± 7.8 *
3	36.0 ± 4.0	25.0 ± 12.0	17.0 ± 14.0 *
4	53.3 ± 10.2	56.8 ± 14.0	2.8 ± 2.5 *
5	85.3 ± 60.6	121.0 ± 11.4	62.0 ± 24.9
6 (after 8 weeks)	29.3 ± 15.5	19.3 ± 11.0	9.3 ± 1.5 *
7	31.3 ± 4.2	14.3 ± 2.3 *	1.3 ± 1.5 *
8 #	120.0 ± 40.5	69.3 ± 25.0 *	17.0 ± 2.6 *

WEIGHT OF SURVIVING ADULTS IN % OF INITIAL WEIGHT

1	156.0 ± 9.0	155.0 ± 19.0	66.0 ± 5.0 *
2	125.0 ± 7.0	127.0 ± 5.0	130.0 ± 7.0
3	90.0 ± 6.0	97.0 ± 8.0	98.0 ± 3.0
4	142.5 ± 12.0	139.9 ± 7.1	146.5 ± 14.3
5	261.1 ± 15.0	245.4 ± 28.9	212.9 ± 32.1 *
6 (after 4 weeks)	115.5 ± 4.1	94.2 ± 9.4 *	92.5 ± 5.3 *
7	121.0 ± 15.0	101.0 ± 12.0	53.0 ± 33.0 *
8 #	153.5 ± 1.73	114.5 ± 7.1 *	76.3 ± 1.9 *

in this laboratory the 1.5-fold resp. 7.5-fold rate was tested
* sign. $p \leq 0.05$, Williams-Test

Table B: Mortality of adults, number of juveniles and weight of surviving adults in the first ringtest with parathion (tested product: E 605 forte)

1-fold application rate: 210 ml product/ha (= 105 g ai/ha)
5-fold application rate: 1050 ml product/ha (= 525 g ai/ha)

MORTALITY OF ADULTS (IN %)

STUDY NUMBER (first ringtest)	CONTROL	1-FOLD RATE	5-FOLD RATE
1	0	0	0
2	0	0	0
3	0	0	0
4	10.0 ± 14.0	5.0 ± 10.0	5.0 ± 10.0
5	0	0	0
6 (after 4 weeks)	7.0 ± 6.0	17.0 ± 12.0	7.0 ± 12.0
7	0	3.0 ± 6.0	27.0 ± 38.0
8 #	0	0	0

NUMBER OF JUVENILES/TEST BOX

1	38.0 ± 16.0	18.0 ± 17.0	11.0 ± 8.0 *
2	36.0 ± 4.0	23.0 ± 17.0 *	22.0 ± 1.0 *
3	53.3 ± 10.2	75.3 ± 26.5	45.0 ± 22.9
4	147.8 ± 31.0	131.5 ± 15.3	88.5 ± 22.0 *
5	95.0 ± 10.1	73.0 ± 15.4	100.0 ± 59.3
6 (after 8 weeks)	29.3 ± 15.5	39.0 ± 5.3	22.3 ± 3.1
7	31.3 ± 4.2	19.7 ± 3.1 *	12.3 ± 2.1*
8 #	148.5 ± 28.1	140.3 ± 14.8	95.5 ± 31.0 *

WEIGHT OF SURVIVING ADULTS IN % OF INITIAL WEIGHT

1	156.0 ± 9.0	175.0 ± 19.0	168.0 ± 15.0
2	90.0 ± 6.0	96.0 ± 9.0	82.0 ± 4.0
3	142.5 ± 11.7	135.3 ± 5.6	72.7 ± 1.2 *
4	95.2 ± 4.9	96.3 ± 2.6	97.8 ± 6.4
5	234.0 ± 4.0	270.0 ± 16.0	254.0 ± 50.0
6 (after 4 weeks)	115.5 ± 4.1	98.3 ± 5.6 *	97.3 ± 3.6 *
7	121.3 ± 1.5	115.3 ± 5.5	93.1 ± 9.6 *
8 #	145.8 ± 19.2	143.0 ± 7.1	138.0 ± 18.1

in this laboratory the 1.5-fold resp. 7.5-fold rate was tested
* sign. $p \leq 0.05$, Williams-Test

