Progress Report 1994 of:

SECOFASE

Third Technical Report

Development, improvement and standardization of test systems for assessing sublethal effects of chemicals on fauna in the soil ecosystem

EU Environmental Research Programme Contract nos. EV5V-CT92-0218 and ERB-CIPD-CT93-0059 (PECO)

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Data Sheet

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Abstract: The results of the second year of the EU R&D project SECOFASE is presented. Test results by the exposure of soil invertebrates with copper, dimethoate and LAS are given. Draft test protocols are presented for springtails, mites, earthworms, enchytraeids, rove beetles, nematodes, centipedes, isopods and millipedes.

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1 Summary report of the project

1.1 General Objectives

The SECOFASE group develops regulatory procedures for evaluation of the environmental risks of chemical substances in terrestrial ecosystems. It also develops a new fundamental approach to the creation of test systems. The work will be directly applicable in the area of soil protection, and of more general benefit in elaborating basic rules for ecotoxicological test method development.

This report presents a sound basis for test protocols for international standardization within regulatory testing frameworks.

1.2 Specific objectives for the reporting period

The following objectives have been dealt with in the different subprojects:

- Establishment of laboratory cultures
- Development of breeding methods for test animals
- Refinement and standardization of test methods
- Drafting test protocols
- Elaboration of different endpoints for sublethal toxicity
- Elaboration of biotic and abiotic test conditions (stage, homogeneity and food selection of test animals, soil type and pretreatment, pH, humidity, temperature, aspects of the chemical treatment), and statistical evaluation
- Intercalibration of the analytical determination of metals in an artificial test soil
- Production of life-history data for all test organisms and development of a common mathematical life-history model (subproject 5)
- Performance of competition studies of two-species tests (subprojects 1, and 5)
- Development and improvement of semifield tests (subproject 2)
- Development of digital image processing tools for observation, enumeration and measurement of the size of individuals (subprojects 1, 7 and 8)

1.3 Main results

In Annex 1 to each detailed report, test system standardization forms for each project are shown. These forms contain central information about each test procedure and have been developed to ensure internal standardization in the SECOFASE project. In Annex 2 to each detailed report, first drafts of test protocols that may be suitable for international review are presented. Overall 14 protocols are being developed. In terms of ecological diversity, taxonomic groups, and range of chemicals tested, this represents the largest body of information to date produced by any international collaborative group within the soil environment.

Uniquely, the work is based on sound ecological principles (life-history theory). This enhances the regulators' ability to interpret the consequences of test results at higher biological organization levels, e.g. the population level.

The ten subprojects have established culturing or maintenance procedures for their organisms and made progress in method development. All participants have already contributed life-history data to participant no. 5 for mathematical analysis and identification of fitness trends in populations subject to pollutant stress.

All groups have participated in an internal review for the purposes of uniformity and the complicance with good laboratory practice (GLP). This progress in collaboration has exceeded our initial objectives because it incorporates all the test organisms despite the many challenges initially presented in terms of culturing and handling. In particular the methods on oribatids should be highlighted because appropriate culturing methods have not previously been available for these organisms. This has been one of the major constraints in the development of comprehensive test batteries for soil organisms.

1.3.1 New subprojects

The new participants from Central and Eastern Europe have all contributed data despite a late start in April, 1994. Of particular note is that the new participants have brought expertise on important organisms into the SECOFASE programme. Most of these test organisms are relatively long-lived and difficult to handle. However, the projects have generated very useful data, and important new life-history information. See project organization, Figure 1.

1.3.2 Intercalibration

Of particular importance is the collaborative exercises which are taking place within the SECOFASE project. All the laboratories undertaking elemental analysis have successfully completed an intercalibration procedure for the determination of copper, zinc and cadmium in their test soils. In the ring test the participants analysed copper, zinc and cadmium in samples prepared by participant no. 4. Though different methods were used, most results were in the same range with a deviation equal to or less than 10% of the added amount of all elements. From the more detailed analysis from participant no. 4 it can be concluded that the water soluble amount does not change during time, and that the concentration of exchangeable metals decreases in the short term, to stabilize after two weeks.

The residue analysis of dimethoate referred to in the Second Technical Report of SECOFASE (Wiles et al., 1994) is now published in Heimbach et al., 1994. The results of this work and further investigations have provided detailed information about the chemical half-life of dimethoate in the different soils used by the SECOFASE participants.

1.3.3 Life history analysis

Progress has been made on the life-history analysis for different species used in the project. Both experimentally obtained date and data derived from literature were collected by each participant to be used for the analysis. At present an extensive data bank exists which will be used to estimate fitness measures for each species and to determine the relative importance of various life stages for the population dynamics of each species. Life-cycle based models are being developed for several organisms and preliminary results indicate that each species has certain specific important stages, which could not be discovered using ordinary life-cycle experiments. The results imply that the impact of contaminants on organisms should be evaluated from a life-history perspective.

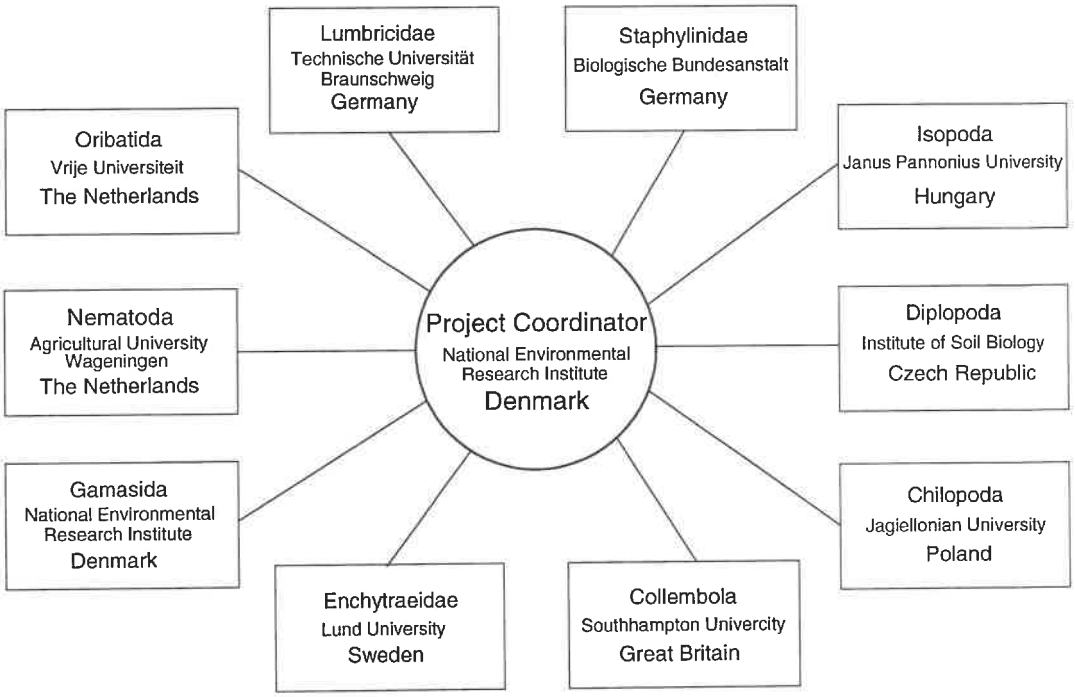

Fig. 1.1. Project organization.

1.3.4 Publishing strategy

During the project two technical reports have been produced (Løkke and van Gestel, 1993; Wiles et al., 1994). Besides the present report the final report will be produced in 1995/1996. The SECOFASE project was presented on 12th April at the EU Session of the Fourth SETAC-Europe Conference in Brussels. A list of publications is included in this part.

The project group aims to publish the main results in high quality international scientific journals with a peer review procedure. Until now 12 papers have been published or accepted as shown in the following list. The participants of SECOFASE continue to present their results on international conferences and workshops.

1.3.5 Co-operation

The SECOFASE project has direct co-operation with the following projects or research programmes:

- BIOPRINT (Biochemical Fingerprint Techniques as Versatile Tools for the Risk Assessment of Chemicals in Terrestrial Invertebrates), EU contract EV5V-CT94-0406
- Danish Center for Ecotoxicologcial Research, the Danish Environmental Research Programme
- ISA (Integrated Soil Assessment), a national Swedish research programme

1.3.6 Acknowledgement

The funding for this work was provided by the EU Programme Environment 1990-1994 (contract no. ERB-EV5V-CT92-0218, and the PECO Programme contract no. ERB-CIPD-CT93-0059) and the Swedish National Research Council.

1.4 Objectives for the next period

- Finalization of culturing methods
- Analyses by participant no. 8 of test soils spiked with copper by each participant
- Complete runs of the test systems for the common test chemicals dimethoate, LAS and copper
- Finalization of method development and standardization
- Finalization of test system protocols for detecting sublethal effects. The PECO partners may only produce preliminary drafts due to their short project time
- Finalization of life-history modelling of data for all test organisms
- Publication of results

1.5 Publications

Augustsson, A. & Rundgren, S. (In press): The enchytraeid worm *Cognettia sphagnetorum* in a metal polluted soil: an outline of a research area. Newsletter on Enchytraeidae.

Hedlund, K. & Augustsson, A. (In press): Effects of enchytraeid grazing on fungal growth and respiration. Soil Biology and Biochemistry.

Heimbach, U., Kula, H., Larink, O., Metge, K. & Siebers, J. (1994): Biologische Wirkungen und Rückstände von Dimethoat in verschiedenen Labortests mit Laufkäferlarven (*Poecilus cupreus*) und Regenwürmern (*Eisenia fetida*). Mitt. Biol. Bundesanst. Land- Forstwirtsch. Berlin-Dahlem, Heft 301. p. 494.

Kammenga, J.E., Van Gestel, C.A.M. & Bakker, J. (1994): Patterns of sensitivity to cadmium and pentachlorophenol among nematode species from different taxonomic and ecological groups. Arch. Environ. Contam. Toxicol. 27:88-94.

Krogh, P.H. (1994): Microarthropods as Bioindicators. A study of disturbed populations. PhD thesis. National Environmental Research Institute, Silkeborg, Denmark 96 pp.

Krogh, P.H. (1995a): Does a heterogeneous distribution of food or pesticide affect the outcome of toxicity tests with Collembola? Ecotoxicology & Environmental Safety 30, 158-163.

Krogh, P.H. (1995b): Effects of pesticides on the reproduction of *Hypoaspis aculeifer* (Gamasida: Laelapidae) in the laboratory. Acta Zoological Fennica. 196, 333-337.

Kula, H. (1994): Auswirkungen von Pflanzenschutzmitteln auf Regenwürmer (Oligochaeta: Lumbricidae) - Zur Problematik der Bewertung letaler und subletaler Effekte in Labor- und Freilandversuchen. PhD.-Thesis, TU Braunschweig. 151 pp.

Kula, H. & Larink, O. (In press): Development and standardization of test methods for the prediction of sublethal effects of chemicals on earthworms. Proc. 5th International Symposium on Earthworm Ecology, Columbus (Ohio), 5.-9.7.1994, in: Soil Biology and Biochemistry (Special Issue).

Metge, K. & Heimbach, U. (1994): Effects of dimethoate on the staphylinid beetle *Philonthus cognatus* STEPH. in the laboratory. 5th European Congress of Entomology, University of York, UK; Abstracts: 287.

Metge, K. & Heimbach, U. (1994): Entwicklung eines Zuchtverfahrens für den Staphyliniden *Philonthus cognatus* STEPH..- Mitt. Biol. Bundesanst. Land-Forstwirtsch.. Berlin-Dahlem, Heft 301: 512.

Sjögren, M., Augustsson, A. & Rundgren, S. (In press): Dispersal and fragmentation of the enchytraeid *Cognettia sphagnetorum* in metal polluted soil. Pedobiologia.

1.6 Other communications

Løkke, H. & van Gestel, C.A.M. (eds.) (1993): Manual of SECOFASE, First Technical Report. 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, 18-19 January, 1993. National Environmental Research Institute, Denmark. 41 pp.

Løkke, H. (1994): Development, improvement and standardization of a test system for assessing sublethal effects of chemicals on fauna in soil ecosystems (SECOFASE). EC Platform Session. Fourth SETAC-Europe Congress, 11-14 April 1994, Brussels, Belgium.

Wiles, J.A., Kammenga, J.E. & Løkke, H. (eds.) (1994): Progress Report 1993 of SECOFASE, Second Technical Report. 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 Braunschweig, Germany, 6-7 December, 1993. National Environmental Research Institute, Denmark. 95 pp.

1.7 Intercalibration of analytical methods

1.7.1 Standardization of artificial soil (OECD): Influence of peat treatment

Artificial soil was prepared by participant 4 as prescribed by OECD (1984) and ISO (1994), using 10% sphagnum peat, 20% kaolin clay, and 70% quartz sand. To determine to what extent the preparation of the peat could influence the properties of the artificial soil (OECD), different methods of peat pretreatment were used: peat was air dried or dried in a stove at 40 °C for 16-24 hours, and sieved. Sieve fractions of < 1 mm, < 2 mm or $1 < x < 2$ mm were used. So, a total of six different peat treatments were tested. Artificial soil (OECD) prepared with these different peat fractions was adjusted to a pH of about 6.0 by adding $CaCO_3$: when oven-dried peat was used, 6.5 g $CaCO_3$ was added per kg, while 7.0 g/kg was added to soil prepared with air-dried peat. To determine differences between the resulting soils, CEC (cation exchange capacity) was determined. This parameter is indicative for the metal binding capacity of the soil. In addition, water holding capacity and pH of the soils were measured. Table 1.1 shows the results.

Table 1.1: Water holding capacity (WHC), cation exchange capacity (CEC) and pH (1M KCl) of artificial soils (OECD) prepared by participant 4, using different peat samples.

Peat pretreatment pH-KCl	Sieve fraction (mm)	WHC (% of dry weight)	CEC (mmol/kg)
air dried 5.84-6.01	< 1	106.3	183
	1 < x < 2	119.4	159
5.84-5.90	< 2	121.2	157
5.89-5.85			
oven dried 5.76-5.94	< 1	90.4	214
	1 < x < 2	133.7	126
5.53-5.79	< 2	133.4	129
5.76-5.81			

From this table it can be concluded that 1 mm sieved peat has the lowest moisture retention capacity but the highest CEC. For the other two peat fractions tested, differences are small. There is however, a difference between the soils prepared with air dried and oven dried peat, which cannot be explained. Differences in soil pH are small; the lower pH of the soils prepared with oven-dried peat can be explained from the lower amounts of $CaCO_3$ used (6.5 g/kg compared to 7.0 g/kg in the soils prepared with air-dried peat).

1.7.2 Ring test on metal analyses in artificial soil (OECD)

To enable a comparison of the copper analyses carried out by each participant, a small ring test was organised. Participant 4 (Vrije Universiteit) prepared an artificial soil (OECD) which was amended with 40.3 mg/kg zinc ($ZnCl_2$), 30.0 mg/kg cadmium ($CdCl_2$,2½H_2O) and 125.0 mg/kg copper ($CuCl_2$,2H_2O) on January 19, 1994. The soil was moistened up to c. 50% of water holding capacity, intensively homogenised, and samples were distributed to all SECOFASE participants in March, 1994.

At Vrije Universiteit (participant 4), soils were analysed three times: immediately after treatment, two weeks later and after 6 weeks. Besides total metal concentrations, water soluble and 0.01 M $CaCl_2$ exchangeable metal concentrations were determined as well. For the latter determinations, soil samples were shaken for two hours at a solution to soil ratio of 10:1, and the aqueous solutions were analysed after filtration over a filter paper.

Soil samples were analysed by all participants except for participant 7. Table 1.2 summarizes all results.

In the analysis 2 and 3 of participant 4, a calcareous loam was included as an internal standard. This soil should contain cadmium, copper and zinc concentrations of 0.30, 31.2 and 70.0 mg/kg, respectively. Upon analysis, it was found to contain 25.3 and 29.1 mg Cu/kg and 62.7 mg Zn/kg (only 2nd analysis); cadmium content was below the limit of detection (~0.5 mg/kg) at both analyses.

From Table 1.2 it can be concluded that most results are within the same range, greatest variation being about 10%. Destruction with only 2M HNO_3, as applied by participant no. 10, does not give complete liberation of zinc from the soil, as is reflected by the somewhat lower concentration found.

HCl/HNO_3 or $HNO_3/HClO_4$ destruction, as applied by participants no. 2, 3, 6 and 8, results in similar concentrations, and there seems to be no difference in AAS and ICP analysis on such destruates (participant no. 8). Also X-ray emmission spectrometry, applied by participant no. 9, gives satisfying results.

Table 1.2: Results of the ring test on metal analysis in artificial soil (OECD)

Participant no.	Destruction method	Analytical method	Conc. in mg/kg dry soil (average±sd) Cu	Zn	Cd	n
1	HNO_3	flame AAS	115.9±4.2	35.6±2.0	-	5
2+3	HCl/HNO_3	AAS	132.8±9.5	37.4±2.97	31.6±2.27	10
4a	$HNO_3/HCl/H_20$	flame-AAS	122.4±4.4	34.0±1.47	31.4±1.05	10
4b	(4:1:1)		110.3±6.9	36.6±2.96	27.3±1.69	8
5a	HNO_3/HCl	flame-AAS	101.1±1.5	32.0±2.1	25.9±0.3	4
5b			135.0±2.0	42.7±2.8	34.5±0.4	4
6	$HNO_3/HClO_4$ (4:1)	flame AAS	116.1	33.2	27.9	-
8	$HNO_3/HClO_4$ (4:1)	AAS	115.7±5.1	35.2±2.11	28.9±1.96	10
		ICP	112.2±4.8	35.0±1.60	29.0±1.13	10
9	X-ray emmision spectrometer		135±5	30±3	24±2	10
10	2M HNO_3	ICP	100	-	-	-

a: Analysis on air dried soil. b: Analysis on soil dried at 105 °C, respectively.

Participant 5 analysed both air-dried and oven-dried soil; this resulted in different values, the difference being explained by differences in soil moisture content. The analysis on oven-dried soil is in agreement with the data of the other participants.

Table 1.3: Water soluble and $CaCl_2$ exchangeable concentrations of cadmium, copper and zinc in artificial soil (OECD), as measured by participant no. 4.

Analysis no.	Time after treatment (weeks)	Fraction	Concentration in mg/kg dry soil (average±sd) copper	zinc	cadmium	n
1	0	H_2O	0.21±0.09	0.40±0.00	n.d.	11
2	2		0.48±0.19	0.59±0.11	0.09±0.02	10
3	6		0.23±0.11	0.36±0.41	0.06±0.02	7
1	0	$CaCl_2$	3.28±0.28	17.96±0.53	18.73±0.60	12
2	2		0.14±0.12	3.89±0.28	4.52±1.41	10
3	6		0.13±0.05	3.08±0.17	5.29±0.39	7

Table 1.3 shows the water soluble and $CaCl_2$-exchangeable metal concentrations in the artificial soil (OECD), as resulting from the three analyses carried out by participant no. 4.

From Table 1.3, it can be concluded that water soluble concentrations remain fairly constant in time, whereas the exchangeable metal concentrations have significantly decreased within the first two weeks after treatment. After that time, exchangeable metal concentrations seem to have stabilized.

2 Sublethal toxicity test with the gamasid mite *Hypoaspis aculeifer* Canestrini (Acari: Gamasida) preying on the collembolan *Folsomia fimetaria* Linné (Collembola: Isotomidae)

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2.1 Objectives for the reporting period

- To perform single species reproduction experiments with *Folsomia fimetaria* Linné (Collembola: Isotomidae) exposed to dimethoate at different temperatures, with different soil types, and soil organic matter contents
- To perform two-species experiments and test the reproducibility of results- when exposed to dimethoate
- To implement routine use of a digital image processing system to enumerate and measure population size and structure

2.2 Objectives for the next period

- To perform tests with the predator and prey as single and two species populations under the influence of Cu and LAS
- To standardise the test procedures
- To initiate the development of methods to discriminate mites and Collembola by digital image processing for the enumeration and size determination of mixed populations

2.3 Main results obtained

2.3.1 Methodology

A methodology to record the growth of isolated individuals using image processing has been developed. Enumeration and size determination of collembolan populations has been undertaken by grabbing video images from a black plaster-charcoal surface mounted on a moving stage. The moving stage makes it possible to take many pictures thus enabling a large surface to be covered and reducing the chance that the collembolans lie close together. If they lie too close to each other the system cannot distinguish single individuals.
A prototype of a predator-prey test system in soil using 10 female and 5 male predatory mites and 100 collembolan prey individuals has been tested.

2.3.2 Results

At the three levels of biological organization studied in the project the following results have been achieved. Some of the main results are summarized in Table 2.1:

Individual

The life-histories of both species used in the laboratory have been described. This information will be used in life-history analysis of fitness within the SECOFASE project. Generally growth has been demonstrated to be most accurately described by a sigmoidal model in contrast to the commonly used Bertalanffy model. No significant effects of copper on growth and reproduction of mites and Collembola have been registered in LUFA 2.2 soil.

Table 2.1: Summary of experiments with single species and predator/prey systems. Figures are in mg compound per kg d.w. soil. Unless otherwise stated, experiments were performed with dimethoate at 20 °C. The Kalø soil is a Danish agricultural sandy loam soil.