Table C: Mortality of adults, number of juveniles and weight of surviving adults in the second ringtest with benomyl (tested product: DuPont Benomyl); trials in brackets are excluded from further evaluations

1-fold application rate: 250 g product/ha (= 125 g ai/ha)
5-fold application rate: 1250 g product/ha (= 625 g ai/ha)

MORTALITY OF ADULTS (IN %)

STUDY NUMBER (second ringtest)	CONTROL	1-FOLD RATE	5-FOLD RATE
1	2.5 ± 5.0	0	60 ± 11.5 *
2	0	0	0
3	0	0	0
4	0	3.1 ± 6.3	3.1 ± 6.3
5	0	(0)	3.0 ± 5.0
6	0	0	0
7	0	0	0
8	0	0	0
9	0	0	0
10	15 ± 10.0	12.5 ± 5.0	12.5 ± 5.0
11	0	0	0
(12)	0	0	0
(13)	15 ± 5.8	17.5 ± 17.0	17.5 ± 23.6
(14)	0	0	0
(15)	0	0	0
(16)	0	0	0

NUMBER OF JUVENILES/TEST BOX

1	29.0 ± 19.7	47.3 ± 21.8	1.3 ± 1.5 *
2	28.3 ± 8.3	29.3 ± 12.5	16.3 ± 3.0
3	34.5 ± 7.8	22.0 ± 3.7 *	4.3 ± 4.7 *
4	44.0 ± 9.2	28.8 ± 11.4 *	16.8 ± 7.4 *
5	167.0 ± 20.0	(173.0 ± 30.0)	92.0 ± 9.0 *
6	48.4 ± 14.1	41.8 ± 16.6	0.5 ± 1.0 *
7	33.3 ± 3.0	6.8 ± 2.0 *	0 *
8	18.5 ± 7.3	15.8 ± 9.6	2.5 ± 0.6 *
9	50.8 ± 6.8	37.3 ± 15.6	19.6 ± 6.2 *
10	78.8 ± 10.0	30.5 ± 7.0	28.3 ± 10.0 *
11	102.0 ± 11.4	73.3 ± 18.8 *	21.0 ± 5.6 *
(12)	108.3 ± 15.2	27.7 ± 19.7 *	113.0 ± 13.0
(13)	1.0 ± 1.5	1.0 ± 2.0	0
(14)	114.7 ± 30.0	157.7 ± 24.0	97.0 ± 10.0
(15)	1.0 ± 0	1.8 ± 1.0	0 *
(16)	88.3 ± 11.9	99.8 ± 9.4	94.8 ± 17.0

WEIGHT OF SURVIVING ADULTS IN % OF INITIAL WEIGHT

1	113.6 ± 7.0	108.1 ± 12.0	82.3 ± 11.6 *
2	90.8 ± 4.8	97.2 ± 5.4 *	96.5 ± 3.6
3	138.7 ± 3.3	146.1 ± 3.8	115.6 ± 8.9 *
4	94.2 ± 4.6	104.4 ± 6.8 *	103.6 ± 6.7 *
5	106.8 ± 4.5	(101.5 ± 2.9)	97.8 ± 5.4 *
6	140.8 ± 3.4	152.8 ± 7.5	103.0 ± 8.5 *
7	112.7 ± 3.8	119.8 ± 3.3	100.2 ± 2.4 *
8	127.6 ± 4.1	123.2 ± 4.5	90.3 ± 6.5 *
9	103.2 ± 2.8	104.3 ± 1.3	101.2 ± 1.0
10	101.8 ± 3.0	103.3 ± 2.5	97.8 ± 6.8
11	111.7 ± 12.2	104.1 ± 7.2	104.1± 5.3
(12)	129.6 ± 12.8.	91.8 ± 11.5	136.0 ± 14.6
(13)	77.7 ± 5.3	81.5 ± 4.5	92.1 ± 4.8 *
(14)	93.6 ± 6.0	101.0 ± 5.2	86.7 ± 5.3
(15)	81.8 ± 6.1	81.5 ± 5.2	76.5 ± 4.7
(16)	104.1 ± 2.7	117.2 ± 11.8	105.0 ± 5.7

* sign. $p \leq 0.05$, Williams-test

Table D: Mortality of adults, number of juveniles and weight of surviving adults in the second ringtest with parathion (tested product: E 605 forte); trials in brackets are excluded from further evaluations

1-fold application rate: 210 g product/ha (= 105 g ai/ha)
5-fold application rate: 1050 g product/ha (= 525 g ai/ha)

MORTALITY OF ADULTS (IN %)

STUDY NUMBER (second ringtest)	CONTROL	1-FOLD RATE	5-FOLD RATE
1	2.5 ± 5.0	0	0
2	0	0	0
3	0	0	0
4	0	0	0
5	0	0	0
6	0	0	0
7	0	0	0
8	0	0	0
9	0	0	0
10	0	0	0
11	15.0 ± 10.0	20.0 ± 18.3	17.5 ± 17.0
12	0	0	0
(13)	0	0	0
(14)	0	0	0
(15)	15.5 ± 5.8	15.0 ± 6.0	17.5 ± 9.6

NUMBER OF JUVENILES/TEST BOX

1	29.0 ± 19.7	36.5 ± 9.7	26.8 ± 22.0
2	28.3 ± 8.3	30.5 ± 5.8	22.3 ± 3.8
3	34.5 ± 7.8	24.0 ± 8.9 *	13.0 ± 4.2 *
4	44.0 ± 9.2	15.8 ± 15.6 *	15.0 ± 8.5 *
5	100.3 ± 8.4	103.5 ± 8.7	83.8 ± 13.3 *
6	62.8 ± 8.5	63.8 ± 9.0	60.0 ± 6.3
7	107.8 ± 20.0	105.8 ± 10.7	51.3 ± 12.0 *
8	55.3 ± 31.1	61.3 ± 17.5	41.8 ± 18.3
9	33.0 ± 2.6	8.0 ± 3.7 *	2.3 ± 1.7 *
10	129.3 ± 20.3	106.0 ± 12.7 *	85.5 ± 19.1 *
11	78.8 ± 10.2	41.8 ± 17.5 *	60.8 ± 15.6 *
12	62.7 ± 27.2	60.0 ± 21.0	52.7 ± 30.6
(13)	114.7 ± 30.0	181.0 ± 19.3 *	187.8 ± 25.1 *
(14)	1.0 ± 0	0.5 ± 0.6 *	0 *
(15)	1.0 ± 1.2	2.8 ± 1.0	2.0 ± 2.3

WEIGHT OF SURVIVING ADULTS IN % OF INITIAL WEIGHT

1	113.6 ± 7.0	98.9 ± 2.3 *	105.1 ± 8.1 *
2	90.8 ± 4.8	82.9 ± 6.3 *	71.1 ± 0.9 *
3	138.6 ± 3.3	128.7 ± 15.4	116.1 ± 15.5 *
4	94.2 ± 4.6	89.7 ± 9.0	96.8 ± 10.0
5	100.9 ± 1.1	99.9 ± 1.1	96.3 ± 0.9 *
6	145.7 ± 3.6	145.1 ± 3.6	145.2 ± 5.3
7	279.5 ± 9.3	281.0 ± 16.0	271.5 ± 5.4
8	158.8 ± 4.4	157.7 ± 1.7	152.6 ± 7.8
9	112.7 ± 3.8	124.2 ± 5.1	110.7 ± 5.2
10	109.0 ± 2.8	110.4 ± 9.1	109.1 ± 1.9
11	101.8 ± 3.0	95.5 ± 1.3 *	104.8 ± 2.5 *
12	129.9 ± 10.8	126.5 ± 6.0	130.1 ± 17.2
(13)	93.6 ± 6.0	88.7 ± 11.8	91.5 ± 9.5
(14)	81.8 ± 6.1	82.0 ± 3.8	79.8 ± 4.3
(16)	77.7 ± 5.3	76.3 ± 6.6	71.6 ± 3.4