Conditions	Parameter	Weeks	Species	LC_{10} EC_{10}	95% C.I.	LC_{50} EC_{50}	95% C.I.
Dimethoate - Temperature experiments, LUFA soil							
10°C	Survival	6	*F. fimetaria*	1.1	1.1-1.2	1.7	1.6-1.9
15°C	Survival	4	*F. fimetaria*	0.6	0.5-0.6	1.0	0.8-1.2
20°C	Survival	3	*F. fimetaria*	1.0	0.8-1.3	.	.
10°C	Reproduction	6	*F. fimetaria*	1.3	1.1-2.6	2.0	1.4-3.0
15°C	Reproduction	4	*F. fimetaria*	0.3	0.07-0.8	1.0	0.7-1.2
20°C	Reproduction	3	*F. fimetaria*	0.8	0.1-1.0	.	.
Dimethoate - Three week predator-prey system, Kalø soil, 20 °C							
Yeast on top	Survival		*F. candida*	0.19	0.032-0.79	0.6	0.23-0.82
Yeast on top	Reproduction		*F. candida*	0.077	0.054-0.16	0.37	0.27-0.71
Yeast on top	Reproduction		*H. aculeifer*	0.37	.	0.86	.
Yeast in lower soil	Survival		*F. candida*	0.14	0.066-0.30	0.63	0.34-0.90
Yeast in lower soil	Reproduction		*F. candida*	0.31	0.010-0.80	0.88	.
Yeast in lower soil	Survival		*H. aculeifer*	>1	.	>1	.
Yeast in lower soil	Reproduction		*H. aculeifer*	0.77	0.35-1.00	>1	.
Yeast on top	Survival		*F. fimetaria*	0.034	0.028-0.044	0.17	0.14-0.25
Yeast on top	Reproduction		*F. fimetaria*	0.057	0.026-0.23	0.28	0.15-0.78
Yeast on top	Survival		*H. aculeifer*	>1	.	>1	.
Yeast on top	Reproduction		*H. aculeifer*	0.67	.	>1	.
Yeast in lower soil	Survival		*F. fimetaria*	0.17	0.0059-0.52	0.55	0.13-0.76
Yeast in lower soil	Reproduction		*F. fimetaria*	0.39	0.37-0.42	0.77	0.68-0.90
Yeast in lower soil	Survival		*H. aculeifer*	>1	.	>1	.
Yeast in lower soil	Reproduction		*H. aculeifer*	>1	.	>1	.
Copper, 20°C							
Soil exposure	Survival	4	*F. fimetaria*	>480	.	>480	.
(Kalø soil)	Reproduction	4	*F. fimetaria*	22.3	5.8-106	156	-65-408
-	Adult weight	4	*F. fimetaria*	13.4	9.3-26.8	169	.
-	Juv. weight	4	*F. fimetaria*	72.2	-0.7-226	302	-18-414
Food exposure	Survival	3	*F. fimetaria*	>5000	.	>5000	.
(LUFA soil)	Reproduction	3	*F. fimetaria*	>5000	.	>5000	.
-	Adult weight	3	*F. fimetaria*	>5000	.	>5000	.
-	Juv. weight	3	*F. fimetaria*	>5000	.	>5000	.

Populations
The different species have been tested in soil from a local agricultural field study site (Krogh (1995a), Krogh (1995b)), and in a European standard soil (LUFA 2.2).

Consequences of standardization have been studied in collaboration with the University of Jyväskylä. The influence of temperature and soil organic matter on the toxicity of dimethoate was studied on the collembolan *F. fimetaria*. In this collaboration, analysis of chemical fate has been included in all soil types and at all temperatures. This study will be published separately.

Collaboration with the Institute of Terrestrial Ecology, ITE, Monks Wood, UK, includes the linking of biomarkers with population parameters. ICP-MS elemental scans have been used to detect disturbances of physiological homeostasis by determination of changes in the main cations and anions and measurement of the copper body burden. Concentrations of copper in *F. fimetaria* increased with copper concentration in soil and resulted in decreased reproduction.

Predator/prey
The design of the predator/prey system has been investigated further. A 60 g soil mass was found suitable because predation was moderate allowing prey to reproduce within the short experimental period of three weeks. EC_{50} values for reproduction for *F. fimetaria* and *Hypoaspis aculeifer* Canestrini (Acari: Gamasida) together were identical to the single species systems, thus the presence of a predator did not affect the toxicity to Collembola.

However, when food was spread as granules in the lower half of the test soil the EC_{50} values for reproduction caused by dimethoate increased about three times. Similar results were obtained with *Folsomia candida* Willem (Collembola, Isotomidae), except that adult survival was not affected by food present in the lower soil layer (possibly due to the large size of this species).

In collaboration with a guest researcher (T. Hamers from RIVM, Bilthoven, The Netherlands) the predator/prey system has been investigated in detail to clarify the key mechanisms governing population dynamics in this system. Basic properties like prey preference, juvenile and adult sensitivity, and temporal changes in susceptibility have been studied.

2.3.3 Discussion
The toxicity studies at the individual level were performed to support the investigations at the two-species level. A joint comparison of results obtained at the individual-, population - and two-species population levels was carried out to evaluate the 'mechanistic link' paradigm which implies that effects at one level of biological organization are linked to those at the next level. This exercise is relevant when arguing for a particular choice of endpoints. It appears that in terms of EC_{10}-values for dimethoate the endpoints of growth, survival and reproduction for individuals are similar to those found for the two-species system. Only species specific sensitivity gives rise to major differences in EC_{10}-values.

ANNEX 2.1 - Test System Standardization Form 1

Sublethal toxicity test with the gamasid mite *Hypoaspis aculeifer* Canestrini (Acari: Gamasida) preying on the collembolan *Folsomia fimetaria* Linné (Collembola: Isotomidae)

General data about the test organism at the start of the experiment

Test organism

Taxonomic group:	Gamasid mite (Acari: Gamasida) and springtail (Collembola: Isotomidae)
Species:	*Hypoaspis aculeifer* (Canestrini) and *F. fimetaria* L.
Life stage:	adult
Sex:	males and females sexually reproducing
Age/weight/size:	about 1 mm
Genetical composition:	not known
Background:	laboratory culture

Type of surroundings and abient climate

Surroundings	
Type:	climate chamber
Ambient climate	
Air temperature:	20 ±1 °C
Relative humidity:	>99% in test container (moist soil).
Light wave length:	no specification needed; normal TL light is suitable
Light intensity:	400-800 lux
Light regime:	light:dark cycle 12:12 h

Type of test system and test conditions

Type:	test container
Material:	plastic ring (inner diameter 6 cm; height 5.5 cm), with a gauze (1 mm mesh) on the bottom and lids at top and bottom in addition.
Openness:	closed. Opened after 14 days.
Spec./test system:	two species test system
Ind./test system:	10 female and 5 male mite predators; 100 adult prey individuals.

Type of habitat

Soil/litter:	artificial soil (OECD), made up with sieved moist peat or LUFA 2.2 soil
Medium:	artificial culture medium
'Habitat characteristics'	
Soil charact.:	artificial soil (OECD) consists of sphagnum peat (10%), kaolin clay (20%), sand (70%), and some $CaCO_3$
Hydrol. charact.:	moisture content 30-40% of water holding capacity
Food supply:	Collembola for the predator and bakers yeast for the prey
Feeding rate:	feeding occurs throughout the incubation period
Type of food:	Collembola cultured in the laboratory for the mites; commercially available bakers yeast for the prey (Collembola)

Exposure characteristics

Exposure route:	via soil
Composition of test soil:	(artificial or natural) soil with deionized water, test substance and auxiliary agent (if used), all constituents well homogenized
Composition of control soil:	a) (artificial or natural) soil and deionized water b) (artificial or natural soil), deionized water and auxiliary agent

Duration of the experiment

Acclimat. time:	prey are allowed to spread in soil for more than 1 hour
Exposure time:	depends on chemical and endpoint. Exposure occurs potentially throughout incubation time
Recovery period:	not relevant

Exposure contrations

Replicates:	4
Concentrations:	6 incl. control
Series:	1
Ref. chemical:	dimethoate

Exposure responses

Effect parameter:	reproduction (number of offspring produced)
Other parameters:	survival of adults
Measurement of variables:	moisture content and pH of the soil at start and end of the test
Toxic endpoint:	EC_{10}, EC_{50} and NOEC for reproduction; LC_{10} and LC_{50} for survival of adults

Unit: mg test substance/ kg d.w. test soil

Working methods:	use modified MacFadyen high gradient extractor for extraction of mites and Collembola from soil at the end of test
Analytical methods:	not relevant yet

Miscellaneous test information

Practicability:	test containers can easily be constructed; extraction apparatus may not belong to the standard equipment of a commercial laboratory. Animals can be obtained from permanent laboratory cultures
Cost-effectiveness:	One test requires between one and two weeks including culturing and final enumeration of animals
Sensitivity:	work in progress
Reproducibility:	mite survival and reproduction seems to be stable while collembolan survival and reproduction seems unstable at present experimental design
Validity of the test:	work in progress
Standardization:	work in progress
Ecological realism:	*Hypoaspis aculeifer* and *F. fimetaria* are common in European soils
Broad chemical responsiveness:	no restrictions are given with respect to the chemical substances that may be evaluated by this test

ANNEX 2.2 - Draft test protocol 1

Sublethal toxicity test with the gamasid mite *Hypoaspis aculeifer* Canestrini (Acari: Gamasida) preying on the collembolan *Folsomia fimetaria* Linné (Collembola: Isotomidae).

1 Scope

This test guideline describes the testing of chemicals on the survival, growth, and reproduction of the predaceous mite *Hypoaspis aculeifer* Canestrini 1883 (Acari: Gamasida) feeding on a limited number of the prey species *Folsomia fimetaria* Linné 1758 (Collembola: Isotomidae).

2 Definitions

2.1 Survival
Mean number of adults surviving until the end of the test period (3 weeks).

2.2 Reproduction
Mean numbers of offspring produced during the test period (3 weeks).

2.3 EC_{50} and EC_{10}
The concentrations where a quantified paramameter has been decreased to 50% and 10% respectively of a control level.

2.4 NOEC (No Observed Effect Concentration):
The highest tested concentration of a substance at which no lethal or other effect is observed, expressed as mass of test substance per dry mass of the test substrate.

3 Principle

Ten female and five male *H. aculeifer* and 100 *F. fimetaria* are added to contaminated test soil. As an alternative *Folsomia candida* (Collembola: Isotomidae) may be used. After 3 weeks the three test parameters survival, growth, and reproduction are quantified.

4 Regents

4.1 Culturing of test animals:
The test animals can be collected in the field and determined by the taxo nomic key of Karg (1993) and Fjellberg (1976).

Mite cultures are synchronized by adding adult mites to a new plaster-charcoal substrate on day 1 and removing the mites on day 4 leaving the produced eggs on the plaster. Juvenile mites 0-3 days old are fed juvenile prey, on day 8 and on day 27 they are used in a test.

Age synchronized Collembola are bred by allowing adult *F. fimetaria* to oviposit on new plaster/charcoal surface on day 1. On day 10 the eggs are collected on filter paper slides impregnated with plaster/charchoal and are hatching from day 10 to 13. At the age of 16-19 days they are used as food for the predator.

4.2 Test substrate

Artificial soil (OECD guideline 207) or one of the LUFA-Speyer soils may be used as the test substrate. The amount of moist soil used per test container should be 60 g. The soil is prepared by wetting the soil substrate with deionized water to reach a water content of approximately 20% of the total water holding capacity (this should be done 1 week before starting the experiment). The total amount of water added should be adjusted to give a porous soil texture making it possible for the animals to live within interstitial cavities.

4.3 Food

Fifteen mg of dried bakers yeast for the collembolan prey are added at the beginning of the test and after 14 days. One hundred collembolan prey 16-19 days old are added at the beginning of the test.

5 Apparatus

5.1 Soil test containers

The containers consist of a cylinder (5.5 cm high, diameter 6 cm) covered with a 1 mm mesh at the bottom. The lid for the bottom should be flush with the mesh. During incubation at 20 ±1 °C the test containers are closed at the top and the bottom with lids. The weight of the test container is determined at the beginning of the test. After 2 weeks they are weighed, water loss is compensated for, and fresh bakers yeast is added.

5.2 Extraction

After termination of the test the lids are removed and the cylinders are placed in a high gradient extractor. The animals are collected either in saturated aqueous benzoic acid (alternatively sodium benzoic acid ajusted to pH = 5) with a few drops of detergent added or at 3 °C on a smooth plaster/charcoal surface. Heat development during extraction may be increased every 12 hours beginning with 25 °C and ending with 40 °C. Extraction onto a plaster/charcoal surface makes chemical analysis and enumeration using digital image processing possible.

6 Test environment

6.1 Climate chamber

Climate chamber controlable to a temperature of 20 ± 1 °C.

6.2 Light source

Light source capable of delivering a constant illumination of approximately 400 lux at a controlled light:dark cycle of 12:12 h.

7 Procedure

7.1 Preliminary test

If it is necessary to determine the range of concentrations for use in the final test, perform a preliminary 1 week acute test for four concentrations of the test substance and a control (for example 0, 1, 10, 100 and 1000 mg/kg, the concentrations being expressed in mg of test substance/kg d.w. soil substrate (4.2.)) on 10 female and 5 male adult mites and 100 prey collembolans per concentration and per test container.

7.2 Final test

7.2.1 Introduction of the test substances:

Use either method a), b) or c), as appropriate.

a) Water soluble substances

Immediately before starting the test, dissolve the quantity of the test substance required for the replicates of one concentration in water (or that portion that is necessary to wet the soil substrate in order to meet the requirements of 4.2.) and mix it thoroughly with the test substrate before introducing it into a test container.

b) 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).

Add it to a small portion of quartz sand or dry soil substrate, mix thoroughly and evaporate the solvent by placing it under a fume hood for 1 h. Then add the remainder of the soil substrate (4.2.) and the water in accordance with 4.2. and mix thoroughly.

c) 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 4.2.) or 10 g of the dry soil substrate and the quantity of the test substance required to obtain the desired concentration. Place the mixture, the remainder of the soil substrate (4.2.) and the water in a beaker and mix thoroughly before introducing it into the test container (5.1).

Mix the test substance into the artificial soil before the mites are introduced.

NOTE - ultrasonic dispersion, organic solvents, emulsifiers or dispersants may be used to disperse substances with low aqueous solubility. When such auxiliary substances are used, all test concentrations and an additional control should contain the same minimum of auxiliary substance. To allow solvents to volatilize, the test containers should remain open for a period of one hour.

The concentrations selected to provide the NOEC are based on the results of the preliminary test (7.1).

NOTE - Substances do not need to be tested at concentrations higher than 1000 mg/kg d.w. of the test substrate.

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

Proceed simultaneously with at least four replicates per concentration and a control test without the test substance and if necessary with an additional one (see 7.2.2), placing the containers in the test environment 6.

Prepare such a series of test and control containers for each determination time (7.2.4).

NOTE: It is advisable to prepare an additional 2 containers per concentration and control to determine recovery immediately after the start of the experiment (t=0). The containers should be extracted (5.2) immediately after mites have been introduced.

7.2.2 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 7.2.1) use additional control containers similar to the test containers without the test substance. Treat these containers in the same way as those without the test substance. Include 2 additional containers for the control and each treatment for pH and soil moisture determination at the end of the test.

7.2.3 Addition of the biological material:
Collembolan prey (100 animals) are added to each container. The animals are allowed to disperse for at least 1 h. Hereafter 10 female and 5 male predator mites are added.

7.2.4 Determination
Maintain the water content of the soil substrate in the test containers during the test period by reweighing the test containers periodically and if necessary replenishing lost water. At the end of the test the water content should not differ by more than 10% from the water content at the beginning of the test.

Feed the prey animals after 2 weeks by placing 15 mg bakers yeast (see food; 4.3) on the soil surface of each test and control container.

After 3 weeks, a complete series of four replicate containers of each treatment and control group is placed in the extractor (5.2) and mites and collembolans are extracted to determine the effect on survival and reproduction.

7.3 Reference substance
The organophosphorus insecticide dimethoate is suggested as reference substance. A concentration series from 0 to 1 mg dimethoate per kg soil will

produce a decreasing survial and reproduction. EC_{50} for reproduction should be within the range of 0.6 to 1.0 mg/kg. In addition a slightly stimulating effect on mite reproduction about 0.4 mg dimethoate/kg soil may be detected.

8 Calculation and expression of results

8.1 Calculation

For each concentration, determine the average survival and number of offspring produced in the final test.

Compare means by suitable statistical methods, e.g. Anova with pairwise comparisons using Dunnett's or Student's t-test (LSD) or Williams test (Gulley *et al.*, 1988) and test for significance (a = 0.05) of difference from control(s).

Calculate LC_{10} and LC_{50} for survival of female adult mites and adult collembolans and EC_{10} and EC_{50} for effects on number of juveniles produced by predator and prey, when results show a consistent concentration-effect relationship. The trimmed Spearman-Karber method (Hamilton et al., 1977/1978) may be used for calculation of LC_{50} values. Non-linear parameter estimation may be performed by fitting procedures choosing the most appropiate model or using a linear interpolation method (Norberg-King 1993).

8.2 Expression of results

Indicate, in mg/kg dry weight soil substrate, LC_{10}, LC_{50}, EC_{10}, and EC_{50} and the highest concentration tested without mortality and significant reduction in numbers of offspring (NOEC) and the lowest concentration with effects (LOEC).

9 Validity of the test

The results are considered to be valid if control mite reproduction is in the range of 20 to 100 juveniles per container and female survival is more than 90%.

10 Test report

The test report shall refer to this standard and, in addition to the results expressed as in 7.2, shall provide the following information:

- complete description of the biological material employed (species, source, etc.)
- method of preparation of the test substrate, and any 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 adult female mites and adult collembolans obtained for each container for each concentration and for the control
- number of offspring of both species per test container at the end of the test
- the LC_{10}, LC_{50} for the effect on adult survival after the end of the test
- the EC_{10} and EC_{50} for the effect on the number of juveniles produced after weeks of exposure
- the highest concentration causing no observed effects (NOEC) and LOEC
- water content and pH of the soil at the start and at the end of the test
- all operating details not specified in the standard, and any occurrences liable to have affected the results: some specimen in the test should be preserved for future reference

11 References

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3 Sublethal toxicity test with the rove beetle *Philonthus cognatus* Steph. 1832. (Coleoptera: Staphylinidae)

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3.1 Objectives for the reporting period

- To improve laboratory culturing of *Philonthus cognatus* Steph. (Coleoptera: Staphylinidae)
- To prepare a first proposal of a standard operating procedure
- To develop a laboratory test system for adult staphylinid beetles to detect effects on egg production and hatchability
- To develop a laboratory test system for larval stages to detect effects on development time and hatching weight
- To investigate effects on *P. cognatus* larvae using a semi-field test with dimethoate incorporating residue analysis (GC)

3.2 Objectives for the next period

- To establish a laboratory culture of *Lathrobium fulvipenne* Grav. (Coleoptera: Staphylinidae) and to investigate the biology and ecology of this species
- To check the standard operating procedure for the adult test system
- To finalize the larval test with *P. cognatus* using LAS and two soils
- To carry out a second semi-field test with *P. cognatus* larval stages
- Tests will be carried out with *Aleochara bilineata* Gyll. (Coleoptera: Staphylinidae) to gain experience with handling this species

3.3 Main results obtained

3.3.1 Methods

Culturing the beetles:
The culturing method for *P. cognatus* described in the "Progress Report 1993 of SECOFASE" was modified slightly:

- for egg laying 5 pairs of mature beetles were mated together in one plastic box with smooth and moistened clay granules
- the artificial winter was prolonged to 16 weeks at 5°C to improve the fertility

In future, males will be separated from the egg laying females after the first week to reduce the percentage of cannibalised eggs. Further matings will be permitted at a later stage. The artificial winter could be reduced to 12 weeks but further investigations are necessary.

Adult and larval test system
Using the larval test system, results were obtained for 1 g copper/kg d.w. LUFA 2.2 soil and a concentration series with dimethoate in LUFA 2.2 and artificial soil (OECD). A further test with LAS in both soils is in progress. The larval test system was also adapted for adult beetles. Mature beetles were exposed for one week to dimethoate treated LUFA 2.2 (0.5 mg and 1 mg/kg d.w. soil) after an artificial winter of more than 15 weeks and an acclimatization period of 1 week in artificial summer conditions. Afterwards, pairs were mated in plastic boxes and the level of reproduction was observed.

Semi-field test
The semi-field test for *P. cognatus* larval stages was carried out in 4 replicates with different concentrations of dimethoate mixed into LUFA 2.2 soil as described in the test protocols for laboratory tests with *P. cognatus* (nos. 2.1 and 2.2). Metal boxes (25 x 22 x 14 cm) with gauze covered bottom and a gauze lid were dug 5 cm deep in the soil of a cereal field. The boxes were filled with treated soil. Nine small holes were prepared in the soil in each box and fenced with plastic rings (5 cm in diameter) to acclimatize larvae at one location in the box. The plastic rings reduce cannibalism. One 1 - 2 days old pre-fed larva was added to each plastic ring. The rings were removed 1 day later. During the test, food was offered 3 times a week in each hole. In the first week observations took place every day, then on all feeding days until the end of the test. For the residue analysis, soil was sampled as a surface fraction and a lower fraction on different days during the test period. Surviving beetles were collected and cultured under laboratory conditions.

3.3.2 Results
Culturing the beetles
The culturing method of *P. cognatus* seems to be suitable. Experiences with 3 laboratory generations producing some thousand eggs, and more than 1000 immature beetles exist. The egg mortality was constant during 3 generations: 20 - 24% for the parental generation, 20 - 24% for the F1- generation and 22% for one group of the F2- generation. Within most culturing experiments more than 40% of the eggs developed to immature beetles.

Larval test system
In the larval treatment with 1 g copper/kg d.w. LUFA 2.2 soil no differences compared to the untreated control in larval mortality, larval development, hatching weight and winter mortality of the adults could be observed. Also reproduction tests with the beetles which had been exposed as larvae did not show significant differences ($p < 0.05$; Tukey) in egg production or numbers of hatching larvae.

Experiments with *P. cognatus* larvae in dimethoate treated soil gave LC_{50}-values of 1.52 mg/kg d.w. LUFA 2.2 and 2.28 mg/kg d.w. artificial soil (OECD) (Table 3.1). No differences could be detected in hatching weights of immature beetles. In both soils a higher winter mortality of 30% (artificial soil (OECD)) and 27% (LUFA 2.2) was observed at 2 mg/kg d.w. soil compared to the control (9% in LUFA 2.2 and 7% in artificial soil (OECD)). Reproduction tests with the low numbers of surviv-

ing beetles did not detect significant differences ($p < 0.05$; Tukey) in egg production or numbers of larvae hatched.

Table 3.1: LC_{50} values of dimethoate treated soil on larvae of *P. cognatus*

test system	soil	LC_{50} values with standard deviation (mg/kg d.w.)
LTS	LUFA 2.2 soil	1.52 (0.145)
LTS	artificial soil(OECD)	2.28 (0.195)
SF	LUFA 2.2 soil	1.74 (0.118)

LTS: larval test system; SF: semi-field test of larvae

Initial results for both soils treated with LAS showed no significant differences ($p < 0.05$; Tukey) in development times of larvae or hatching weights of immature beetles between treatments and control. Further investigations are in progress.

Adult test system
Mature beetles exposed for 1 week to dimethoate treated LUFA 2.2 (0.5 mg and 1 mg/kg d.w. soil) showed significantly reduced weekly egg production compared to the control ($p < 0.05$; Wilcoxon).

Semi-field test
The semi-field test using dimethoate treated LUFA 2.2 soil and larvae of *P. cognatus* resulted in an LC_{50}-value of 1.74 mg/kg d.w. soil (Table 3.1). Surviving beetles were collected and cultured in the laboratory for further investigation of winter mortality. Residue analysis is in progress.

3.3.3 Discussion

Culturing the beetles
The culturing method of *P. cognatus* seems to be established. No difficulties arose during the culturing. However, further biological investigations are necessary to interpret the consequences of results obtained from the test systems for population performance. This is especially the case for the different dates at which females died during the reproductive period.

Larval and adult test system
The experiences and results obtained from the laboratory test systems of larvae indicate that long-term effects (complete life cycle) and short-term effects (until adult stage) might be detected with the larval test system (duration of the experiment for long-term effects: 38 weeks). It is possible to shorten the test duration if the larval stage is used for short-term investigations only or effects of chemicals are so severe that long-term effects on reproduction cannot be observed. With a duration of 11 weeks the adult test system is relatively short but long-term effects cannot be detected.

Semi-field test
First experiences with the semi-field test of *P. cognatus* larvae were obtained. The LC_{50}-value of 1.74 mg dimethoate/kg d.w. soil as a first result indicates that the laboratory test system may be a realistic mimic of field conditions. The residue analysis is not completed.

3.3.4 Deviations from the workplan in 1994

- Because of many problems in culturing *Tachyporus hypnorum* L. (Coleoptera, Staphylinidae) under laboratory conditions in 1993 and 1994 and the unexpected high parasitism by braconid wasps in field-collected beetles in 1994 we decided to cease the investigations with this species
- For *Lathrobium fulvipenne* Grav. it has not been possible to establish a laboratory culturing procedure to date

ANNEX 3.1 - Test System Standardization Form 2.1

Sublethal toxicity test with mature beetles of *Philonthus cognatus* Steph. 1832. (Coleoptera: Staphylinidae)

General data about the test organism at the start of the experiment

Test organism

Taxonomic group:	Rove beetle (Staphylinidae: Coleoptera)
Species:	*Philonthus cognatus*, Steph. 1832.
Life stage:	adult
Sex: male and female	
Age/ weight/ size:	> 15 weeks as adult (minimum of 12 weeks under short day conditions with a light dark cycle of 8:16 hours and 5 °C)
Gen. composition:	not known
Background:	the animals are from a laboratory culturing programme

Type of surroundings and ambient climate

Surroundings	
Type:	climatic chamber
Ambient climate	
Air temperature:	15 ± 1 °C
Relative humidity:	85-100%
Light wave length:	no specific needed
Light intensity:	> 400 lux
Light regime:	light:dark cycle 16:8 h

Type of test system and test conditions

Type:	19 cm^3 glass tubes (7 cm high, 2.8 cm in diameter)
Material:	glass
Openess:	closed, open only for feeding
Spec./ test system:	single species test system
Ind./ test system:	1 individual/glass tube

Type of habitat

Soil/ litter:	LUFA 2.2 soil, homogeneously mixed
'Habitat characteristics'	
Soil/ litter charact.:	dried soil is stored at 5 °C.
Hydrol. charact.:	50% WHC
Food supply:	during the complete test
Feeding rate:	continously 3 times a week
Type of food:	deep frozen half *Calliphora* spec. pupae for each animal and feeding date

Exposure characteristics

Exposure route:	via soil
Composition of test soil:	1 week before the test begins the soil is moistened with destilled water up to 20% WHC. 1 day before the adults are put into the soil the chemical with an amount of water, corresponding to 10 - 20% of the WHC, is mixed for 15 minutes into the soil. Afterwards the soil is wetted up to 50% WHC and stored at 5 °C. At the day the test starts soil amounts corresponding to 20 g d.w soil are filled into 19 cm^3 glass tubes.
Composition of control soil:	same procedure without chemical (see above)

Duration of the experiment

Acclimat. time:	1 week at long day conditions
Exposure time:	1 week
Recovery period:	adults are mated in plastic boxes (10.5 x 6 x 2 cm) filled with a mixture of plaster of paris and active coal (5 : 1) and some expanded clay granules.

Exposure concentration

Replicates:	10 females and 10 males per concentration and control
Concentrations:	4 concentrations and 1 control
Series:	1
Ref. chemical:	work in progress

Exposure responses

Effect parameters:	affected animals (legs or mandible trembling, lying on the back, uncoordinated movement, dead), egg production, egg mortality and weight of hatching larvae
Frequency of measurement:	observations after 1, 2, 3, 4, 5 and 7 days during the treatment. Searching for eggs 3 times a week during 6 - 10 weeks after discovery of the first egg.
Measurement of variables:	moisture content and pH of the soil at the start and end of the test
Toxic endpoint:	LOEC, NOEC, egg production, hatching rate
Unit: mg test substance/kg	d.w. test soil
Working methods:	counting eggs, determination of dead eggs, counting hatched larvae
Analytic methods:	not relevant yet

Miscellaneous test information

Practicability:	The test can be performed without specific training by the technical staff. Materials are normal laboratory equipment for rearing *Philonthus cognatus*
Cost-effectiveness:	time for culturing the beetles, no expensive material
Sensitivity:	work in progress
Reproducibility:	work in progress
Validity of the test:	the mortality of the females in the control and the treatment should not differ too much within the same week.
Standardization:	work in progress
Ecological realism:	work in progress
Broad chemical responsiveness:	not known

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ANNEX 3.2 - Test System Standardization Form 2.2

Sublethal toxicity test with the larval stage of the beetle *Philonthus cognatus*, Steph. 1832. (Coleoptera: Staphylinidae)

General data about the test organism at the start of the experiment

Test organism

Taxonomic group:	Rove beetle (Staphylinidae: Coleoptera)
Species:	*Philonthus cognatus* STEPH. 1832.
Life stage:	larvae LI
Sex:	not determinable for larvae
Age/ weight/ size:	1 - 2 days old
Gen. composition:	not known
Background:	animals from laboratory culture

Type of surroundings and ambient climate

Surroundings	
Type:	climate chamber
Ambient climate	
Air temperature:	15 ± 1 °C
Relative humidity:	85-100%
Light wave length:	-
Light intensity:	-
Light regime:	darkness

Type of test system and test conditions

Type:	19 cm^3 glass tubes (7 cm high, 2.8 cm in diameter)
Material:	glass
Openess:	open, only closed for some days before the hatching of the beetles
Spec./ test system:	single species test system
Ind./ test system:	1 individual/glasstube

Type of habitat

Soil/ litter:	LUFA 2.2 soil, homogeneously mixed
'Habitat characteristics'	
Soil/ litter charact.:	dried soil is stored at 5°C.
Hydrol. charact.:	50% WHC
Food supply:	during the complete test
Feeding rate:	continously 3 times a week
Type of food:	deep frozen half *Calliphora* spec. pupae for each animal at each feeding date

Exposure characteristics

Exposure route:	via soil
Composition of test habitat:	1 week before the test begins the soil is moistened with distilled water up to 20% WHC. 1 day before the larvae are put into the soil the chemical with an amount of water, corresponding to 10 - 20% of the WHC, is mixed for 15 minutes into the soil. Afterwards the soil is wetted up to 50% WHC and stored at 5 °C. At the day the test starts soil amounts corresponding to 20 g d.w. soil are filled into 19 cm^3 glass tubes.
Composition of control habitat:	same procedure without chemical (see above)

Duration of the experiment

Acclimat. time:	no acclimatization time
Exposure time:	the complete time of larval development
Recovery period:	hatching adults are kept in uncontaminated habitat

Exposure concentration

Replicates:	30 per concentration and control
Concentrations:	4 concentrations in a logarithmic series and 1 control
Series:	1
Ref. chemical:	work in progress

Exposure responses

Effect parameters:	survival, affected animals (legs or mandible trembling, lying on the back, uncoordinated movement, dead) sublethal effects (weight of hatching beetles, development time, sex, winter mortality, later reproduction)
Frequency of measurement:	observations after 1, 2, 3, 4, 5, 7, 10, 14, days and weekly thereafter, until the first black pupae is observed. Daily observations follow.
Measurement of variables:	moisture content and pH of the soil at the start and end of the test.
Toxic endpoint:	LC_{50}, EC_{50}, development time, hatching weight of males and females

Unit: mg test substance/kg d.w. test soil

Working methods:	counting survivals, measurement of weight, determination of sex
Analytic methods:	not relevant yet

Miscellaneous test information

Practicability:	The test can be performed without specific training by the technical staff. Materials are normal laboratory equipment for rearing *Poecilus cupreus* L. and *Philonthus cognatus*.
Cost-effectiveness:	time for culturing the beetles, no expensive material
Sensitivity:	work in progress
Reproducibility:	work in progress
Validity of the test:	mortality of the control should not be higher than 30%
Standardization:	work in progress.
Ecological realism:	the test method mimics quite well the natural habitat
Broad chemical responsiveness:	work in progress

References

Eghtedar, E. (1970): Zur Biologie und Ökologie der Staphyliniden *Philonthus fuscipennis* und *Oxytelus rugosus*. Pedobiologia 10: 169-179.

Heimbach, U. (1989): Massenzucht von *Poecilus cupreus* (Coleoptera, Carabidae). 19. Jahrestagung der Gesellschaft für Ökologie, Tagungsbericht 19/1: 228-229.

Metge, K. & Heimbach, U. (1994): Effects of dimethoate on the staphylinid beetle *Philonthus cognatus* , Steph. in the laboratory. 5th European Congress of Entomology, University of York, UK; Abstracts: 287.

Metge, K. & Heimbach, U. (1994): Entwicklung eines Zuchtverfahrens für den Staphyliniden *Philonthus cognatus*, Steph.. Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft Berlin-Dahlem, Heft 301: 512.

ANNEX 3.3 - Draft test protocol 2.1

Sublethal toxicity test with mature beetles of *Philonthus cognatus*, Steph. 1832 (Coleoptera: Staphylinidae)

1 Scope

This draft describes a method to determine effects on egg production and hatching rate of mature *Philonthus cognatus*, Steph. 1832 (Coleoptera: Staphylinidae). It may be used to determine EC- and LC-values of pesticides and other chemicals. Also the influence of different types of soil may be studied. Mature beetles are exposed to the chemical which is mixed into the soil.

2 Definitions

2.1 Egg production
Measured as the mean number of eggs laid per week and female alive.

2.2 Hatching rate
Percentage of offspring hatched from eggs laid.

2.3 NOEC (No Observed Effect Concentration)
The highest tested concentration of a substance at which no lethal or other effect is observed, expressed as mass of test substance per dry mass of the test substrate.

3 Principle

Effects on beetles exposed for 1 week to contaminated substrate are observed. After the exposure the beetles are kept in reproduction containers and mated in pairs. The eggs laid have to be collected and cultured to determine the hatching rate. Over a period of 6 - 10 weeks the number of eggs laid and the number of offspring are counted.

4 Reagents

4.1 Biological material
The biological material consists of adult staphylinid beetles of the species *P. cognatus* cultured in the laboratory. After hatching the young beetles have to live 2 - 4 weeks under long day conditions with a light:dark cycle of 16 : 8 hours at 20 ± 1 °C. An artificial winter of 12 - 20 weeks with a light:dark cycle of 8 : 16 hours at 5 ± 1 °C. is necessary for the development of gonads in the following artificial summer. Before using the beetles in the reproduction assay they have to be acclimatized under long day conditions for 1 week.

NOTE: The species is easy to identify within the genus *Philonthus*. The determination follows Freude et al. 1971. The first antenna segment is light yellow coloured at the lower side. Sexual difference between the males and females can be seen externally as an angled part at the 6th sternit of the male.

4.2 Soil
Soil should be a sandy soil such as LUFA 2.2.

4.3 Food
Deep frozen *Calliphora* spec. pupae are a good food supply. They are cut in two pieces when given as food.

5 Apparatus

Standard laboratory equipment, and:

- 2 litre glass containers with lid for mixing the chemical into the soil can be used
- 19 cm^3 glass tubes (7 cm high, 2.8 cm in diameter) closeable with a lid to prevent the beetles from escaping are used during the exposure of the beetles. The lids are punctured to allow air exchange
- plastic boxes (10.5 x 6 x 2 cm) filled with a mixture of plaster of paris and active coal (5 : 1) and some expanded clay granules are used during the reproduction period

6 Test environment

6.1 Exposure
The ambient climate during the exposure should be 15 ± 1 °C., relative humidity of 85 - 100% and a controlled light:dark cycle of 16 : 8 h.

6.2 Reproduction period
6.2.1 Mating of beetles:
During the reproduction period the climatic conditions for the mated beetles should be 20 ± 1 °C, a relative humidity of 85 - 100% and a controlled light:dark cycle of 16 : 8 h.

6.2.2 Egg culturing:
The ambient climate for the collected eggs should be 20 ± 1 °C, a relative humidity of 85 - 100% and darkness.

7 Procedure

7.1 Premilimary test
To determine the range of concentrations in the final test, perform a preliminary acute test with the test substance in four concentrations in a logarithmic series (eg.: 1, 10, 100, 1000 mg/kg d.w. soil) and a control. This test should be done with 3 males and 3 females per concentration. The final test will be carried out with concentrations below the LC_{50}-value.

7.2 Final test

7.2.1 Introduction of the test substances:
The soil is heated for 2 hours at 80 °C and stored at 5 °C in the dark. 1 week before the test begins, moist the soil with dest. water up to 20% of the WHC.

Use either method a), b) or c), as appropriate.

a) Water soluble substances
1 day before the adults are placed into the soil the chemical is dissolved in an amount of water corresponding to 10 - 20% WHC and mixed 5 minutes by hand using a spoon and 10 minutes using a rotation machine. Afterwards the soil is moistened up to 50% WHC and stored at 5 °C for 20 hours. Large soil lumps are broken up with a spoon during the last wetting. At the day the test starts soil amounts corresponding to 20 g dry weight soil are filled into the 19 cm^3 glass tubes.

b) 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). Add it to a small portion of dry substrate, mix thoroughly and evaporate the solvent by placing it under a fume hood for 1 hours. Then add the remainder of the soil substrate and the water and mix thoroughly. Store at 5 °C for 20 hours. Soil amounts corresponding to 20 g dry weight soil are filled into the 19 cm^3 glasstubes. Further work is in progress.

c) Substances insoluble in water or organic solvents
For a substance insoluble in a volatile solvent, prepare a mixture of 40 g dry soil (per kg d.w. soil substrate) and the quantity of the test substance required to obtain the desired concentration. Place the mixture, the remainder of the soil substrate and the water into a glass vessel (5.1.) and mix thoroughly. Store at 5 °C for 20 hours. At the day the test starts soil amounts corresponding to 20 g dry weight are filled into the 19 cm^3 glass tubes (5.2.). Further work is in progress.

NOTE: ultrasonic dispersion, organic solvents, emulsifiers or dispersants may be used to disperse substances with low aqueous solubility. When such auxiliary substances are used, all test concentrations and an additional control should contain the same minimum of auxiliary substance. To allow solvents to volatilize, the test containers should remain open for a period of one hour.

NOTE: Substances do not need to be tested at concentrations higher than 1000 mg/kg d.w. of the test substrate.

Determine the water content and the pH in the presence of 1mol/l KCl of the soil at the beginning and the end of the test.

7.2.2 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 use additional control containers similar to the containers without the test substance. Treat these containers in the same way as those without the test substances. Include an additional container for the control and each treatment for pH and soil moisture determination at the end of the test.

7.2.3 Addition of the biological material:
For each concentration and the control weigh 10 females and 10 males and distribute them so that in all concentration series equal numbers of beetles of the same size are used. Place one beetle on the soil surface of each glasstube and close it with a lid.

7.2.4 Duration of exposure:
The duration of exposure is 7 days. The beetles are observed after 2, 4, 6 and 24 hours, then at day 2, 3, 4, 5 and 7. Beetles are fed with one half *Calliphora* spec. pupae on day 0, 2, 4 and 6. Further work is in progress.

7.2.4 Reproduction test:
After 7 days exposure to the chemical the beetles are mated in pairs (5.3.). The egg production should be observed for at least 6 weeks after the first eggs have been found. Also the emerging larvae should be observed for 2 weeks after collecting the eggs.

8 Calculation and expression of results

8.1 Calculation

For each concentration and female alive determine the egg production per week and hatchability of the eggs laid. Observe the mortality of females and males during the egg laying period, the number of cannibalized eggs and the numbers of hatchings per female.

NOTE: Eggs will be counted as 'cannibalized egg' if an egg is empty and has a visible hole in the egg shell.

Compare the results by suitable statistical methods, e.g. Wilcoxon test for pair differences or Tukey's multiple t-test and test for significance ($p<0.05$) of difference from controls.

8.2 Expression of results

Indicate, in mg/kg d.w. soil substrate, significant changes in reproduction and number of offspring.

9 Validity of the test

Work in progress.

10 Test report

The test report shall refer to this standard and, in addition to the results expressed as in 8., shall provide the following information:

- complete description of the biological material employed (species, source, etc.)
- method of preparation of the test substrate, and any auxiliary substances used for a low-/non-water-soluble substance

- results obtained with the reference substance, if performed
- detailed conditions of the test enviroment
- table giving the egg production obtained for each concentration and for the control, mean number of eggs per week and female alive for each concentration and control, number of dead females for every week for each concentration and for the control
- number of offspring per female at the end of the test
- the highest concentration causing no observed effects (NOEC) and LOEC
- water content and pH of the soil at the start and at the end of the test
- all operation details not specified in the standard, and any occurrences liable to have affected the results
- Some specimens used in the test should be preserved for future reference

11 References

Eghtedar, E. (1970): Zur Biologie und Ökologie der Staphyliniden *Philonthus fuscipennis* und *Oxytelus rugosus.* Pedobiologia 10: 169-179.

Freude, H., Harde, K.W. & Lohse, G.A. (1971): Die Käfer Mitteleuropas. Band 4; Goecke und Evers, Krefeld.

Heimbach, U. (1989): Massenzucht von *Poecilus cupreus* (Coleoptera, Carabidae). 19. Jahrestagung der Gesellschaft für Ökologie, Tagungsbericht 19/1: 228-229.

Metge, K. & Heimbach, U. (1994): Effects of Dimethoate on the staphylinid beetle *Philonthus cognatus* , Steph. in the laboratory. 5th European Congress of Entomology, University of York, UK; Abstract: 287.

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Sublethal toxicity test on the larval stage of the beetle *Philonthus cognatus*, Steph. 1832 (Coleoptera: Staphylinidae)

1 Scope

This draft for an International Standard describes a method to determine sublethal and lethal effects on larval stages of *Philonthus cognatus*, Steph. 1832. Sublethal effects on hatching weight of immature beetles, development time and winter mortality are measured. If the number of hatched females per concentration is higher than 10, a reproduction test can be carried out to examine long term effects.