* sign. $p \leq 0.05$, Williams-test

Table E: Coefficient of variation (in %) of juvenile numbers in the control in the first ringtest; controls marked with an (i) were identical for both pesticides; values in brackets are excluded from further comparison because of low juvenile numbers

STUDY NUMBER (first ringtest)	CONTROL BENOMYL	CONTROL PARATHION
1	42.6 (i)	42.6 (i)
2	19.5	11.1 (i)
3	11.1 (i)	19.1 (i)
4	19.1 (i)	20.9
5	71.1	10.6
(6)	(52.9 (i))	(52.9 (i))
7	13.4 (i)	13.4 (i)
8	33.8	18.9

* sign. $p \leq 0.05$, Williams-test

Table F: Coefficient of variation (in %) of juvenile numbers in the control in the second ringtest; controls marked with an (i) were identical for both ringtests; values in brackets are excluded from further comparison because of low juvenile numbers

STUDY NUMBER (second ringtest)	CONTROL BENOMYL	CONTROL PARATHION
1	(67.9) (i)	(67.9) (i)
2	(29.3) (i)	(29.3) (i)
3	22.6 (i)	22.6 (i)
4	20.9 (i)	20.9 (i)
5	12.0	8.4
6	29.2	13.5
7	9.0	18.6
8	39.5	56.2
9	13.4	7.9
10	12.7	15.7
11	11.2	12.9
12	14.0	43.4
13	(1.5)	26.2 (i)
14	26.2 (i)	/
15	/	(1.2)
16	13.5	/

* sign. $p \leq 0.05$; Williams-test

IV.2 Method (BBA-Guideline VI, 2-2, January 1994)

Federal Biological Research Centre for Agriculture and Forestry, Germany

Guidelines for the Testing of Plant Protection Products Within Registration

Part VI

January 1994 2-2

EFFECTS OF PLANT PROTECTION PRODUCTS ON REPRODUCTION AND BODY WEIGHT OF EISENIA FETIDA/EISENIA ANDREI

Published by the Department for Plant Protection Products
and Application Techniques, Braunschweig

---

non-official translation - German text is binding

---

by

C. Kula
Biologische Bundesanstalt
Fachgruppe Biologische Mittelprüfung
Messeweg 11/12
D 38104 Braunschweig

in cooperation with:

C. Bauer
Danneckerstr. 8
D 60594 Frankfurt

P. Dohmen
BASF AG
Landwirtschaftliche Versuchsstation
Postfach 220
D 67117 Limburgerhof

F. Heimbach
Bayer AG
Pflanzenschutzzentrum
Pf.F(CE-OE-Geb. 6620)
D 51368 Leverkusen-Bayerwerk

T. Kampmann
Biologische Bundesanstalt
Fachgruppe Biologische Mittelprüfung
Messeweg 11/12
D 38104 Braunschweig

H. Kula
Zoologisches Institut
TU Braunschweig
Pockelsstr. 10a
D 38092 Braunschweig

F. Riepert
Institut für Ökotoxikologie im
Pflanzenschutz Berlin
Königin-Luise-Str. 19
D 14195 Berlin

J. Römbke
ECT Ökotoxikologie GmbH
Böttgerstr. 2-14
65439 Flörsheim am Main

C.A.M. van Gestel
Department of Ecology and Ecotoxicology
Vrije Universiteit Amsterdam
De Boelelaan 1087
NL 1081 HV Amsterdam

Obtainable from:

Saphir-Verlag
Gutsstr. 15
38551 Ribbesbüttel
Tel. 05374/6576 Fax 05374/6577

CONTENTS

1. Introduction

When testing side effects of plant protection products on earthworms in the laboratory the test on acute toxicity should be followed by a test of side effects on reproduction described in this guideline. The goal of the test is to determine whether a plant protection product has any influence on the reproduction or body weight of earthworms. A similar method is described in an ISO-draft (ISO/DIS 11268-2).

The following endpoints are evaluated in this test:

- number of surviving juveniles after 8 weeks of testing
- change of body weight of adults after 4 weeks relative to the weight at the beginning
- mortality of adults after 4 weeks
- feeding activity and behaviour of test organisms during the test

2. Test design

2.1 Preparation

2.1.1 Test organisms

Adult animals of the earthworm species Eisenia fetida (Savigny 1826) or *Eisenia andrei* (Andre 1963) are used in the test. These species are regarded as subspecies of *Eisenia fetida* by some authors (e.g. BOUCHE, 1972). Only one subspecies should be used in a single test.

Only test animals from a synchronized laboratory culture should be used. A synchronisation of breeding can be achieved by introducing adult animals into a breeding substrate. These animals are removed from the substrate not later than after a period of 4 weeks. When the juveniles which hatch from this substrate have reached the adult stage, they can be used in the test.

The test animals should be at least 2 months old, but not older than one year. Only adult animals having a well developed clitellum are used. The individual weight of the animals should be between 250 and 600 mg. The range of weight of the earthworms should not exceed 200 mg within one experiment.

Before the test the animals are preincubated for 1 to 7 days in the test substrate in a suitable container. The food, which is also used as food source in the test (see chapter "food"), should be given in a sufficient amount. The animals have to be weighed individually before the test. They are dunked in water to clean them from soil particles and then dabbed to remove the excess water.

2.1.2 Test substrate

Artificial soil (OECD, 1984) is used as a test substrate. The substrate consists of the following components (% dry weight):

- 10 % sphagnum peat (air dried, finely ground, with no visible plant remains)
- 20 % kaolin clay (kaolinit content if possible > 30 %)
- 1 % food (see chapter "food")
- 68-69 % quartz sand (depending on the amount of calcium carbonate needed)

  (fine sand, more than 50 % with a particle size of 0.05-0.2 mm)
- calcium carbonate ($CaCO_3$, fine powder, pure) for adjustment of pH to 6.0 ± 0.5)

Before the experiment the test substrate has to be mixed with food. When dried dung is used (see chapter "food") an amount of 5g dried dung/500g dry weight of soil is recommended.

Deionized water has to be used for moistening the test substrate. The water content of the substrate should be 40 to 60 % of the water holding capacity. The water holding capacity should be determined when starting the test with three mixed samples (example for determining water holding capacity see Annex 1). The substrate should be moistened for at least 24 hours before determining water holding capacity. During the test the water holding capacity might change due to the food added. Therefore an adjustment of the water content to the upper margin of 60 % is recommended.

The water content of the substrate at the end of the test should not be less than 10 % of that at the beginning. Moisture content should be evaluated at the end of the test for each test box separately and recorded in % of dry weight (ISO-guideline 114611). A reduction of moisture content in the test boxes should be measured when weighing and should be replenished by deionized water. For this purpose the weight of each test box including soil and earthworms has to be determined at the beginning of the test.