2 Definitions

2.1 LOEC (Lowest Observed Effect Concentration)
The concentration of a test substance at which an effect is determined, expressed as mass of the test substance per dry mass of the test substrate.

2.2 NOEC (No Observed Effect Concentration)
The highest tested concentration of a substance at which no lethal or other effect is observed, expressed as mass of test substance per dry mass of the test substrate.

2.3 Hatching weight
Weight of surviving animals, measured not later than 1 - 2 days after hatching of the beetles and before the first feeding.

2.4 Development time
Development time of larvae measured in days from hatching from the egg to the hatching as an immature beetle.

2.5 Winther mortality
Expressed as percent of dead beetles after more than 12 weeks under short day conditions with a light:dark cycle of 8 : 16 hours at 5 ± 1 °C and a following 1 week period under long day conditions with a light:dark cycle of 16 : 8 hours and 20 ± 1 °C.

2.6 Reproduction
2.6.1 Egg production:
Mean number of eggs laid per week and female survived.

2.6.2 Hatching rate:
Expressed as percentage of offspring hatched from collected eggs.

3 Principles

The first larval stage of the species is placed in the soil treated with different concentrations of the test substance. The mortality during the development to an immature beetle is observed. Effects on development time, hatching weight and sex are determined. Investigations on winter mortality and reproduction can follow. A reproduction test is possible if the number of hatched females is higher than 10.

4 Reagents

4.1 Biological material

The biological material consists of 1-2 days old larvae of the species *P. cognatus* cultured in the laboratory. Larvae are fed before use in experiments and randomly selected from different rearing boxes.

NOTE: The species is easy to identify within the genus *Philonthus*. The determination follows Freude et al. (1971). At the adult beetles the first antenna segment is light yellow coloured at the lower side. Sexual difference between the males and females can be seen externally as an angled part at the 6^{th} sternit of the male.

4.2 Soil

The test substrate should be a sandy soil such as LUFA 2.2.

NOTE: Artificial soil (OECD) can be used but the larvae may have problems to build moulting chambers big enough, especially the pupae chamber. Occasionally the hatching beetles may not emerge successfully.

4.3 Food

Deep frozen *Calliphora* spec. pupae are a good food supply. They are cut in two pieces when given as food.

5 Apparatus

Standard laboratory equipment, and:

- 2 litre glass containers with lid for mixing the chemical into the soil can be used
- 19 cm^3 glass tubes (7 cm high, 2.8 cm in diameter) closeable with a lid to prevent the beetles to escape are used during the exposure of the beetles. The lids are punctured to allow air exchange
- If further investigations on winter mortality and reproduction follow, plastic boxes (19 x 19 x 6 cm) with moistened peat will be used during the artificial winter period. For the reproduction test beetles are mated after the overwintering period in plastic boxes (10.5 x 6 x 2 cm) filled with a mixture of plaster of paris and active coal (5 : 1) and some expanded clay granules. In these reproduction containers the egg production can be measured

6 Test environment

6.1 Exposure
The ambient climate during the exposure of the larval stage should be 15 ± 1 °C, a relative humidity of 85 - 100% and darkness.

6.2 Reproduction period
6.2.1 Mating of beetles:
During the reproduction period the climatic conditions for the mated beetles should be 20 ± 1 °C, a relative humidity of 85 - 100% and a controlled light:dark cycle of 16 : 8 hours.

6.2.2 Egg culturing:
The ambient climate for the collected eggs should be 20 ± 1 °C, a relative humidity of 85 - 100% and darkness.

7 Procedure

7.1 Preliminary test
To determine the range of concentrations in the final test, perform a preliminary acute test with the test substance in four concentrations in a logarithmic series (eg.: 1, 10, 100, 1000 mg/kg d.w. soil) and a control. This test should be done with 6 - 10 larvae per concentration. The final test will be carried out with at least 3 concentrations near and below the LC_{50}-value and a control to examine sublethal effects.

7.2 Final test
7.2.1 Introduction of the test substances:
The soil is heated for 2 hours at 80 °C and stored at 5 °C in the dark. 1 week before the test begins moisten the soil with dest. water up to 20% of its water holding capacity (WHC).

Use either method a), b) or c), as appropriate.

a) Water soluble substances
1 day before the larvae are placed into the soil the chemical is dissolved in an amount of water corresponding to 10 - 20% WHC and mixed 5 minutes by hand using a spoon and 10 minutes using a rotation machine. Afterwards the soil is moistened up to 50% WHC and stored at 5 °C for 20 hours. Large soil lumps are broken up with a spoon during the last wetting. At the day the test starts soil amounts corresponding to 20 g dry weight are filled into the 19 cm^3 glasstubes (5.2.).

b) 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). Add it to a small portion of dry substrate, mix thoroughly and evaporate the solvent by placing it under a fume hood for 1 hour. Then add the remainder of the soil substrate and the water and mix thoroughly and store for 20 hours at 5 °C. Soil amounts corresponding to 20 g d.w. soil are filled into the 19 cm^3 glass tubes. Further work is in progress.

c) Substances insoluble in water or organic solvents
For a substance insoluble in a volatile solvent, prepare a mixture of 40 g dry soil (per kg d.w. soil substrate) and the quantity of the test substance required to obtain the desired concentration. Place the mixture, the remainder of the soil substrate and the water into a glass vessel (5.1.) and mix thoroughly. Store at 5 °C for 20 hours. At the day the test starts soil amounts corresponding to 20 g d.w. are filled into the 19 cm^3 glass tubes (5.2.). Further work is in progress.

NOTE: ultrasonic dispersion, organic solvents, emulsifiers or dispersants may be used to disperse substances with low aqueous solubility. When such auxiliary substances are used, all test concentrations and an additional control should contain the same minimum of auxiliary substance. To allow solvents to volatilize, the test containers should remain open for a period of one hour.

NOTE: Substances do not need to be tested at concentrations higher than 1000 mg/kg d.w. of the test substrate.

Determine the water content and the pH in the presence of 1 mol/l KCl of the soil at the beginning and the end of the test.

7.2.2 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 use additional control containers similar to the containers without the test substance. Treat these containers in the same way as those without the test substances. Include an additional container for the control and each treatment for pH and soil moisture determination at the end of the test.

7.2.3 Addition of the biological material:
For each concentration and control use 30 larvae randomly selected from the hatching boxes. The larvae should be fed once before they are introduced in the glasstubes.

7.2.4 Duration of exposure:
During the exposure the larvae are observed after 2, 4 and 6 hours then after 1, 2, 3, 4, 5, 7, 10, 14 days and weekly thereafter until the first black pupae are observed. Hereafter daily observation is necessary. Larvae are fed 3 times a week until they have finished building their pupa chamber.

7.2.5 Determination:
Maintain the water content of the soil substrate in the test glasstubes during the test period by reweighing the glasstubes periodically (once a week) and if necessary replenish lost water.

7.2.6 Artificial winter:
Immature beetles are separated according to sex and concentration used. They are cultured for 2 - 4 weeks under long day conditions, more than 12 weeks under short day conditions and 1 week under long day conditions again. Thereafter determine the winter mortality. During the artificial winter feed the beetles once a week with cut *Calliphora* spec. pupae.

7.2.7 Reproduction test:
After the artificial winter and 7 days of long day conditions mate the beetles in pairs and culture them at 20 ± 1 °C. The egg production should be observed for at least 6 weeks after the first eggs have been found. Also the emerging larvae should be observed for 2 weeks after collecting the eggs.

NOTE: Only if the number of hatched females in a concentration is higher than 10 the reproduction test (7.2.7.) can be carried out to examine long term effects.

8 Calculation and expression of results

8.1 Calculation

For each concentration determine the mortality of larvae. The mean hatching weight and the mean development time for each concentration is pointed out. If possible winter mortality, egg production and hatching rate of eggs are observed.

Compare the results by suitable statistical methods, e.g. Wilcoxon test for pair differences or Tukey's multiple t-test and test for significance ($p<0.05$) of difference from controls.

8.2 Expression of results

Indicate significant changes in development time of larvae, hatching weight of immature beetles, winter mortality, egg production of the mature beetles and hatching rate of their eggs. State NOEC and LOEC levels.

9 Validity of the test

Work in progress.

10 Test report

The test report shall refer to this standard protocol and, in addition to the results expressed as in 7.2., shall provide the following informations:

- complete description of the biological material employed (species, age, culturing conditions, food supply, etc.)
- method of preparation of the test substrate
- detailed conditions of the test environment
- table giving the numbers and the percentage of dead larvae, number of males and females hatched, mean hatching weights for both sexes and concentration, mean of development time of larvae
- table giving the egg production obtained for each concentration and for the control, mean number of eggs per week and female alive for each concentration and control, number of dead females for every week for each concentration and for the control
- number of offspring per female at the end of the test

- the highest concentration causing no observed effects (NOEC) and LOEC
- water content and pH of the soil at the start and at the end of the test
- all operation details not specified in the standard, and any occurrences liable to have affected the results: Some specimens used in the test should be preserved for future reference

11 References

Eghtedar, E. (1970): Zur Biologie und Ökologie der Staphyliniden *Philonthus fuscipennis* und *Oxytelus rugosus*. Pedobiologia 10: 169-179.

Freude, H., Harde, K.W. & Lohse, G.A. (1971): Die Käfer Mitteleuropas. Band 4; Goecke und Evers, Krefeld.

Heimbach, U. (1989): Massenzucht von *Poecilus cupreus* (Coleoptera, Carabidae). 19. Jahrestagung der Gesellschaft für Ökologie, Tagungsbericht 19/1: 228-229.

Metge, K. & Heimbach, U. (1994): Effects of Dimethoate on the staphylinid beetle *Philonthus cognatus* , Steph. in the laboratory. 5th European Congress of Entomology, University of York, UK; Abstract: 287.

Metge, K. & Heimbach, U. (1994): Entwicklung eines Zuchtverfahrens für den Staphyliniden *Philonthus cognatus*, Steph.. Mitt. Biol. Bundesanst. Land-Forstwirtsch. Berlin-Dahlem, Heft 301: 512.

4 Sublethal toxicity tests with earthworms (Annelida: Oligochaeta)

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Detailed report of the contractor/sub-contractor

4.1 Objectives for the reporting period

- To determine sublethal effects of the three SECOFASE test compounds (dimethoate, copper and LAS) on *Eisenia fetida* (Savigny 1826) (Annelida: Oligochaeta) in artificial soil (OECD) and LUFA 2.2 soil
- To select sensitive test parameters for the characterization of sublethal effects
- To compare the sensitivity of *Eisenia fetida* and *Eisenia andrei* (André 1963)
- To compare the effects of total soil contamination and surface contamination with dimethoate
- To characterize the fate of dimethoate in the test system by residue analysis
- To characterize the microbial activity in the test system
- To determine optimum conditions for the use of LUFA 2.2 soil as test substrate
- To improve culturing methods for the indigenous earthworm species *Aporrectodea caliginosa* (Savigny 1826) and *Allolobophora chlorotica* (Savigny 1826)

4.2 Objectives for the next period

- To determine sublethal effects of the three SECOFASE test compounds (dimethoate, copper and LAS) on *A. caliginosa* and *A. chlorotica* in artificial soil (OECD) and LUFA 2.2 soil
- To continue characterization of microbial activity in the test system
- To finalize test system protocols for the evaluation of sublethal effects of chemicals on growth and reproduction of earthworms

4.3 Main results obtained

4.3.1 Reproduction toxicity tests

A brief description of the methodology of reproduction toxicity tests is given in the second technical report of SECOFASE (Wiles et al., 1994). Sublethal effects of the insecticide dimethoate were investigated in artificial soil (OECD) using two

different ways of substrate contamination. The test substance was either mixed homogeneously into the soil (TC=total contamination) or an equivalent amount was sprayed homogeneously onto the soil surface (SC=surface contamination).

With *E. fetida* the same NOEC (5.0 mg/kg) for cocoons and number of juveniles was observed with TC and SC. *E. andrei* was slightly more susceptible to TC (NOEC: 2.5 mg/kg). In contrast to previous results body weight development showed the same sensitivity as the reproduction parameters in both species.

Reactions of both test species to TC with copper were also different. With *E. fetida* the same NOEC (10 mg/kg) was observed in artificial soil (OECD) and LUFA 2.2 soil. Effects on *E. andrei* in contrast were dependent on the soil. In artificial soil (OECD) *E. andrei* was less susceptible (NOEC: 100 mg/kg), but in LUFA 2.2 soil significant effects on the number of juveniles were observed at the lowest test concentration of 3.2 mg/kg.

Table 4.1: Effects of dimethoate on sexual development of juveniles of *Eisenia fetida* after 58 days of exposure (total contamination) in artificial soil (OECD) and LUFA 2.2 soil

	Mortality [%]	Body weight development [%]1)	Mature animals [%]	Total number of cocoons
Art. soil				
Control	0	2546±271	90	4.0±2.9
1.8 mg/kg	0	2515±159	90	1.5±1.9
3.2 mg/kg	2.5	3037±353	72	2.0±2.8
5.6 mg/kg	5.0	2350±176	63 *	1.0±0.8
10 mg/kg	7.5	2186±439 *	36 *	0
LUFA 2.2.				
Control	5.0	2082± 88	34	0
1.8 mg/kg	7.5	2123±235	27	0
3.2 mg/kg	5.0	1310±299 *	16 *	0
5.6 mg/kg	40.0	1235±289 *	5 *	0
10 mg/kg	92.5	175±181 *	0 *	0

* significant difference, $p \leq 0.05$, multiple t-test by TUKEY
1) initial body weight = 100%

The effect of dimethoate on sexual development of 2 week old juvenile *E. fetida* was investigated in order to check the suitability of further test parameters. The usual experimental design (TC) was used but exposure time had to be prolonged. After 28 days all treatments were checked in weekly intervals for mature animals. Test evaluation took place 8 weeks after test start when most control animals were mature.

Sexual development in LUFA 2.2 soil was slower than in artificial soil (OECD) and very high mortality rates were observed at 5.6 and 10 mg/kg (Table 4.1). Maturation of juveniles showed a sensitivity similar to the reproduction parameters of adults (number of cocoons and juveniles). Body weight development was less sensitive in artificial soil (OECD). Overall results with LUFA 2.2 soil indicate that this soil is not suitable for tests with juvenile *E. fetida*.

In reproduction toxicity tests with LAS more infertile cocoons were observed in LUFA 2.2 soil than in artificial soil (OECD). The mean number of juveniles/cocoon was 4.0 in artificial soil (OECD) and 3.1 in LUFA 2.2 soil. The NOEC for cocoons was 1800 mg/kg in artificial soil (OECD) and 1000 mg/kg in LUFA 2.2 soil,

whereas the NOEC for surviving juveniles was 1000 mg/kg in both soils. In reproduction toxicity tests with food supply significant effects on body weight development were only observed at the two highest concentration levels. In unfed LC_{50}-studies however significant differences in body weight development were observed at low concentration levels of 100 mg/kg but a clear decrease in body weight occured only at 5000 mg/kg. The acute toxicity of LAS to *E. fetida* in artificial soil (OECD) was very low (LC_{50}: 5781 mg/kg).

4.3.2 Characterization of microbial activity in the test system

Dimethoate residues and microbial activity were determined simultaneously in one reproductive toxicity test with *E. fetida* (in cooperation with participant no. 2 (BBA)). The initial concentration was 10 mg/kg of soil. Test animals were introduced 1 and 8 days after test start. The same experimental set-up was conducted with and without earthworms in order to estimate the effect of earthworm burrowing on microbial activity and dimethoate degradation.

Development of microbial activity in the test system was investigated by measuring dehydrogenase activity (TTC-method, modified after Malkomes, 1993). Results are shown in Fig. 4.1. The soil had been remoistened one week before test start. One day after test start all five treatments with artificial soil (OECD) showed a similar level of dehydrogenase activity, whereas with LUFA 2.2 soil differences up to 30% were observed. In controls with LUFA 2.2 soil initial dehydrogenase activity was about 5.6 times higher compared to controls with artificial soil (OECD). This difference diminished to about 1.4 times after 22 days. At that moment highest activity was observed in controls of both soils. All treatments with earthworms showed a steady increase in microbial activity. In contrast to this a distinct activity peak was observed in all treatments without earthworms on day 4. Activity then decreased to low levels just above the initial activity.

Although distinct differences were observed in the activity level between both soils, overall trends in development of microbial activity were very similar. The fast disappearance of dimethoate in LUFA 2.2 soil which was observed in former experiments might be explained by the higher microbial activity. Although LUFA 2.2 soil was stored air dried at low temperatures it seems that this natural soil shows more variability than artificial soil (OECD). This might be of importance for further standardization.

4.3.3 Establishment of laboratory cultures

A. caliginosa was cultured in artificial soil (OECD) and a natural loamy soil (pH (0.01 M $CaCl_2$): 7.5; O.M. content: 4.2%; clay: 23.9%, silt: 54.6%, sand: 21.5%) which originated from an orchard where the earthworms were sampled. Finely ground air dried grass meal which was mixed into the topsoil was accepted as food source. In preliminary experiments at 15 °C most of the earthworms entered a resting stage. This could be prevented by lowering the culture temperature to 12 °C.

Reproduction in artificial soil (OECD) was not very successful. Cocoon production was better in the natural soil but still too low (1-2 cocoons/animal/month) to generate sufficient numbers of test animals. At present only field sampling of adult animals in uncontaminated sites can be recommended in order to obtain test animals. They should be maintained for at least one week under laboratory conditions before being used in tests.

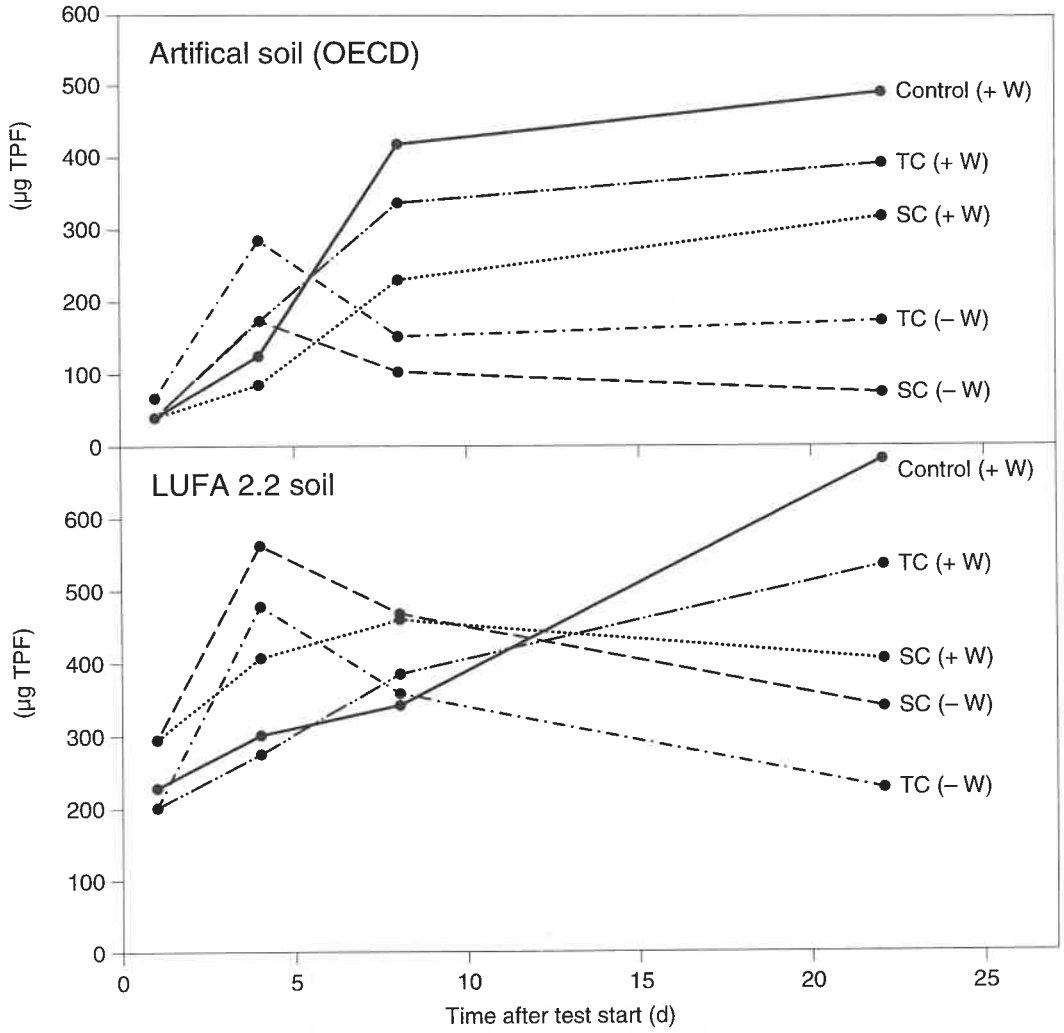

Figure 4.1: Dehydrogenase activity (µg TPF) in artificial soil (OECD) and LUFA 2.2 soil with different types of soil contamination with dimethoate (10 mg/kg).
TC = total contamination, SC = surface contamination
+ W = with earthworms, - W = without earthworms

4.4 References

Malkomes, H.P. (1993): Eine modifizierte Methode zur Erfassung der Dehydrogenaseaktivität (TTC-Reduktion) im Boden nach Herbizidanwendung. Nachrichtenbl. Deut. Pflanzenschutzd., 45, 180-185.