The pH of the soil has to be determined according to standard methods (e.g. ISO-standard 103091)

2.1.3 Test containers

Glass boxes, high-grade steel boxes or one-way-plastic boxes are suitable test containers. When plastic material is used, plastic containers of 18.3 x 13.6 cm and 6 cm height (surface about 200 cm^2) are suitable. The box must be closeable. A transparent cover has to be used so that the earthworms can adapt to the light:dark-cycle. It is necessary to ventilate the test boxes. As adult and juvenile earthworms might escape from the test boxes a lid with some small ventilation holes should be fixed by an adhesive tape.

The height of the moistened substrate in a container with a surface area of 200 cm^2, should amount about to 5 cm. This corresponds to 500 to 600 g dry weight/test box depending on the test substrate.

As a rule of thumb for the number of animals per test box, an amount of 50 to 60 g substrate per test organism should be used. This sums up to 10 animals per test box. If the amount of test substrate is more than 500 to 600 g per test box the animal number has to be adapted.

2.1.4 Food

To achieve sufficient reproduction the earthworms must be fed during the experiment. A suitable food is finely ground cow dung, dried at room temperature. Cow dung can be collected or be obtained from garden suppliers. It is recommended to test each charge of food whether the earthworms feed on it. They should gain weight or produce cocoons in the test compared to an unfed control, because a toxic effect might appear (e.g. by ammonia).

2.2 Test procedure

2.2.1 Extent of experiment

Each experiment consists of water-treated controls and boxes with the test substance. Each concentration including control has to be replicated four times.

2.2.2 Test substance

The formulation of the pesticide which will be used in the field has to be tested in a rate corresponding to the maximum applied dose in the field. For the rate tested it is assumed that the whole applied amount will reach the soil.

For up to 5 applications the highest recommended amount for one application and the fivefold of one application has to be tested.

For more than 5 applications per year, the highest recommended and the x-fold of one application is tested. In justified cases this regulation can be modified with consultation of the regulatory body.

When an active ingredient has to be tested, the range of concentrations has to be chosen according to the procedure for chemicals testing in ISO/DIS 11268-2.

2.2.3 Reference compound (toxic standard)

The toxic standard should be applied in a concentration which causes a reduction in the number of juveniles of at least 30 % after 8 weeks when compared to the control. Suitable substances for this purpose are pesticides with the active ingredients benomyl or carbendazim. The dosage recommended for benomyl or carbendazim should amount to 750g ai/ha. The toxic standard should be tested at least twice a year.

2.2.4 Application

The test substrate is filled into the test boxes following to the preparation. The weighed animals are put on the test substrate. It should be waited until the animals have moved into it. Between the on-putting of the animals and the application of the test substance there should be at least half an hour. The animals which remain on the surface after this time are defined as damaged and have to be substituted.

Before the application the cover of the test box has to be removed and replaced by an inlet protecting the side walls, e.g. a plastic test box without the bottom.

As far as possible the pesticide should be applied in the same manner as in the field. Pesticides which are applied with a spraying equipment should be applied with a suitable laboratory spraying equipment to achieve a distribution as homogenous as possible.

The application should take place at a temperature of 20 °C, the water amount should correspond to 300 to 800 l water/ha. The spraying equipment should be calibrated by re-weighing the amount applied. Granules or treated seeds are to be used as in the field. The test box size should be adjusted, if necessary, to test the dose of practical relevance.

After the application the inlet protecting the side-walls has to be removed and the test boxes to be left open with illumination for half an hour. Then the boxes are closed with a transparent lid which allows an exchange of air but ensures that the test organisms cannot escape.

2.2.5 Test environment

The test boxes are stored in a climatic chamber with 20 ± 2 °C and normal illumination of about 400 to 800 Lux. The light:dark-cycle should be adjusted between 12:12 hours and 16:8 hours light:dark.

2.2.6 Feeding during the experiment

One day after application animals are fed by distributing 5 g dried food on the surface of each test box. The food should be spread out as even as possible. Then the food is moistened by a fine sprayer with about 5-6 ml of demineralized water per test box.

Normally animals have to be fed weekly. If there are test boxes with a low feeding activity the amount of food has to be adjusted. Food on the soil surface should not get mouldy but disappear from the soil surface before the next feeding date.

2.3 Evaluation

The whole experiment takes 8 weeks. The adult earthworms are exposed for 4 weeks and are fed weekly. While feeding, attention has to be paid to dead animals or affected ones crawling on the soil surface. Feeding activity should be reported.

Four weeks after the start of the experiment the surviving adult worms of each test box are sorted from the substrate and weighed. These animals are not placed back in the test boxes. The juveniles which have been produced in the test boxes are fed by mixing 5 g food carefully into the substrate. They stay for another 4 weeks in the substrate and are no longer fed.

After a total of 8 weeks the experiment is stopped. The number of juveniles is determined by an adequate method (see annex 2) and reported for each test box. The water content is determined for each replicate and for the replicate of each concentration the pH has to be determined.

The number of adults and juveniles which have survived has to be reported in tabular form and statistically analysed.

2.4 Criteria of validity

For the untreated control the following criteria have to be fulfilled:

- mortality of adults $\leq$ 10 %
- change of body weight of adults $\leq$ 20 %
- at least 30 juveniles per test box with 10 adults after 8 weeks
- coefficient of variance for the mean number of juveniles $\leq$ 50 %

3. Reporting

The test report should provide the following information:

- test animals used (species, age, breeding substrate, breeding conditions)
- preparation of the test substrate
- food used in the experiment
- test conditions (light intensity, light:dark-cycle, temperature)
- results obtained with the reference substance
- pH, water content and water holding capacity of the artificial soil
- results of calibration of the spraying equipment

- number of surviving juveniles per test box after 8 weeks
- number of adults per test box after 4 weeks
- body weight of adults per test box after 4 weeks relative to the weight at the beginning of the experiment
- amount of food given per test box and description of feeding activity per evaluation date

In addition to the original data the mean and standard deviation for each concentration have to be given. Other observations within the experiment (e.g. behavioural changes) and deviations from the test protocol must be explained.

4. Bibliography

BOUCHE, M.B., 1972: Lombriciens de France. Ecologie et systematique; INRA Publ. 72-2, Institut National des Recherches Agriculturelles, Paris.
KOKTA, C., 1992: A laboratory test on sublethal effects of pesticides on Eisenia fetida. In: Greig-Smith, P.W., Becker, H., Edwards, P.J. & Heimbach, F. (eds.): Ecotoxicology of earthworms. Intercept, Andover, 213-216.
OECD, 1984: Guideline for testing of chemicals no. 207. Earthworm, acute toxicity tests. Adopted 4 April 1984

5. Annex

5.1 Determination of water holding capacity

For the determination of the water holding capacity a defined amount of moistened test substrate is saturated with water. The bottom of a tube with holes in it has to be closed with filter paper. The tube has to be put in a water bath. The water level should first be beneath the rim of the tube, later above this rim. The substrate sample should be left in the water for about 3 hours.

As not all water absorbed from the substrate capillary can be retained, the sample should be placed for a period of two hours on very wet fine quartz sand for draining.

The sample should be weighed, dried to a constant mass at 105 °C and re-weighed.

Calculation of the water capacity (WC):

$$WC \text{ (in \% of dry weight)} = \frac{S - T - D}{D} \times 100$$

S = water saturated substrate + mass of tube + mass of filter paper

T = tare (mass of tube + mass of filter paper)

D = dry mass of substrate

5.2 Methods for the determination of number of juveniles

One possibility is the heat extraction in a water bath. The test boxes are opened and placed in water, so that the surface of the substrate is at the same level as the surface of the water. The water bath should have a temperature of 50 to 60 °C. Within 20 minutes the juveniles will come to the surface and can be counted.

Juveniles may also be searched by hand in the substrate but the substrate has to be checked atleast twice for hidden juveniles.