Wiles, J.A., Kammenga, J.E. & Løkke, H. (1994): Progress report 1993 of SECOFA-SE, Second technical report. NERI, Denmark. 94 pp.

ANNEX 4.1 - Test System Standardization Forms 3.1

Sublethal toxicity test with the earthworm *Eisenia fetida* (Savigny 1826) (Annelida: Oligochaeta)

General data about the test organism at the start of the experiment

Test organism

Taxonomic group:	Earthworms (Annelida: Oligochaeta)
Species:	*Eisenia fetida* (Savigny 1826)
Life stage:	adult
Sex:	hermaphrodite
Age/weight/size:	adult age: min. 2 months, max. 12 months, weight: 250-600 mg
Genetical composition:	not known
Background:	laboratory culture

Type of surroundings and ambient climate

Surroundings	
Type:	climate chamber
Ambient climate	
Air temperature:	20 ± 1 °C
Relative humidity:	≥ 80%
Light wave length:	artificial light
Light intensity:	400-800 lx
Light regime:	light:dark cycle 8:16-16:8 h

Type of test system and test conditions

Type:	container (1000 cm^3)
Material:	plastic
Openess:	perforated transparent cover (allowing continous gas exchange)
Spec./test system:	single species test system
Ind./test system:	10

Type of habitat

Soil/litter:	artificial soil (OECD), homogeneously mixed
Medium:	artificial culture medium
'Habitat characteristics'	
Soil charact.:	artificial soil (OECD) consists of sphagnum peat (10%), kaolin clay (20%) and sand (70%) plus calcium carbonate (about 1%, to adjust pH to 6.0 ± 0.5)
Hydrol. charact.:	moisture content 50-60% of water holding capacity
Food supply:	during acclimatization and test
Feeding rate:	0.5 g/ind./week (work in progress)
Type of food:	finely ground dried cattle manure

Exposure characteristics

Exposure route:	via soil
Composition of test habitat:	artificial OECD soil, deionized water, test substance and auxiliary agent (if used), all constituents well homogenized
Composition of control habitat:	a) artificial OECD soil, deionized water b) artificial OECD soil, deionized water and auxiliary agent

Duration of the experiment

Acclimat. time:	1 day (min.) - 7 days

Exposure time:	28 days
Recovery period:	not relevant

Exposure concentrations

Replicates:	4
Concentrations:	at least 3 concentrations (work in progress)
Series:	1
Ref. chemical:	benomyl

Exposure responses

Effect parameters:	weight development of adults, cocoon production, hatching rate, number of juveniles
Other parameters:	survival of adults, general behaviour (burrowing, repellent effects, feeding behaviour)
Measurement of variables:	soil moisture content and pH (0.01 M $CaCl_2$) at start and end of the test
Toxic endpoint:	NOEC, LOEC and EC_{50}
Unit:	mg test substance/kg d.w. test soil
Working methods:	-
Analytical methods:	not relevant

Miscellaneous test information

Practicability:	the required materials must be obtained from commercial agents of laboratory equipment, test animals must be cultured or purchased from commercial bait dealers. The test protocol can be performed by technical and academic personnel without necessity to follow specific courses.
Cost-effectiveness:	the amount of material, energy, test organisms and time to prepare and conduct the test are usual
Sensitivity:	work in progress
Reproducibility:	work in progress
Validity of the test:	A) standard statistical methods (ANOVA) B) 1) rate of mortality within the control group should be less than 10% 2) during the experiment the water content of the soil substrate should not differ by more than 10% from the water content at the start of the test
Standardization:	this draft test protocol integrates several methods described in literature or as test guidelines
Ecological realism:	occurrence of *E. fetida* is restricted to places rich in organic matter (dung heaps), but responses to chemicals in most cases resemble those of indigenous earthworm species (ref. to literature)
Broad chemical responsiveness:	no restrictions are given with respect to the chemical substances that may be evaluated by this test

References

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

Kula, H. (1994): Auswirkungen von Pflanzenschutzmitteln auf Regenwürmer (Oligochaeta: Lumbricidae) - Zur Problematik der Bewertung letaler und subletaler Effekte in Labor- und Freilandversuchen. PhD.-Thesis, TU Braunschweig, 151 pp.

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

ANNEX 4.2 - Test System Standardization Forms 3.2

Sublethal toxicity test with the earthworm *Aporrectodea caliginosa* (Savigny 1826) and *Allolobophora chlorotica* (Savigny 1826) (Annelida: Oligochaeta)

General data about the test organism at the start of the experiment

Test organism

Taxonomic group:	Earthworms (Annelida: Oligochaeta)
Species:	*Aporrectodea caliginosa* (Savigny 1826) or *Allolobophora chlorotica* (Savigny 1826) (work in progress)
Life stage:	juvenile and adult (work in progress)
Sex:	hermaphrodite
Age/weight/size:	work in progress
Gen. composition:	not known
Background:	laboratory culture or sampled in uncontaminated field sites

Type of surroundings and ambient climate

Description of surroundings	
type:	climate chamber
Ambient climate	
Air temperature:	12 ±1 °C (15 °C may induce inactivity)
Relative humidity:	≥ 80%
Light wave length:	light not required
Light intensity:	-
Light regime:	-

Type of test system and test conditions

Description of test system	
Type:	container (1000 cm^3)
Material:	plastic
Openess:	perforated transparent cover (allowing continous gas exchange)
Spec./test system:	single species test system
Ind./test system:	at least 6 (work in progress)

Type of habitat

Soil/litter:	artificial soil (OECD) or LUFA 2.2 soil, homogeneously mixed (work in progress)
Medium:	artificial culture medium
'Habitat characteristics'	
Litter/soil charact.:	- artificial soil (OECD) consists of sphagnum peat (10%), kaolin clay (20%) and sand (70%) plus calcium carbonate (about 1%, to adjust pH to 6.0 ± 0.5) - LUFA 2.2 soil is a commercially obtainable natural soil, O.M. content: ca 4%, pH 6.0 ± 0.5
Hydrol. charact.:	moisture content 50-60% of water holding capacity (work in progress)
Food supply:	during acclimatization and test
Feeding rate:	work in progress
Type of food:	finely ground dried cattle manure or finely ground grass meal (work in progress)

Exposure characteristics

Exposure route:	via soil
Composition of test habitat:	artificial soil (OECD) or LUFA 2.2 soil, deionized water, test substance and auxiliary agent (if used), all constituents well homogenized
Composition of control habitat:	a) artificial soil (OECD) or LUFA 2.2 soil, deionized water b) artificial soil (OECD) or LUFA 2.2 soil, deionized water and auxiliary agent

Duration of experiment

Acclimat. time:	1 day (min.) - 7 days
Exposure time:	min. 28 days (work in progress)
Recovery period:	not relevant

Exposure contrations

Replicates:	4
Concentrations:	at least 3 concentrations (work in progress)
Series:	1
Ref. chemical:	benomyl

Exposure responses

Effect parameters:	weight development of adults, cocoon production, hatching rate, number of juveniles (work in progress)
Other parameters:	survival of adults, general behaviour (burrowing, repellent reaction, feeding behaviour)
Measurement of variables:	soil moisture content and pH (0.01 M $CaCl_2$) at start and end of the test
Toxic endpoint:	NOEC, LOEC and EC_{50}
Unit:	mg test substance/kg d.w. test soil
Working methods:	-
Analytical methods:	not relevant

Miscellaneous test information

Practicability:	the required materials must be obtained from commercial agents of laboratory equipment, test animals must be cultured or obtained by field sampling. The test protocol can be performed by technical and academic personnel without necessity to follow specific courses.
Cost-effectiveness:	the amount of material, energy, test organisms and time to prepare and conduct the test are big
Sensitivity:	work in progress
Reproducibility:	work in progress
Validity of the test:	A) standard statistical methods (ANOVA) B) 1) rate of mortality within the control group should be less than 10% 2) during the experiment the water content of the soil substrate should not differ by more than 10% from the water content at the start of the test
Standardization:	this draft guideline integrates several methods described in literature
Ecological realism:	*A. caliginosa* is a common earthworm species of great importance especially for agro-ecosystems *A. chlorotica* is a common earthworm species especially in grassland
Broad chemical responsiveness:	no restrictions are given with respect to the chemical substances that may be evaluated by this test

References

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

Kula, H. (1994): Auswirkungen von Pflanzenschutzmitteln auf Regenwürmer (Oligochaeta: Lumbricidae) - Zur Problematik der Bewertung letaler und subletaler Effekte in Labor- und Freilandversuchen. PhD.-Thesis, TU Braunschweig, 151 pp.

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

Sublethal toxicity tests with earthworms (Annelida: Oligochaeta)

1 Scope

This test protocol describes a method for determining the effects on mortality, growth and reproduction of earthworms 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. Either *Eisenia fetida* (Savigny 1826), *Aporrectodea caliginosa* (Savigny 1826) or *Allolobophora chlorotica* (Savigny 1826) might be used as test species.

2 Definitions

2.1 Growth
Increase in biomass i.e. the fresh mass of organisms expressed as a percentage of the fresh mass of organisms at the start of the test.

2.2 Reproduction
Mean number of offspring surviving till the end of the test period per adult earthworm alive after four weeks.

2.3 NOEC (No Observed Effect Concentration)
The highest tested concentration of a substance at which no lethal or other effect is observed, expressed as mass of test substance per dry mass of the test substrate.

2.4 LOEC (Lowest Observed Effect Concentration)
The lowest tested concentration of a substance with observed effects, expressed as mass of test substance per dry mass of the test substrate.

3 Principle

The percentage mortality, and the effects on growth and reproduction of adult earthworms placed in a defined artificial soil substrate containing the test substance in different concentrations are determined. The mortality and the effects on growth are determined after 4 weeks. The fertility is measured by counting the number of cocoons. The number of offspring hatching from the cocoons is determined during an additional period of at least 4 weeks.

For substances without a defined toxicity it is useful to conduct the test in two steps:

- a preliminary toxicity test as described in ISO 11268-1 to give an indication of the concentrations for total mortality and for the absence of mortality, for determination of the concentrations to be tested in the final test
- the final test on reproduction to determine both the concentration of a chemical which causes no significant effects on numbers of offspring hatched from cocoons compared with the control (NOEC) and the lowest concentration causing effects (LOEC)

4 Reagents

4.1 Biological material

The test can either be run with the compost worm *Eisenia fetida* (Savigny 1826) or with the indigenous species *Aporrectodea caliginosa* (Savigny 1826) or *Allolobophora chlorotica* (Savigny 1826). Field collected animals should be determined according to Sims & Gerard (1985).

4.1.1 *Eisenia fetida*:
The biological material consists of adult earthworms of the species *Eisenia fetida* at least two months old but not older than 1 year, with a clitellum and a wet mass between 250 mg and 600 mg. Within one test, fresh masses of individuals shall not differ by more than 200 mg.

Earthworms to be used in the test are selected from a synchronized culture to form a relatively homogeneous population in terms of age, size and mass. A synchronized culture is obtained by placing adult earthworms in breeding containers (see Supplement) and removing them after not more than 4 weeks. Offspring of the remaining cocoons have reached an adult stage after at least 2 months.

4.1.2 *Aporrectodea caliginosa, Allolobophora chlorotica*:
Adult earthworms either from laboratory cultures or collected in the field from uncontaminated soil can be used. Field collected animals should be maintained at least one week under laboratory conditions in artificial soil. Depending on environmental variables (mainly temperature and soil moisture) indigenous earthworm species may enter resting phases. Temperature therefore should not exceed 12 ± 1 °C.

Selected earthworms are conditioned for between 1 and 7 days before introduction into an artificial soil substrate containing the appropriate amount of food.

It is advisable to preserve some biological material for later identification of the test species.

4.2 Test substrate

For each test container (5.1), the quantity of substrate (artificial soil (OECD 1984)) per test container should be eqv. to 500 g dry mass. Percentage (expressed on dry weight basis):

10%	Sphagnum peat (finely ground and with no visible plant remains)

20% Kaolinite clay (containing not less than 30 % kaolinite)
70% Industrial quartz sand (dominantly fine sand with more than 50 % of particle size 0.05 - 0.2 mm)

Add sufficient (about 1%) calcium carbonate ($CaCO_3$), pulverized recognized analytical grade to bring the pH (0.01 M $CaCl_2$) to 6.0 ± 0.5.

The substrate shall be moistened with deionized or distilled water to reach 50 to 60% of the total water holding capacity determined in accordance with ISO 11274. Determine the water content of the substrate in accordance with ISO 11461. The substrate shall be moistened one week before starting the experiment.

4.3 Food
With *Eisenia fetida* finely ground cattle manure is spread homogeneously onto the soil surface (0.5 g/earthworm/week). Finely ground air dried grass meal mixed into the topsoil is used with the indigenous species *Aporrectodea caliginosa* or *Allolobophora chlorotica*.

5 Apparatus

Standard laboratory equipment, and:

5.1 One-way plastic containers of about 1 liter with a cross-sectional area of about 200 cm^2, so that a depth of 5 - 6 cm of the soil substrate contains 500 g dry mass. Test containers shall permit adaption to the light:dark cycle and gaseous exchange between the medium and the atmosphere (e.g. by means of a perforated transparent cover) and shall have provisions to prevent earthworms from escaping (e.g. by using a tape to fix the cover).

5.2 Apparatus to determine the dry mass of the substrate in accordance with ISO 11461.

6 Test environment

6.1 Enclosure, controllable at a temperature of 20±1 °C (*Eisenia fetida*) or 12±1 °C (*Aporrectodea caliginosa, Allolobophora chlorotica*).

6.2 Light source (e.g. white fluorescent tubes) capable of delivering a constant light intensity of 400 - 800 lux on the containers at a controlled light:dark cycle of between 8:16 h and 16:8 h.

7 Procedure

7.1 Preliminary test
If it is necessary to determine the range of concentrations for use in the final test on reproduction, perform a preliminary acute test in accordance with

ISO 11268-1 for four concentrations of the test substance and a control (e.g., 0, 1, 10, 100, 1000 mg/kg) on 6 (*A. caliginosa, A. chlorotica*) or 10 (*E. fetida*) earthworms per concentration and test container.

7.2 Final test

7.2.1 Introduction of the test substance:
Mix the test substance into the artificial soil before earthworms are introduced. Use either method a), b) or c), as appropriate.

a) Water soluble substances
Immediately before starting the test, dissolve the quantity of the test substance required for the replicates of one concentration in the water or that portion of it necessary to wet the soil substrate in order to meet the requirements of 4.2, and mix thoroughly with the test substance before introducing it into a test container.

b) 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).
Add it to a small portion of quartz sand or dry soil substrate, mix thoroughly and evaporate the solvent by placing the container under a fume hood for 1 h. Then add the reminder of the soil substrate (4.2) and the water in accordance with 4.2 and mix thoroughly.

c) 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 4.2) or 10 g of the dry soil substrate and the quantity of the test substance required to obtain the desired concentration. Place the mixture, the remainder of the soil substrate (4.2) and the water into a beaker and mix thoroughly before introducing it into the test container.

NOTE - Ultrasonic dispersion, organic solvents, emulsifiers or dispersants may be used to disperse substances with low aqueous solubility. When such auxiliary substances are used, all test concentrations and an additional control should contain the same minimum of auxiliary substance. To allow solvents to volatilize, the test containers should remain open for a period of 1 h.

The concentrations selected to provide the NOEC are based on the results of the preliminary test (7.1).

NOTE - Substances do not need to be tested at concentrations higher than 1000 mg/kg d.w. of the test substrate.

Determine the water content and the pH (0.01 M $CaCl_2$) of the artificial soil at the beginning and end of the test (when acid or basic substances are added, do not adjust the pH).

Proceed simultaneously with at least four replicates per concentration and a control test without the test substance and if necessary with an additional solvent control (see 7.2.2).

7.2.2 Control container:
Prepare control containers in the same way as the test containers. If the preparation of the

test requires the use of a solvent (see 7.2.1) use an additional control prepared with solvent but without the test substance.

7.2.3 Introduction of the biological material:
For each test container and the control container 10 (*Eisenia fetida*) or 6 earthworms (*Aporrectodea caliginosa, Allolobophora chlorotica*) are carefully cleaned in water, softly blotted on adsorbent paper to remove excess water and weighed individually. Then add them to the test container.

Cover the container as indicated in 5.1. Place the container in the test enclosure (6).

7.2.4 Determination:
One day after application of the test substance, spread 0.5 g per earthworm of air dried finely ground food (see 5.3) on the soil surface and moisten it with water (about 5-6 cm^3 per container). Food for *A. caliginosa* and *A. chlorotica* should then be mixed in the topsoil. Feed once a week during the test period. If food consumption is low, reduce feeding. Record feeding behaviour and the quantity of food applied over the test period for each test container.

Maintain the water content of the soil substrate in the test containers during the test period by reweighing the test containers periodically and if necessary replenishing lost water. Due to an increase in biomass the weight of the test containers at the end of the test should not be lower than at the start of the test.

Keep adult earthworms over a period of 4 weeks in the test substrate. At the end of this period remove adults and record the total number and mass of living adults earthworms, the water content and the pH of the artificial soil for each container. Keep the test containers for another period of 4 weeks in the test environment to allow offspring to develop. Continue feeding at reduced rates according to the amounts of food consumed during the preceeding week.

After this period, count by a suitable method the number of offspring per test container hatched from the cocoons. Alternatively cocoons can be sorted out of the test substrate after 4 weeks together with the adults. They should be transfered to petri dishes containing moistened filter paper. Record the number of hatching juveniles and the number of infertile cocoons during the next 4 weeks.

7.3 Reference substance
The concentration of the reference substance should reduce the number of juveniles produced after a period of 8 weeks by at least 30% compared to the control. Benomyl and Carbendazim, at a concentration of 2-2.7 mg active ingredient/kg d.w. soil have been shown in a ring test to affect reproduction.

8 Calculation and expression of results

8.1 Calculation

For each concentration, determine the percentage mortality and percentage loss or increase in biomass of the adults and number of offspring produced in the final test. Compare means by suitable statistical methods and test for significance ($a \leq 0.05$) of difference from control.

8.2 Expression of results

Indicate, in mg/kg d.w. soil substrate, the highest concentration tested without mortality, significant change in biomass of adults and significant reduction in number of offspring (NOEC) and the lowest concentration with effects (LOEC).

9 Validity of the test

The results are considered to be valid if:

- The percentage mortality of the adults observed in the control is less than 10%
- A loss in biomass of the earthworms in the control does not exceed 20%
- A minimum reproduction of 30 juveniles per control container is achieved
- The coefficient of variance does not exceed 50% in the control

10 Test report

The test report shall refer to this standard and, in addition to the results expressed as in 7.2, shall provide the following information:

- complete description of the biological material employed (species, age, mass range, breeding conditions, supplier)
- method of preparation of the test substrate, and any auxiliary substances used for a low-/non-water-soluble substance
- description of the food and quantities applied; results obtained with the reference substance, if performed; detailed conditions of the test environment
- table giving the percent mortality obtained for each container for each concentration and for the control
- mass of live adult earthworms at the beginning of the test and after a period of 4 weeks
- number of offspring per test container at the end of the test
- the highest concentration causing no observed effects (NOEC)
- the lowest concentration causing effects (LOEC)
- description of obvious pathological symptoms or distinct changes in behaviour (e.g. reduced feeding activity) observed in the test organisms per test container
- description of the feeding activity in the course of the study

- water content and pH of artificial soil at start, after 4 weeks and at end of the test for the control, the lowest and the highest concentration
- all operating details not specified in the standard, and any occurrence liable to have affected the results
- Some specimens used in the test should be preserved for future reference

11 References

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

Sims, R.W. & Gerard, B.M. (1985): Earthworms. In: Kermack, D.M. Barnes, R.S.K. (Eds.): Synopses of the British Fauna, No. 31. E.J. Brill Publishing Company, Leiden, The Netherlands.

12 Supplement

Example of a breeding technique for *Eisenia fetida*

Eisenia fetida can be bred in a wide range of animal wastes. The recommended breeding medium is a 50 : 50 mixture or horse or cattle manure and peat. The medium should have a pH value of about 6 to 7 (regulated with calcium carbonate), a low ionic conductivity (less than 6 ms or less than 0.5% salt concentration) and should not be contaminated excessively with ammonia or animal urine. The substrate should be moist but not too wet. Breeding boxes or any other shallow containers of 10 litre to 50 litre volume are suitable.

To obtain earthworms of standard age and mass, it is best to start the culture with cocoons. Therefore adult earthworms are put in a breeding box with fresh substrate to produce cocoons and are removed after a period of 14 to 28 days. These must be used for further breeding batches. The earthworms hatched from the cocoons are used for testing when mature.

Breeding is preferably carried out in a climatic chamber at 20 ± 1 °C. At this temperature earthworms become mature after 2 to 3 months.