IV.3 List of Experts for the Earthworm Ringtests

First ringtest 1990/91

P. Dohmen
BASF AG
Landw. Versuchsstation
Postfach 220
67117 Limburgerhof

R. Fischer
Hoechst Roussel Agri Vet Company
c/o NOR-AM Chemical Company
Rt. 2. Country Rd 1324
Pikeville, North Carolina 27863
U.S.A.

F. Heimbach
Bayer AG
Pflanzenschutzzentrum
Pf.F(CE-OE-Geb. 6620
51368 Leverkusen-Bayerwerk

H. Kula
Zoologisches Institut
TU Braunschweig
Spielmannstr. 8
38092 Braunschweig

F. Riepert
Biologische Bundesanstalt für Land- und Forstwirtschaft
Institut für Ökotoxikologie im Pflanzenschutz
Königin-Luise-Str. 19
14195 Berlin

J. Römbke
ECT Ökotoxikologie GmbH
Böttgerstr. 2-14
65439 Flörsheim am Main

U. Thielemann
GEFAÖ Gesellschaft für
angewandte Ökokologie mbH
Ringelsgasse 1-3
69226 Nußloch bei Heidelberg

C.A.M. van Gestel
Department of Ecology and Ecotoxicology
Vrije Universiteit Amsterdam
De Boelelaan 1087
The Netherlands

Second ringtest 1991/92

K. Barrett
Schering Agrochemicals Limited
Chesterford Park Research Station
Saffron Walden
GB-Essex CB10 1 XL

P. Edwards, M. Coulson
Zeneca Agro
Environmental Science Department
Jealotts Hill Research Station
Bracknell
GB-Berkshire RG 12 GEY

P. Dohmen
BASF AG
Landw. Versuchsstation
Postfach 220
67117 Limburgerhof

D. Hänisch
Institut für Pflanzenschutz
Saatgutuntersuchung und Bienenkunde
Nevinghoff 40
48135 Münster

F. Heimbach
Bayer AG
Pflanzenschutzzentrum
Pf.F(CE-OE-Geb. 6620
51368 Leverkusen-Bayerwerk

R. Heusel
Hoechst Schering AgrEvo GmbH
Entwicklung, Umweltforschung Ökologie
Postfach 80 03 20
65926 Frankfurt/Main 80

I. Kraffcyk-Mansouri
Urania Agrochem GmbH
Versuchsstation Christinenthal
Loofterstr. 9
Christinenthal

B. Lang
Biochem GmbH Karlruhe
Labor Cunnersdorf
Am Wieseneck 7
04451 Cunnersdorf

J. Notenboom
National Institute of Public Health
and Environmental Protection
Antonie van Leuwenhoeklaan 9
3720 BA Bilthoven
The Netherlands

R. Petto
Institut für Biologische Analytik und
Consulting
IBACON GmbH
Industriestr. 1
64380 Roßdorf

F. Riepert
Biologische Bundesanstalt für Land- und
Forstwirtschaft
Institut für Ökotoxikologie im
Pflanzenschutz
Königin-Luise-Str. 19
14195 Berlin

J. Römbke
ECT Ökotoxikologie GmbH
Böttgerstr. 2-14
65439 Flörsheim am Main

S. Thun
EBRC
Feodor-Lynen-Str. 23
30625 Hannover

Dr. C.A.M. van Gestel
Vrije Universiteit Amsterdam
Faculteit Biologie de Boelelaan
NL-1081 HV Amsterdam
The Netherlands

Danksagung

Allen, die im Verlaufe der Methodenentwicklung sowohl experimentell als auch auf verschiedenen Besprechungen mit ihren Diskussionsbeiträgen entscheidend mitgeholfen haben, das zur Methodenentwicklung nötige Hintergrundwissen zusammenzutragen, wollen die Autoren hiermit ganz herzlich danken. Besonderer Dank gebührt dabei den technischen Assistentinnen Frau Silvia Baas und Frau Carola Behrend.