5 Sublethal toxicity test with the oribatid mite *Platynothrus peltifer* (Koch, 1839) (Oribatida: Acari)

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Detailed report of the contractor/subcontractor

5.1 Objectives for the reporting period

- To further develop an extraction technique allowing for the recovery of adult and juvenile mites from artificial soil (OECD) and LUFA 2.2 soil
- To perform acute and sublethal toxicity tests with copper, LAS and dimethoate in artificial soil (OECD)
- To improve the culturing method of *Platynothrus peltifer* (Koch 1839) (Oribatida: Acari)
- To collect data on the life history of *Platynothrus peltifer*

5.2 Objectives for the next period

- To finish sublethal toxicity studies with dimethoate in artificial soil (OECD)
- To perform acute and sublethal toxicity studies with copper, LAS and dimethoate in LUFA 2.2 soil
- To improve methods for maintaining and culturing *Platynothrus peltifer* in the laboratory
- To investigate the influence of abiotic conditions on the sensitivity of *Platynothrus peltifer* for LAS and dimethoate
- To finalize the test protocol for *Platynothrus peltifer*
- To publish the results of all studies on *Platynothrus peltifer*

5.3 Main results obtained

5.3.1 Development of culturing methods:

First attempts to start a culture showed that *Platynothrus peltifer* will produce eggs on plaster of Paris and juveniles emerge from these eggs. Unfortunately, due to excessive growth of fungi, the culture broke down and the juveniles did not mature. New attempts to start a culture were more succesful, but only few juveniles (less than 1%) reached the adult stage. Especially in their first life stages, juveniles appeared to be very vulnerable to treatment, and is was therefore difficult to clean the culture boxes without harming the juveniles. Furthermore, cleaning the culture boxes and at regular intervals transferring animals to new culture boxes appeared to be very time consuming.

P. peltifer can easily be collected in the field in large numbers, and can be maintained in the laboratory for long time periods before testing. The condition of the animals and their suitability for testing however, appeared to depend on the sampling time (biological synchronization).

5.3.2 Further development of the extraction methods

As described in the Second Technical Report (Wiles et al., 1994), test containers were designed, which allow for an efficient extraction of the mites from the test soil at the end of an experiment without making it necessary to transfer the soil to other containers. In 1994, one of our Tullgren extraction apparatus was rebuilt to allow for a more efficient extraction of animals from test soils.

Several experiments were performed to determine optimal conditions for the recovery of adult mites from both artificial soil (OECD) and LUFA 2.2 soil. Recovery of juveniles could only be determined for tritonymphs. In artificial soil (OECD), recovery of tritonymphs was on average 96% (90-100%; n=6). Recovery of juveniles from LUFA 2.2 soil still has to be determined.

Long-term experiments with artificial soil (OECD) and LUFA 2.2 soil showed a decreasing recovery of adult mites with time, and only in one experiment with artificial soil (OECD) juveniles appeared in the extracts. This demonstrates that *P. peltifer* can reproduce in this soil. For both soils amendment with green algae enhanced recovery of adults. Despite this, recovery in both soils decreased with time with only 30-50% of the adults being recovered after a 10 week incubation period.

To find a way to increase survival of *P. peltifer* in the course of a soil test, experiments were performed with different food sources. Amendment of artificial soil (OECD) with fungi (*Cladosporium* sp.) or 1% green algae 4 weeks before or immediately before the test showed that food supply does not affect recovery of *P. peltifer*. The number of juveniles was significantly higher with green algae as a food source. Fresh green algae appeared to give more juveniles than old algae, and much more than fungi, but the best result was obtained when additional algae were given in the course of the experiment.

To determine the influence of soil pH on *P. peltifer*, artificial soil (OECD) was made up with soil pH values of approximately 4.4, 5.5, 6.3, 6.6 and 6.9. After 6 weeks recovery of adult mites had decreased to 15-40% and was 5-20% after 10 weeks. Although differences between the soils were small, recovery was highest at pH 4.4. After 6 weeks, a few juveniles were found at all pH levels except for pH 6.9, while after 10 weeks some juveniles were found at pH 4.4, 6.3 and 6.9. The juvenile numbers however, were too low to allow for a conclusion about pH effect on reproduction.

5.3.3 Toxicity tests in soil

Toxicity of LAS and copper to *P. peltifer* was determined in artificial soil (OECD). In the copper experiment, metal analyses were carried out to check soil concentrations and to determine copper levels in the surviving animals. In both experiments, control survival decreased with time and was approx. 25 and 44% after 8 weeks in the copper and LAS experiments, respectively. LC_{50} values for both copper and LAS decreased with time, but tended to stabilize after 6 weeks. For copper, a lethal body concentration (internal LC_{50}) of 250 mg Cu/kg dry body weight could be estimated.

In these experiments, no juveniles were produced. Therefore, it is not possible to draw conclusions about the effects of copper and LAS on the reproduction of *P. peltifer* in artificial soil (OECD).

Range-finding toxicity experiments with dimethoate were performed in both artificial soil (OECD) and LUFA 2.2 soil. Results are included in Table 5.1.

5.3.4 Additional toxicity experiments

A food choice experiment was carried out with copper, using concentrations of 0, 2000 and 6300 mg/kg. *P. peltifer* significantly avoided food with 2000 or 6300 mg Cu/kg and preferred the low concentration when only treated food was offered. Significant mortality occurred when the mites were offered food with the highest copper concentration, and copper concentrations in the mites increased with increasing copper concentrations in the food. Although the mites were able to avoid food, they did consume contaminated food and strongly accumulated copper resulting in a lethal body concentration of 250-300 mg/kg dry body weight. This value is in agreement with the one found in the soil tests.

A study on the influence of temperature on the toxicity of copper on *P. peltifer* was carried out on plaster of Paris, using temperatures of 12, 16 and 20 °C. From the preliminary results, it can be concluded that toxicity slightly increases with increasing temperature. Internal copper concentrations in the mites seemed, however, not to be affected by temperature. Lethal body concentration was provisionally estimated to be 200-400 mg/kg dry body weight, which is in agreement with earlier findings. It also became evident that a test temperature of 20 °C is too high for *P. peltifer*, as control mortality at this temperature was much higher than at the lower ones. Results on egg production and food consumption still have to be evaluated.

Table 5.1: Summary of data on the toxicity of copper, LAS and dimethoate for *Platynothrus peltifer* obtained on plaster of Paris and in two different soils (duration of all tests is between 8 and 11 weeks).

Chemical	parameter	OECD artificial soil (mg/kg dry soil)	LUFA 2.2 soil (mg/kg soil)	plaster of Paris (mg/kg d.w. food)
Copper	LC_{50}	320	-	1600
	NOLC	200	-	-
	NOECreprod.	-	-	200
LAS	LC_{50}	1720	-	> 10.000
	NOLC	1000	-	10.000
	NOECreprod.	-	-	1.000
Dimethoate	LC_{50}	1.3#	1.0	-

5 weeks

The toxicity of LAS for *P. peltifer* was also determined on plaster of Paris, with LAS provided in the food (green algae) over a period of 8 weeks. No significant effect on survival was found at the highest concentration tested. Egg production showed a dose-related decrease, but was not significantly reduced at the highest concentration. The number of juveniles was higher than the number of eggs counted, demonstrating that some eggs hidden in holes and crevices in the plaster of Paris were overlooked. The average egg hatching time was 25-30 days. The number of juveniles was significantly reduced ($p=0.05$) at 10.000 mg LAS/kg dry food.

From Table 5.1, it can be concluded that both copper and LAS are much more toxic for *P. peltifer* when mixed in with the soil than when provided in the food. Maybe this is due to a reduced bioavailability of the chemicals in food since algae is 100% organic matter compared to 10% organic matter in soil. These results might however, also suggest that additional exposure of mites may occur via the soil pore water.

ANNEX 5.1 - Test System Standardization Form 4

Sublethal toxicity test with the oribatid mite *Platynothrus peltifer* (Koch, 1839) (Oribatida: Acari)

General data about the test organism at the start of the experiment

Test organism

Taxonomic group:	Oribatid mites (Oribatida: Acari)
Species:	*Platynothrus peltifer* (Koch, 1839)
Life stage:	adult (in future, also juveniles may be used for the test)
Sex:	parthenogenetic
Age/weight/size:	about 1 mm
Genetical composition:	not known
Background:	sampled in the field

Type of surroundings and ambient climate

Surroundings	
Type:	enclosure
Ambient climate	
Air temperature:	18 ± 1 °C (the suitability of a temperature of 15 °C needs further investigation)
Relative humidity:	75 ± 10%
Light wave length:	no specification needed; normal TL light is suitable
Light intensity:	400-800 lux
Light regime:	light:dark cycle 12:12 h

Type of test system and test conditions

Description of test system	
Type:	test container
Material:	plastic ring (diameter 5 cm; height 3.5 cm), with gauze on the bottom (1 mm mesh size)
Openness:	lid with an opening covered with plankton gauze
Spec./test system:	single species test system
Ind./test system:	20

Type of habitat

Soil/litter:	artificial soil (OECD), made up with sieved air-dried peat and modified by adding green algae, or natural soil (LUFA 2.2)
Medium:	artificial culture medium
'Habitat characteristics'	
Soil charact.:	modified artificial soil (OECD) consists of sphagnum peat (9%), kaolin clay (20%), sand (70%), green algae (1%), and some $CaCO_3$; work in progress on the influence of soil characteristics.
Hydrol. charact.:	moisture content 30-40% of water holding capacity
Food supply:	yes, algae mixed in the soil (see above)
Feeding rate:	not applicable
Type of food:	green algae, sampled in the field from tree bark

Exposure characteristics

Exposure route:	via soil
Composition of test soil:	artificial or natural soil with deionized water, test substance and auxiliary agent (if used), all constituents well homogenized
Composition of control soil:	a) artificial or natural soil and deionized water b) artificial or natural soil, deionized water and auxiliary agent

Duration of the experiment

Acclimat. time:	not relevant
Exposure time:	needs further investigation; depends on endpoints (survival or reproducion)
Recovery period:	not relevant

Exposure concentrations

Replicates:	5
Concentrations:	5
Series:	1
Ref. chemical:	work in progress

Exposure responses

Effect parameter:	reproduction (number of offspring produced)
Other parameters:	survival of adults
Measurement of variables:	moisture content and pH of the soil at start and end of the test
Toxic endpoint:	NOEC and EC_{50} for reproduction; LC_{50}
Unit:	mg test substance/kg d.w test soil
Working methods:	use Tullgren apparatus for extraction of mites from soil at end of test
Analytical methods:	not relevant yet

Miscellaneous test information

Practicability:	test containers can easily be constructed; Tullgren apparatus may not belong to the standard equipment of a commercial laboratory. Animals cannot be obtained from commercial hatcheries; at present, best way to obtain animals is by extraction from leaf or needle litter sampled in the field. Work on a culture method is in progress. Identification of the mites, and especially of the different juvenile stages needs some training.
Cost-effectiveness:	at present, both culture and test are rather laborious.
Sensitivity:	work in progress
Reproducibility:	work in progress
Validity of the test:	work in progress
Standardization:	work in progress
Ecological realism:	*Platynothrus peltifer* is a common oribatid species, which may be found in most deciduous and coniferous forests in Europe.
Broad chemical responsiveness:	no restrictions are given with respect to the chemical substances that may be evaluated by this test.

ANNEX 5.2 - Draft test protocol 4.1

Sublethal toxicity test with the oribatid mite *Platynothrus peltifer* (Koch, 1839) (Oribatida: Acari) in soil

1 Scope

This guideline describes a method for determining the effects on survival and reproduction of *Platynothrus peltifer* (Koch, 1939) (Balogh & Mahunka, 1983) exposed to a substance in a defined artificial soil (OECD) substrate treated with a defined amount of that substance.

2 Definitions

2.1 Survival
Mean number of adults surviving till the end of the test period (10 weeks).

2.2 Reproduction
Mean numbers of offspring produced during the test period (10 weeks).

2.3 NOEC (No Observed Effect Concentration)
The highest tested concentration of a substance at which no lethal or other effect is observed, expressed as mass of test substance per dry mass of the test substrate.

3 Principle

Adult mites are placed in a defined artificial soil (OECD) substrate containing the test substance in different concentrations. The percentage of survival and reproduction of adult mites (*Platynothrus peltifer*) is determined. Surviving adults are counted after exposure periods of 2, 6 and 10 weeks, and number of juveniles produced are counted after 6 and 10 weeks.

Optionally, the test may be considered an acute toxicity test with assessment of effects on survival after only 2 weeks.

4 Reagents

4.1 Sampling and culturing of animals
The oribatid mite *Platynothrus peltifer* may be collected from soil cores or litter, taken in a pine or deciduous wood, and extracted using Tullgren-type equipment, as described, for example, by Van Straalen & Rijninks (1982). To collect the mites alive, the funnels of the Tullgren apparatus (5.2) are equipped with vials with a moist plaster of Paris bottom. Following daily

inspection of the vials under a microscope, mites are collected using a fine brush and transferred to culture pots.

Platynothrus peltifer may also be cultured in the laboratory, but due to its long life cycle and low rate of reproduction, it is difficult to obtain sufficient numbers for experiments.

Before use in experiments the animals have to be kept in the laboratory for at least two weeks, to ensure acclimatization. Cultures have to be kept at a temperature of approximately 18 ± 1 °C.

It is advisable to preserve some biological material for later identification of the test species.

4.2 Test substrate

4.2.1 Soil substrate:
The soil substrate should be composed of (on dry weight basis):

9%	Sphagnum peat (air dry) (mesh size <1 mm)
1%	Algae (see food), (when starting the experiment)
20%	Kaoline clay
70%	Industrial quartz sand, (predominantly fine sand with more than 50% of particle size 0.05 - 0.2 mm)

Add sufficient (about 0.5%) calcium carbonate ($CaCO_3$), pulverized recognized analytical grade to bring the pH (measured in 1 mol/l KCl solution) to 6.0 ± 0.5 .

The mass of soil substrate should be 7.5 g (dry weight) per test container.

4.2.2 Artificial soil (OECD):
Artificial soil (OECD) is prepared by wetting the soil substrate with deionized water to reach a water content of 50% of the total water holding capacity (this should be done about 1 week before starting the experiment).

4.3 Food

The food used in experiments with *P. peltifer* is green algae, which have to be sampled from a non-contaminated site by scraping them from trees and collecting them in a container. The algae must be air-dried for 72 hours spread on a tray. After drying and picking out small pieces of wood, the algae have to be sieved through a sieve, mesh size 0.5 mm. The throughfall is collected and stored at a temperature of 4 °C in the dark. In this way the algae can be stored for about 4 months.

5 Apparatus

Standard laboratory equipment, and:

5.1 Containers

Containers with a diameter of 5 cm and a height of 3 cm. The bottom con-

tains gauze with a mesh size of 2 mm (sealed with plastic foil). The top is a closed lid (which may have a hole in it covered with cheese cloth for aeration).

5.2 Tullgren apparatus
Tullgren apparatus to extract the mites from the soil. This apparatus has a temperature of 30 °C in the upper compartment and of 5 °C in the lower one.

5.3 Apparatus
Apparatus capable of measuring the dry mass of the substrate.

6 Test environment

6.1 Enclosure
Enclosure, controllable to a temperature of 18 ± 1 °C.

6.2 Light source
Light source capable of delivering a constant illuminance of approximately 400 lux at a controlled light:dark cycle of 12:12 h.

7 Procedure

7.1 Preliminary test
If necessary to determine the range of concentrations for use in the final test, perform a preliminary acute test for four concentrations of the test substance and a control (for example 0, 1, 10, 100 and 1000 mg/kg, the concentrations being expressed in milligrams of test substance per kilogram of dried soil substrate (4.2.1)) on 20 adult mites per concentration and per test container.

7.2 Final test
7.2.1 Introduction of the test substances:
Use either method a), b) or c), as appropriate.

a) Water soluble substances
Immediately before starting the test, dissolve the quantity of the test substance required for the replicates of one concentration in water (or that portion that is necessary to wet the soil substrate in order to meet the requirements of 4.2.2 and mix it thoroughly with the test substrate before introducing it into a test container.

b) 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).

Add it to a small portion of quartz sand or dry soil substrate, mix thoroughly and evaporate the solvent by placing it under a fume hood for 1 hour. Then add the remainder of the soil substrate (4.2.1) and the water in accordance with 4.2.2. and mix thoroughly.

c) 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 4.2.1) or 10 g of the dry soil substrate and the quantity of the test substance required to obtain the desired concentration. Place the mixture, the remainder of the soil substrate (4.2.1) and the water into a beaker and mix thoroughly before introducing it into the test container (5.1).

Mix the test substance into the artificial soil (OECD) before the mites are introduced.

NOTE - ultrasonic dispersion, organic solvents, emulsifiers or dispersants may be used to disperse substances with low aqueous solubility. When such auxiliary substances are used, all test concentrations and an additional control should contain the same minimum of auxiliary substance. To allow solvents to volatilize, the test containers should remain open for a period of 1 h.

The concentrations selected to provide the NOEC are based on the results of the preliminary test (7.1).

NOTE - Substances do not need to be tested at concentrations higher than 1000 mg/kg d.w. of the test substrate.

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

Proceed simultaneously with at least five replicates per concentration and a control test without the test substance and if necessary with an additional one (see 7.2.2) and placing the containers in the test environment (see item 6). Prepare such a series of test and control containers for each determination time (7.2.4).

NOTE: It is advisable to prepare an additional 2 containers per concentration and control to determine recovery immediately after the start of the experiment (t=0). The containers should be extracted in the Tullgren apparatus (5.2) immediately after the mites have been introduced.

7.2.2 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 7.2.1) use additional control containers similar to the test containers without the test substance. Treat these containers in the same way as those without the test substance. Include 2 additional containers for the control and each treatment for pH and soil moisture determination at the end of the test.

7.2.3 Addition of the biological material:
For each test container and the control container, take 20 adult mites (4.1); use a fine brush to transfer them from the culture or laboratory stock to the test or control soil. Place them on the substrate surface in each test container and the control container. Close the containers as indicated in 5.1, and determine the mass of each container.

7.2.4 Determination:
Maintain the water content of the soil substrate in the test containers during the test period by reweighing the test containers periodically (once a week) and if necessary replenishing lost water. At the end of the test the water content shall not differ by more than 10% from that at the beginning of the test.

Feed the animals every 2 weeks by placing 0.1 g algae (see food; 4.3) on the soil surface of each test and control container.

After 2 weeks, a complete series of five replicate containers of each treatment and control group is placed in the Tullgren apparatus (5.2) and adult mites are extracted to determine the effect on survival. This procedure is repeated after 6 and 10 weeks; at these times also juveniles may appear in the extracts. Count the number of adults and juveniles extracted from each sample.

7.3 Reference substance
Work in progress.

8 Calculation and expression of results

8.1 Calculation
For each concentration, determine the percent mortality and number of offspring produced in the final test.

Compare means by suitable statistical methods, e.g. Williams, Dunnett's or Student's t-test and test for significance (a = 0.05) of difference from control(s).

Calculate LC_{50} for survival and EC_{50} for effects on number of juveniles produced, when results show a consistent concentration-effect relationship. The trimmed Spearman-Karber method (Hamilton et al., 1977/1978) may be used for calculation of LC_{50} values at each sampling time; for calculation of a time-related LC_{50} the method proposed by Kooijman (1981) may be used. For calculation of EC_{50} values, logistic models such as the one proposed by Haanstra et al. (1985) are recommended.

8.2 Expression of results
Indicate, in mg/kg dry weight soil substrate, the highest concentration tested without mortality and significant reduction in numbers of offspring (NOEC) and the lowest concentration with effects (LOEC).

9 Validity of the test

Work in progress.

10 Test report

The test report shall refer to this standard and, in addition to the results expressed as in 7.2, shall provide the following information:

- complete description of the biological material employed (species, source, etc.)
- method of preparation of the test substrate, and any 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 percentage mortality obtained for each container for each concentration and for the control
- number of offspring per test container at the end of the test (not if the test is restricted to an exposure period of 2 weeks)
- the LC_{50} for the effect on survival after 2 weeks (in case of an acute toxicity test) and if possible also after 6 and 10 weeks
- the EC_{50} for the effect on the number of juveniles produced after 6 and 10 weeks of exposure
- the highest concentration causing no observed effects (NOEC) and LOEC
- water content and pH of the artificial soil (OECD) at start and at the end of the test
- all operating details not specified in the standard, and any occurrences liable to have affected the results
- some specimens used in the test should be preserved for future reference

11 References

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Sublethal toxicity test with the oribatid mite *Platynothrus peltifer* (Oribatida: Acari) on plaster of Paris with dietary exposure

1 Scope

This guideline describes a method for determining the effects on survival and reproduction on *Platynothrus peltifer* (Koch, 1839) (Balogh & Mahunka, 1983) exposed to a chemical substance via dietary exposure to green algae treated with a defined amount of that chemical substance.

2 Definitions

2.1 Survival
Mean number of adults surviving till the end of the test period (10 weeks).

2.2 Reproduction
Mean numbers of eggs produced during the test period (10 weeks).

2.3 NOEC (No Observed Effect Concentration)
The highest tested concentration of a substance at which no lethal or other effect is observed, expressed as mass of test substance per dry mass of the test substrate.

3 Principle

Adult mites are placed on plaster of Paris and provided with green algae containing the test substance in different concentrations. The percentage of survival and reproduction of adult mites is determined. Surviving adults and number of eggs produced are counted at weekly intervals for a period of 10 weeks.

NOTE: It should be noted that the number of eggs may be difficult to assess because the mites tend to hide the eggs in holes and crevices in the plaster of Paris. A more reliable estimate of reproduction may be obtained by incubating test containers for another 4-6 weeks and counting the number of juveniles emerging from the eggs. The disadvantage of this method, however, is that new test containers have to be prepared for each determination time.

Optionally, the number of faecal pellets produced can be counted; this seems to give an estimate of toxicity which is as reliable as the number of eggs produced.

4 Reagents

4.1 Sampling and culturing of animals

The oribatid mite *Platynothrus peltifer* may be collected from soil cores or litter, taken in a pine or deciduous wood, and extracted using Tullgren-type equipment, as described, for example, by Van Straalen & Rijninks (1982). To collect the mites alive, the funnels of the Tullgren apparatus are equipped with vials with a moist plaster of Paris bottom. Following daily inspection of the vials under a microscope, mites are collected using a fine brush and transferred to culture pots.

Platynothrus peltifer may also be cultured in the laboratory, but due to its long life cycle and low rate of reproduction, it is difficult to obtain sufficient numbers for experiments.

Before use in experiments the animals have to be kept in the laboratory for at least two weeks, to ensure acclimatization. Cultures have to be kept at a temperature of approximately 18 ± °C.

4.2 Food

4.2.1 Sampling green algae:
The food used in experiments with *P. peltifer* is an algal suspension. Green algae have to be sampled from a non-contaminated site, by scraping them from trees and collecting them in a container. The algae must be air-dried for 72 hours spread on a tray. After drying and picking out small pieces of wood, the algae have to be sieved over 0.5 mm. The throughfall is collected and stored at a temperature of 4 °C in the dark. In this way the algae can be stored for about 4 months.

4.2.2 Preparation of algal paste:
Before using the algae for contamination of the food, they have to be moistened. Press algae through a gauze with a mesh size of 0.05 mm in deionized water. The algae solution is filtered through a filter paper using a "Buchner-funnel", equipped with a tap water-driven vacuum pump, until cracks appear in the algae paste on the filter paper. Determine the water content of the paste, to facilitate calculations for the solutions to be prepared for contamination.

5 Apparatus

Standard laboratory equipment, and:

5.1 Containers

Containers consisting of perspex rings with a diameter of 1.5 cm and a bottom of plaster of Paris. The top is a closed lid, which may have a hole in it covered with cheese cloth for aeration.

NOTE: It is advisable to use pink plaster of Paris (e.g. used by dentists), as this may facilitate the counting of the white eggs produced by *P. peltifer*.

6 Test environment

6.1 Enclosure

Enclosure, controllable to a temperature of 18 ± 1 °C.

6.2 Light source

Light source capable of delivering a constant illumination of approximately 400 lux at a controlled light:dark cycle of 12:12 h.

7 Procedure

7.1 Preliminary test

If it is necessary to determine the range of concentrations for use in the final test, perform a preliminary acute test for four concentrations of the test substance and a control (for example 0, 1, 10, 100 and 1000 mg/kg, the concentrations being expressed in milligrams of test substance per kilogram of dried algal substrate (4.2)), 5 adult mites per test container, 10 test containers per concentration.

7.2 Final test

7.2.1 Introduction of the test substances:
Concentrations have to be based on mg/kg d.w. of the algae. For each treatment a solution of the test substance in water or, when not water soluble, in acetone or alcohol is prepared, and kept in a refrigerator. Calculate the amounts of substance necessary per gram dry weight of food, to obtain the desired dose, taking the water content of the algal paste (4.2.2) into account (typically 80%). Mix 0.5 g of fresh (moist) paste with 2 ml of deionized water (or solvent) to obtain a suspension with a suitable consistency. Apply the suspension to filter paper discs (diameter 0.5 cm), 10 µl per disc, 10 discs per concentration. Excess of water should be absorbed by the filter paper. The discs with algae paste are then ready for transfer to the experimental containers (5.1); use one disc per container.

When the substance to be tested is not water-soluble, contaminated algae suspensions have to be prepared one day before use. Place the suspensions for 1 hour in a fume hood to evaporate the solvent. Mix the algal paste with 2 cm^3 of deionized water and prepare for application to filter paper discs and transfer to the containers.

NOTE - The amount of contaminant solution per treatment necessary depends on the duration of the experiment. For each week 2 cm^3 solution per treatment is needed. When stability of the test substance can be guaranteed, contaminant solutions may be prepared for the whole test period and kept in the refrigerator. If stability cannot be guaranteed, prepare new solutions every week.

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

The concentrations selected to provide the NOEC are based on the results of the preliminary test (7.1).

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

Proceed simultaneously with at least 10 replicates per concentration and a control test without the test substance and if necessary with an additional one and placing the containers in the test environment 6.

OPTION - as an alternative, 5 replicates per concentration each containing 10 mites may be used.

7.2.2 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 7.2.1) use additional control containers similar to the test containers without the test substance. Treat these conditions in the same way as those without the test substance.

7.2.3 Addition of the biological material:
For each test container and the control container, take 5 adult mites (4.1); use a fine brush to transfer them from the culture or laboratory stock to the test or control containers.

7.2.4 Determination:
Determine the number of dead animals each week. To ascertain the death of an individual, place it on a clean place of the substrate and observe whether it has moved from its place after 5 min. Place the adults in new test containers, and replace the food discs once a week with freshly prepared ones (see 7.2.2).

NOTE - A test duration of 10 weeks is recommended. When the LC_{50}s, estimated for two successive weeks, do not differ by more than a factor of 2, the experiment may however, be finished sooner.

After transfer of the adults to new test containers, count the number of eggs produced.

NOTE - Instead of counting the eggs, the test containers can be incubated for another 4-5 weeks after which the number of juveniles emerging from the eggs can be counted. Optionally, the number of faecal pellets can be counted as a measure of consumption.

7.3 Reference substance
Work in progress.

8 Calculation and expression of results

8.1 Calculation
For each concentration, determine the percentage mortality and number of eggs (or juveniles and/or faecal pellets) produced in the final test.

Compare means by suitable statistical methods, e.g. Williams, Dunnett's or Student's t-test and test for significance (a = 0.05) of difference from control(s).

Calculate LC_{50} for survival and EC_{50} for effects on number of eggs produced. The trimmed Spearman-Karber method (Hamilton et al., 1977/1978) may be used for calculation of LC_{50} values at each sampling time; for calculation of a time-related LC_{50} the method proposed by Kooijman (1981) may be used. For calculation of EC_{50} values, logistic models such as the one proposed by Haanstra et al. (1985) are recommended.

8.2 Expression of results
Indicate, in mg/kg d.w. of algal substrate, the highest concentration tested without mortality and significant reduction in numbers of eggs (or juveniles or faecal pellets) (NOEC) and the lowest concentration with effects (LOEC).

9 Validity of the test

Work in progress.

10 Test report

The test report shall refer to this standard and, in addition to the results expressed as in 7.2, shall provide the following information:

- complete description of the biological material employed (species, source, etc.)
- method of preparation of the test substrate, and any auxiliary substances used for a low-water-soluble substances
- results obtained with the reference substance, if performed
- detailed conditions of the test environment
- table giving the percentage mortality obtained for each container for each concentration and for the control
- number of eggs (or juveniles and/or faecal pellets) per test container at the end of the test
- the LC_{50} for the effect on survival
- the EC_{50} for the effect on the number of eggs (or juveniles and/or faecal pellets) produced
- the highest concentration causing no observed effects (NOEC) and LOEC
- all operating details not specified in the standard, and any occurrences liable to have affected the results
- some specimens used in the test should be preserved for future reference

11 References

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6 Sublethal toxicity test based on the competition between two bacterivorous nematodes

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6.1 Objectives for the reporting period

- To measure the effect of copper, dimethoate and LAS on life-history traits for two bacterivorous nematodes *Plectus acuminatus* (Bastian 1865) and *Heterocephalobus pauciannulatus* (Marinari 1967) (Nematoda: Plectidae) in complete life cyle toxicity tests in agar
- To conduct toxicity tests in artificial soil (OECD) for both species
- To gather life-history data for all species used in the SECOFASE project in order to estimate the fitness for each species

6.2 Objectives for the next period

- To estimate the effect of LAS on life-cycle variables of *P. acuminatus*
- To study the effect of copper and LAS on the competition between *P. acuminatus* and *H. pauciannulatus* in artificial soil and to identify suitable effect parameters
- Based on experimentally obtained life-history data and data from literature, a model approach will be applied to determine which life cycle variables are important from a life-history perspective
- To finalise the test protocol and standardization forms

6.3 Main results obtained

6.3.1 Methodology

Life cycle experiments

Toxicity experiments were conducted in multi compartment plates (Greiner) each containing 24 agar droplets (80 µl, 0.5% agar) with a bacterial density (*Acinetobacter johnsonion*) of 2.10^8 cells/ml. The following concentration (µM) ranges were used for i) copper: 0.0, 12.5, 22.4, 40.4, 72.8 and 131.0 for *H. pauciannulatus* and 0.0, 9.7, 17.5, 31.4, 56.6 and 101 for *P. acuminatus*, ii) LAS: 0.0, 13.8, 24.9, 44.9, 80.7, 145.3 for *H. pauciannulatus*. Fourth stage juveniles were randomly selected from stock cultures and individually transferred to the agar plates. Each droplet contained one female which was transferred to fresh plates every 5 days. Females

were allowed to produce eggs for 24 h, after which they were removed to new plates. The number of eggs produced were recorded for each female during the complete reproductive period, and longevity of adults was registered. Eggs and juveniles were observed druing the complete juvenile period using a stereo-microscope (magnification 40 - 64). Fitness, defined as the intrinsic rate of population increase was estimated using specific algorithms in SAS.

Soil toxicity test
At the start of the test, 50 nematodes (*H. pauciannulatus*) were transferred to 5-6 g d.w. artificial soil (OECD) and 14 g d.w. LUFA 2.2 soil in a Petridish (6 cm diameter). Copper was mixed dry with sand after which it was homogenized with the other ingredients in tubes on a roller bank for 5 h. The following concentration ranges were used: 0, 32, 100, 320, 1000 mg/kg d.w.

Experiments were carried out at optimal conditions e.g. 20°C, 70% soil moisture content, pH_{KCl}=5.5, and two replicates were run in the dark. After three weeks the soil was washed and sieved and the number of juveniles and adults were counted in water using an inverted microscope.

The ratio between number of juveniles and adults was used as an effect parameter.

6.3.2 Results
From preliminary experiments it appeared that dimethoate did not have a significant effect on the nematode species used (Wiles et al., 1994). It was therefore decided not to proceed with this compound but instead focus on copper and LAS.

Life cycle experiments
Table 6.1 shows the values for the different life cycle variables and estimates for fitness (*P. acuminatus*) at different concentrations of copper. Significant effects were found on reproduction, NOEC=9.7 µM, reproductive period, NOEC=17.5 µM, juvenile survival NOEC=9.7 µM, and juvenile period, NOEC=31.4 µM. Fitness decreased from 0.15 d^{-1} in the control to 0.09 d^{-1} at 56.6 µM.

Table 6.1: Effect of copper (µM) on life-history traits of *P. acuminatus*. t_j: juvenile period (days), S_j: juvenile survival, n_t: daily reproduction (day^{-1}), t_r: reproductive period (days), S_r: survival over the reproductive period, r: fitness (day^{-1}). (*: significantly different from control p<0.05).

Copper µM	0	9.7	17.5	31.4	56.6	101
t_j	19.9	16.9	16.3	17.1	21.8 *	∞
n_t	8.6	7.5	5.3 *	4.9 *	2.5 *	-
S_j	0.73	0.79	0.64 *	0.49 *	0.33 *	-
S_r	0.57	0.80	0.78	0.98	0.99	-
t_r	51.8	56.1	44.2	19.4 *	17.6 *	-
r	0.15	0.18	0.16	0.14	0.09	-

Table 6.2 shows these data for *H. pauciannulatus*. Significant effects were found for juvenile period, NOEC=72.8 µM, and reproductive period, NOEC=40.4 µM. Fitness decreased from 0.41 d^{-1} in the control to 0.24 d^{-1} at 131.1 µM.

Table 6.2: Effect of copper (µM) on life-history traits of *H. pauciannulatus*. t_j: juvenile period (days), S_j: juvenile survival, n_t: daily reproduction (day^{-1}), t_r: reproductive period (days), S_r: survival over the reproductive period, r: fitness (day^{-1}). (*: significantly different from control $p<0.05$).

Copper µM	0	12.4	22.4	40.4	72.8	131
t_j	7.5	7.3	8.0	7.7	8.1	10.2 *
n_t	11.7	9.2	11.2	9.3	10.3	9.8
S_j	0.92	0.82	0.89	0.82	0.81	0.86
S_r	0.50	0.65	0.73	0.55	0.56	0.59
t_r	40.9	36.9	40.8	35.4	31.3 *	24.2 *
r	0.41	0.40	0.39	0.39	0.34	0.24

Complete life-cycle studies for *H. pauciannulatus* which focused on the effect of LAS revealed a NOEC=24.9 µM for reproduction.

Soil toxicity test
After extraction from artificial soil (OECD) and LUFA 2.2 soil, it appeared that the ratio between juveniles and adults was not clearly dose-related in the artificial soil (OECD). However, in LUFA 2.2 soil, a strong response was found with the ratio decreasing from 15.1 in the control to 3.1 in the highest concentrations (NOEC=32 mg/kg).

6.3.3 Discussion
The results obtained indicate a marked difference between the susceptibility of the two nematode species. Life cycle studies were conducted successfully and it appears that for copper a strong discrepancy was observed in the effects between artificial soil (OECD) and LUFA 2.2 soil. Preliminary results obtained recently show great promise for a two-species nematode toxicity test in artificial soil (OECD) which is based on the present results of single species experiments.

ANNEX 6.1 - Test System Standardization Form 5

Sublethal toxicity test based on the competition between two bacterivorous nematodes

General data about the test organism at the start of the experiment

Test organism

Taxonomic group:	Nematode (Nematoda: Plectidae)
Species:	*Plectus acuminatus* (Bastian, 1865), *Heterocephalobus pauciannulatus* (Marinari, 1967)
Life stage:	adult
Sex:	only female, parthenogenetic
Age/weight/size:	*P. acuminatus* 20 days, *H. pauciannulatus* 8 days
Gen. composition:	-
Background:	laboratory

Type of surroundings and ambient climate

Surroundings	
Type:	climate chamber
Ambient climate	
Air temperature:	20 ± 1 °C
Relative humidity:	not relevant
Light wave length:	not indicated
Light intensity:	darkness
Light regime:	not indicated

Type of test system and test conditions

Type:	Petri dish
Material:	plastic
Openness:	air-tight closed
Spec./test system:	two species test system
Ind./test system:	work in progress

Type of habitat

Soil/litter:	artificial soil (OECD)
'Habitat characteristics'	
Soil:	10% peat, 20% kaolin clay, 70% sand, some $CaCO_3$ (d.w.)
Hydrol. charac.:	work in progress
Food supply:	yes
Feeding rate:	continuously
Type of food:	soil bacteria *Acinetobacter johnsonian* (2.10^8 cells/g)

Exposure characteristics

Exposure route:	via soil
Composition of test soil:	artificial soil (OECD) with water, test substance
Composition of control soil:	artificial soil (OECD) with water

Duration of the experiment

Acclimat. time:	work in progress
Exposure time:	work in progress
Recovery period:	work in progress

Exposure concentrations

Replicates:	work in progress
Concentrations:	work in progress
Series:	work in progress
Ref. chemical:	not proposed

Exposure responses

Effect parameters:	ratio of adults of the two species
Other parameters:	work in progress
Measurement of variables:	work in progress
Toxic endpoint:	EC-value, NOEC
Unit:	mg test substance /kg d.w. test soil
Working methods:	work in progress
Analytical methods:	not relevant

Miscellaneous test information

Practicability:	work in progress
Cost-effectiveness:	work in progress
Sensitivity:	work in progress
Reproducibility:	work in progress
Validity of the test:	work in progress
Standardisation:	work in progress
Ecological realism:	The two bacterivorous nematodes occur next to each other in the top layer of the soil. They are supposed to compete for bacterial food which is the main factor in regulating nematode community densities in the field
Broad chemical responsiveness:	work in progress

11 References

Oostenbrink, M. (1960): Estimating nematode populations by some selected methods. In: Sasser J.N. and Jenkins W.R. (eds.) Nematology. University of North Carolina Press, Chapel Hill, pp. 85-102.

Zell, H. (1993): Die Gattung Plectus, Bastian, 1865. Andrias 11, Staatliches Museum für Naturkunde Karlsruhe.

ANNEX 6.2 - Draft test protocol 5

Sublethal toxicity test based on the competition between the nematodes *Plectus acuminatus* (Bastian 1865) and *Heterocephalobus pauciannulatus* (Marinari, 1967), (Nematoda: Plectidae)

1 Scope

This guideline describes a method for the determination of the effects of a pollutant on the competition between two bacterivorous nematodes using a defined artificial soil substrate treated with a defined amount of that pollutant.

2 Definitions

2.1 Competition

The competition between two bacterivorous nematode species for one food source, namely *Acinetobacter johnsonion*. Measured as the ratio between the number of adults from each species after extraction from the soil.

2.2 NOEC (No Observed Effect Concentration)

The highest tested concentration of a test substance at which no lethal or other effect is observed, expressed as mass of test substance per dry mass of the test substrate.

3 Principle

The ratio between adult nematodes of the species *Plectus acuminatus* and *Heterocephalobus pauciannulatus* is determined from the total number of nematodes extracted from the test substrate. The test substance is added in a single step. The ratio is determined after 3 weeks of exposure to the chemical.

4 Reagents

4.1 Sampling and culturing of animals

The nematodes may be collected from arable soil or forest litter and extracted using Oostenbrink equipment (Oostenbrink, 1960).

Nematodes to be used for the test are obtained from age synchronized stock cultures. Therefore gnotobiotic stock cultures are reared in sloppy water agar (0.5%) in plastic Petridishes (6 cm diameter) with *Acinetobacter johnsonion*

mixed through the agar at an initial density of 2.10^8 cells/ml. Stock cultures are transferred to fresh agar plates every week to maintain fresh cultures.

The biological material for the test consists of adult females of the parthenogenetic species *P. acuminatus* (3 weeks old) and *H. pauciannulatus* (8 days old).

4.2 Test substrate
4.2.1 Soil substrate:
The soil substrate should be composed of (on d.w. basis):

10%	Sphagnum peat (air dry) (mesh size < 1 mm)
20%	Kaoline clay
70%	Industrial quartz sand (oven dry), (predominantly fine sand with more than 50 % of particle size 0.05 - 0.2 mm)

Add sufficient (about 0.5 %) calcium carbonate ($CaCO_3$), pulverized recognized analytical grade to adjust pH (measured in 1 M KCl) to 6.0 ± 0.5.

The mass of soil substrate used to make the artificial soil shall be 5.0 g per test container.

4.2.2 Artificial soil (OECD):
Artificial soil (OECD) is prepared by wetting the soil substrate with deionized water to reach a water content of 50% of the total water holding capacity (this should be done 3 weeks prior to testing).

Before testing, the artificial soil is heated for 2 minutes in a micro-wave oven to 80 °C in order to eliminate rid of unwanted nematodes which might be present in the substrate.

4.3 Food
The soil bacterium *Acinetobacter johnsonion* is used as food source, applied at an initial density of 2.10^8 cells/g d.w. on the onset of the test. The bacteria are applied together with the water.

5 Apparatus

Standard laboratory equipment and:

5.1 Petri dishes
Sterile Petri-dishes (6.0 cm diameter) which can be closed to prevent infections.

5.2 Dry mass measuring
Apparatus capable of measuring dry mass of the substrate.

5.3 Photospectrometer
Photospectrometer to measure the densities of bacteria at a wavelength of 560 nm.

5.4 Micro-wave oven
Micro-wave oven to heat the soil before testing to eliminate other unwanted nematodes.

5.5 Oostenbrink extraction
Oostenbrink-extraction method for the extraction of nematodes from the soil.

6 Test environment

6.1 Climatic chamber
Climatic chamber to control the temperature at 20 °C ± 1 °C in the dark for 24 h/day.

7 Procedure

7.1 Preliminary test
A range finding test is advised in accordance with the ISO 11268-1 for four concentrations of the test compound.

The number of nematodes used is approx. 50 - 100. This has yet to be determined exactly.

7.2 Final test
7.2.1 Introduction of the test substances:
Use either method a), b) or c), as appropriate:

a) Water soluble substances
Immediately before starting the test, dissolve the quantity of the test substance required for the replicates of one concentration in water (or that portion that is necessary to wet the soil substrate in order to meet the requirements of 4.2.2.) and mix it thoroughly with the test substrate before introducing it into the test dish.

b) 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). Add it to a small portion of quartz sand or dry soil substrate, mix thoroughly and evaporate the solvent by placing it under a fume hood for 24 hours. Then add the remainder of the soil substrate (4.2.1) and the water in accordance with 4.2.2. and mix thoroughly.

c) Substances insoluble in water and organic solvents
For a substance insoluble in a volatile solvent, prepare a mixture of 10 g of finely ground industrial quartz sand (see 4.2.1) or 10 g of the dry soil substrate and the quantity of the test substance required to obtain the desired concentration. Place the mixture, the remainder of the soil substrate (4.2.1) and the water into a beaker and mix thoroughly before introducing it into the test dish (5.1).

Mix the test substance into the artificial soil before the nematodes are introduced.

NOTE: ultrasonic dispersion, organic solvents, emulsifiers or dispersants may be used to disperse substances with low aqueous solubility. When such auxiliary substances are used, all test concentrations and an additional control should contain the same minimum of auxiliary substance. To allow solvents to volatilize, the test containers should remain open for a period of one hour.

The concentrations selected to provide the NOEC are based on the results of the preliminary test (7.1).

NOTE: Substances do not need to be tested at concentrations higher than 1000 mg/kg d.w. of test substrate.

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

Proceed simultaneously with at least five replicates per concentration and a control test without the test substance and if necessary with an additional one (7.2.2) and place each container in the test environment (see item 6). Prepare such a series of test and control dishes for each determination time (7.2.4)

7.2.2 Control:
Prepare dishes in the same way as the test dishes without the test substances. Any auxiliary compounds which are used in the tests should also be added to the control dishes.

7.2.3 Addition of biological material:
Work in progress.

7.2.4 Determination:
Work in progress.

7.3 Reference substance
To be determined.

8 Calculation and expression of results

Work in progress.

9 Validity of the test

Work in progress.

10 Test report

The test report shall refer to this standard and, in addition to the results expressed as in 7.2, shall provide the following information.

- complete description of the biological material employed (species, age, breeding conditions, supplier)
- method of preparation of the test substrate, and any 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 percent effect obtained for each dish for each concentration and for the control
- determination of the NOEC
- water content and pH of the artificial soil at start and end of the test
- all operating details not specified in the standard, and any occurrences liable to have affected the results
- some specimens used in the test should be preserved for future reference

11 References

Oostenbrink, M. (1960): Estimating nematode populations by some selected methods. In: Sasser J.N. and Jenkins W.R. (eds.) Nematology. University of North Carolina Press, Chapel Hill, pp. 85-102.

Zell, H. (1993): Die Gattung Plectus, Bastian, 165. Andrias 11, Staatliches Museum für Naturkunde Karlsruhe.

7 Sublethal toxicity test with the enchytraeid worm *Cognettia Sphagnetorum* (Vejdovsky, 1878), (Enchytraeidae: Oligochaeta)

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7.1 Objectives for the reporting period

- To establish a medium suitable for culturing the enchytraeid *Cognettia sphagnetorum* (Vejdovsky, 1878) (Enchytraeidae: Oligochaeta)
- To establish an optimum culturing temperature and soil moisture
- To study food selection of the species in order to be able to follow the responses over a long period of time
- To standardise handling and extraction of adult worms and fragments
- To define and quantify fragmentation
- To quantify survival of the P-generation in Cu-polluted soil
- To quantify fragmentation rate
- To establish a technique to follow the individual growth of fragments (F_1-generation)
- To determine the time period for the fragments to reach an adult stage
- To determine the time needed for a test on survival, fragmentation, and growth of the enchytraeid *C. sphagnetorum*
- To start a long-term test on the responses of *C. sphagnetorum* to Cu-polluted soil

7.2 Objectives for the next period

- To finish the studies on mortality, fragmentation rate and growth rate in *C. sphagnetorum* when exposed to dimethoate and LAS
- To establish culturing medium, optimum temperature and soil moisture, and handling and extraction techniques for a sexually reproducing enchytraeid species
- To follow survival of P- and F_1-generations and fitness parameters of a sexually reproducing enchytraeid species under chemical stress

7.3 Main results obtained

7.3.1 Methods:

A study of the responses of the enchytraeid worm *C. sphagnetorum* (Vejdovsky, 1878) after exposure to copper was carried out. This species is asexual and reproduces by fragmenting. The worms were maintained individually in 5 cm^3 glass vials containing 2.5 cm^3 of soil. The vials (n of each concentration = 40) were incu-

bated in darkness at 15 °C for ten weeks. Specimens of *Cognettia* were extracted from coniferous forest soil using a wet funnel extraction method (O'Connor 1967) and placed individually on the soil surface of a vial. The development of worms was checked once a week by flushing the soil with water into a Petri dish. Worms and fragments were collected, examined under light microscope (x10) and put back in vials provided with fresh soil obtained from the stock of mixed experimental soils.

The substrate used was a modified standard soil. It was made by mixing 70 %vol sieved *Sphagnum* peat and 30 %vol of the LUFA 2.2 soil, both of which had been dried at 80 °C for 2 hrs. One week before the start of the experiment, the soils were inoculated with a spore suspension of the fungus *Mortierella isabellina* (Oudem), upon which the worms are known to feed (K. Hedlund and A. Augustsson, unpubl.). The amount of metabolically active mycelium was checked by FDA-staining (Söderström 1977).

A series of soils with increasing Cu-concentration was produced. Copper was added as a solution of $CuCl_2$. The soils were mixed in a bench roller for 5 hrs to give a homogeneous substrate. The amount of water needed for adding the fungal spores and the Cu-solution was set to give a soil water content of 50% d.w. This water content was kept constant over the experimental period by storing the treated soils not needed at start of the experiment individually in glass beakers wrapped in plastic bags at 4 °C.

Two size classes of worms were used, i.e. one batch consisting of large worms (40±1 segments) considered to be adult and able to fragment and another batch consisting of large immatures (30±1 segments) considered to become adult within 5 weeks after the start of the experiment. Care was taken when handling the fragile specimens.

7.3.2 Results:
Mortality of adult worms was low in all soils, but increased over the series from 0% (control) to 13% (400 mg Cu/kg soil d.w.). There were no indications of an increased mortality of the fragments due to exposure to copper; the fragment mortality varied only slightly, between 0 and 5%.

The average size of adult worms at fragmentation decreased with increasing Cu concentration and differed significantly for specimens of the batch of small worms between the control and the soil of 50 mg Cu/kg ($p<0.05$, ANOVA). The rate of fragmentation (number of fragments produced per day) also decreased significantly with increasing Cu concentration, from 0.01214 (batch of large worm) in the control soils to 0.00429 in 400 mg Cu/kg soils.

The number of fragments produced by a worm decreased with increasing Cu concentration. Whereas worms in the control soil produced up to five fragments, worms in soil of a Cu concentration of 400 mg Cu/kg soil only produced two and in soils of 50 and 100 mg Cu/kg soil produced at the most three fragments. There was, however, no significant difference in the production between specimens in control soil and in either 50 or 100 mg Cu/kg soil, but between specimens kept in the control soil and soils of 400 mg Cu/kg soil ($p<0.05$).

Growth rate was severely impeded by copper and the growth rates of worms in the different soils differed significantly for both kinds of batches ($p<0.001$,

ANOVA). Worms kept in control soil grew on average 14.75±3.2 segments over the experimental period, whereas those maintained in soils of 400 mg Cu/kg soil only grew 1.30±1.06 over the same period (pooled data of both batches). The growth rates of small worms maintained in soil of 50 mg Cu/kg soil and the control differed significantly ($p<0.05$, ANOVA) as did the growth rates of those kept in soil of 100 mg Cu/kg soil ($p<0.001$, ANOVA). Specimens of the batch of large worms grew significantly slower in soils of 50 and 100 mg Cu/kg soils than in control soil ($p<0.05$ and $p<0.001$, respectively).

7.3.3 Discussion:
The results obtained in this experimental set-up partly contradict the findings presented by Sjögren et al. (in press). In the latter case the worms were maintained in natural coniferous forest soils collected from unpolluted and polluted sites near a brass mill and mixed to produce a series of polluted experimental soils. The main difference between the two series was that in the coniferous forest soils the fragmentation increased with increasing metal content of the soil, whereas it decreased over the series in the modified LUFA soil. This may depend on direct causes (the differences in availability of the metals in soils exposed to metal emission over a long period of time and the experimental soils to which the metal was added as a salt) but also on indirect causes (e.g. food deficit).

The coniferous soil represents a complex environment and may contain other fungal species that may serve as alternative food supply, whereas in the modified LUFA soil there was only one fungal species present, though in a rather high amount (90 m of fungal mycelium/g soil, d.w.) and though normally being preferred, it may be that the fungus incorporating copper is less palatable.

Worms in soils of a high Cu concentration were conspicuously thinner than worms maintained in other soils. The low fragmentation rate might be a result of energy deficit in the worms, which is emphasized by the slow development of the fragments. Whether a lack of energy is resulting in the low numbers of fragments produced in soils of high Cu concentration is unknown. However, fragments that have to regenerate a head clearly need a longer period of time to do so in highly polluted soil, which means that the fragments *per se* are exposed to copper for a shorter period of time. Moreover, the fragments seem to be less active and thus less prone to feed, which may imply a low degree of exposure, if the metal is taken up by the test organism via food and not through the skin.

7.4 References

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Sjögren, M., Augustsson, A. & Rundgren, S. (In press): Dispersal and fragmentation of the enchytraeid *Cognettia sphagnetorum* in metal polluted soil. Pedobiologia.

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ANNEX 7.1 - Test System Standardization Form 6

Sublethal toxicity test with the enchytraeid worm *Cognettia Sphagnetorum* (Vejdovsky, 1878), (Enchytraeidae: Oligochaeta)

General data about the test organism at the start of the experiment

Test organism

Taxonomic group:	Enchytraeidae: Oligochaeta
Species:	*Cognettia sphagnetorum* (Vejdovsky, 1878)
Life stage:	adult, immature
Sex:	not revelant, asexual reproduction
Size:	about 10 mm; > 35 segments (adults), < 30 segments (immatures)
Gen. composition:	not known
Source:	sampled in the field

Type of surroundings and ambient climate

Surroundings	
Type:	climate chamber
Ambient climate	
Air temperature:	15 ± 1 °C
Relative humidity:	not applicable
Light regime:	darkness

Type of test system and test conditions

Type:	vial, 5 cm^3
Material:	glass
Openness:	capped
Spec./test system:	single species
Ind./test system:	1
Fragments per test system:	1

Type of habitat

Soil:	modified LUFA 2.2 soil
'Habitat characteristics'	
Soil:	70 vol% sieved (1 mm) *Sphagnum* peat, 30 vol% LUFA 2.2 soil
Hydrol. charac.	
Soil moisture:	50% d.w.
pH:	4.1
Soil per vial:	2.5 cm^3 (the vial should be half-filled)
Food supply:	The fungus *Mortierella isabellina* (Oudem) added as a spore suspension (750,000 cells per cm^3) to the soil to be used one week before the start of the experiment
Feeding rate:	not applicable
Type of food:	*Mortierella isabellina* (Oudem) from stock culture

Exposure characteristics

Exposure route:	via soil and food
Composition of test soil:	modified LUFA 2.2 soil with deionized water, test substance, all constituents well homogenized in a bench roller for 5 hrs.
Composition of control soil:	modified LUFA 2.2 soil with deionized water, homogenized as above

Duration of the experiment

Acclimat. time:	not relevant
Exposure time:	70 days
Recovery period:	not relevant

Exposure concentrations

Replicates:	40 specimens (20 adult worms and 20 immature worms); each concentration and control
Series:	1
Reference chemical:	work in progress

Exposure responses

Effect parameters:	reproduction: fragmentation rate and number of fragments at fragmentation
Other parameters:	growth of adults, immatures and fragments: increase of segments over test period. Survival: number of adults, immatures, and fragments surviving over test period
Measurement of variables:	moisture content and pH of the soil at start and end of the test
Toxic endpoints:	NOEC LOEC EC_x
Unit:	mg test substance/kg d.w. test soil
Working methods:	extraction of specimens/fragments by flushing test soil into a Petri dish; determination of the number of fragments and the number of segments per fragment
Analytical methods:	not relevant

Miscellaneous test information

Practicability:	test containers needed are standard laboratory equipment and extraction apparatus is easily constructed. Required biological material can be obtained by field collection; the test organism is commonly found (Abrahamsen 1972, Lundkvist 1981) in litter and mor soils. The test protocol can be performed by technical and academic personnel after some taxonomic training
Cost-effectiveness:	the work that must be done before, during, and after the performance of the experiment has not yet been estimated; the amount of material, energy and test organisms required is normal
Further data:	work in progress
Sensitivity:	work in progress
Reproducibility:	work in progress
Validity of the test:	work in progress
Standardization:	work in progress
Ecological realism:	*C. sphagnetorum* is a common species that inhabits mor soil of coniferous forests (Abrahamsen 1972). The temperature and soil moisture regime is set to correspond to a regime that in the field results in optimum life conditions (Standen 1973, Lundkvist 1981, Makulec 1983). *C. sphagnetorum* inhabits humus rich soil (Standen 1973). Thus, the test soil has to be modified and more organic matter has to be incorporated in the LUFA 2.2 soil. Food has to be supplied. It is not yet known how *C. sphagnetorum* differs from other enchytreid species with respect to ecological requirements. Its reproduction mode (asexual) distinguish this species from many other commonly occurring enchytraeid species
Broad chemical responsiveness:	work in progress

References

Abrahamsen, G. (1972): Ecological study of Enchytraeidae (Oligochaeta) in Norwegian coniferous forest soils. Pedobiologia 12:26-82.

Lundkvist, H. (1981): Enchytraeidae (Oligochaeta) in pine forest soils: Population dynamics and response to environmental changes. Acta Universitatis Upsaliensis. Abstracts of Uppsala Dissertations from the Faculty of Science 606.

Makulec, G. (1983): Enchytraeidae (Oligochaeta) of forest ecosystems: I. Density, biomass and production. Ekol. Polska 31:9-56.

O'Connor, F.B. (1967): The Enchytraeidae. In: Soil biology. A. Burges and F. Raw (eds). Academic Press, London and New York. pp. 212-257.

Standen, V. (1973): The production and respiration of an enchytraeid population in blanket bog. J. Anim. Ecol. 42:219-245.

Hedlund, K. & Augustsson, A. (In press): Effects of enchytraeid grazing on fungal growth and respiration. Soil Biology and Biochemistry.

ANNEX 7.2 - Draft test protocol 6

Sublethal toxicity test with the enchytraeid worm *Cognettia Spahgnetorum*, Vejdovsky, 1878, (Enchytraeidae: Oligochaeta)

1 Scope

This test protocol describes a method for the determination of the effects on mortality, growth, and asexual reproduction of *Cognettia sphagnetorum* (Vejdovsky, 1878) (Enchytraeidae: Oligochaeta) when exposed to a pollutant in a defined modified LUFA 2.2 soil substrate treated with a defined amount of that pollutant.

2 Definitions

The following definitions apply:

2.1 Adult worms
Worms that have more than 35 segments are considered to be adult, i.e. prone to fragment.

2.2 Growth
Increase in number of segments of the worm body, the head part not included. Growth rate is here defined as the increase in number of segments over the experimental period of time.

2.3 Reproduction success
Mean number of fragments following upon a fragmentation. The number of fragments surviving over the experimental period of time.

2.4 Fragmentation rate
The average number of fragments produced per day.

2.5 NOEC (No Observed Effect Concentration)
The highest tested concentration of a test substrate at which no lethal or sublethal effect is observed, expressed as mass of test substance per dry mass of the test substrate.

3 Principles

The percentage mortality and effects on growth rate of adults and fragments, and the fragmentation rate of adults of *Cognettia sphagnetorum* placed in a defined soil substrate containing different concentrations of the test substance are determined. The test substance is added in a single step. The

mortality and the effects on growth and fragmentation are determined once a week over 10 weeks. The reproductive success is determined by counting the number of surviving fragments after 10 weeks.

4 Reagents

4.1 Biological material

The enchytraeid worm *Cognettia sphagnetorum* can be collected from the mor layer of coniferous forest soil and extracted using the wet funnel method (O'Connor 1967). Field collected animals should be determined according to Nielsen and Christensen (1959). The biological material consists of adult worms (> 35 segments) or immatures (< 30 segments) of the species. Worms of the different size classes are randomly selected from a population. Worms selected for testing should be of a similar size, for instance one batch of specimens all being 30 segments and another consisting of specimens of 40 segments. Care should be taken to select healthy individuals and to handle the extracted worms carefully since otherwise they will easily fragment.

Selected worms can be stored in soil in plastic containers at 15 °C for some days to acclimatize.

4.2 Test substrate

4.2.1 Soil substrate:

Commercial *Sphagnum* peat is dried (80 °C for 2 hrs). The peat should be sifted using a screen (mesh size 1 mm). The peat is mixed with LUFA 2.2 soil dried to 80 °C for 2 hrs. Mixing: 70 vol% of peat soil, 30 vol% of LUFA soil. The two soils are homogeneously mixed in glass beakers (3 litre).

The volume of soil substrate to produce the modified LUFA soil needed for each concentration of the following series is approximately 1.5 litre.

4.2.2 Modified LUFA soil:

The modified LUFA soil will be wetted in two steps. Firstly, when the fungal spore suspension is added (see below). This implies that a water content of 30% d.w. is obtained. Secondly, when the chemical solution is added and the soil thus reaches its final water content, 50% d.w.

4.3 Food

As a food source a spore suspension of the fungus *Mortierella isabellina* (Oudem) should be used. *M. isabellina* is aseptically inoculated on agar (2% Bacto agar, 2% Oxoid malt extract) in sterilized Petri dishes. After 2-3 weeks the Petri dish is flushed with sterile water. 1 g of glucose and 43 ml of this spore suspension, approximately 750,000 cells per ml, is added to 100 g of test soil one week before the start of the experiment.

Soils not used at start of the experiment but needed in the course of the test period should be stored in a refrigerator at 4 °C in the glass beakers used for the mixing of the test soils. The beakers are individually wrapped in plastic bags to maintain soil moisture constant.

5 Apparatus

Standard laboratory equipment, and:

5.1 Glass vials 5 cm^3 provided with a screw cap are used as enclosures.

5.2 Bench roller.

5.3 Rearing chambers.

5.4 Sterile cabinet.

6 Test environment

6.1 Culturing chamber
Culturing chamber, controllable to a temperature of 15 ± 1 °C.

7 Procedure

7.1 Preliminary test
If necessary to determine the range of concentrations for use in the final test, a preliminary test for four concentrations of the test substance and a control may be performed. 5-10 replicates of each concentration may be used.

7.2 Final test
7.2.1 Introduction of test substance:
Use either method a) or b) as appropriate:
a) Water soluble substances
Dissolve the test substance in deionized water. The amount of the solution is calculated to give the desirable concentration and a final soil water content of 50% The solution is added to 500 g of the modified LUFA soil. The soil is homogenized in plastic bottles in a bench roller for 5 hrs.

2.5 cm^3 of the mixed experimental soil is added to each glass vial (5 cm^3), i.e. the vial will be approximately half-filled.

b) Substances insoluble in water but soluble in organic solvents.
Work in progress.

7.2.2 Controls:
Prepare the control soil in the same way as the test soils. Deionized water is added in the second step of wetting to obtain equivalent water content.

40 vials are used as control.

7.2.3 Addition of biological material:
A single worm is transferred to each test vial. The vials should be numbered, since the individual worm will be followed. Before adding the specimen, its

number of segments should be determined using a light microscope (x10), one well microscope slide and cover glass. The worm is then placed on the soil surface of a test vial and the cap is screwed on. 40 vials are used for each concentration, 20 replicates for worms 30 segments of size and 20 replicates for worms 40 segments of size.

7.2.4 Determination:
After one week the worms are extracted from the soil by flushing the vials separately with water and pouring the content of the vial into a Petri dish. Worms and fragments are removed by using a hooked needle.

The number of fragments found and the number of segments of each fragment are immediately determined using a microscope, as above.

After the determination of the numbers of fragments of still unfragmented individuals, the worms are transferred to vials with fresh soil out of the stored stock and of the same concentration as they previously experienced. Fragments are transferred to individual vials provided with fresh, treated soil out of the stock in order to establish their survival and growth.

After another week, all vials are checked and survival, fragmentation, and growth are determined. The same procedure is repeated once a week for 10 weeks.

7.3 Reference substance
Work in progress.

8 Calculation and expression of results

8.1 Calculation
For each concentration, the percentage mortality of adults and the growth, expressed as the increase in the number of segments over time, should be calculated. The number of surviving fragments at the end of the 10 week period is determined.

Compare means by suitable statistical methods, e.g. ANOVA.

Determine differences in slope coefficients of growth curves using ANOVA.

8.2 Expression of results
Indicate in mg/kg soil substrate (d.w.), the highest concentration tested without significant mortality of adults and fragments, significant change in growth rate, and significant number of fragments formed (NOEC) and the lowest concentration with any significant effects (LOEC).

9 Validity of the test

The results are considered to be valid if:

9.1 Rate of fragmentation
Work in progress.

9.2 Percentage mortality of adults
The percent mortality of the adults observed in the controls is: work in progress.

10 Test report

The test report shall refer to this standard and, in addition to the results expressed as in 7.2, shall provide the following information:

- complete description of the biological material employed (species, size in terms of number of segments, culturing conditions)
- method of preparation of the test substrate
- detailed conditions of the test environment
- table giving the percentage mortality obtained for each vial for each concentration and for the control
- number of fragments per test container over the time period
- the highest concentration causing no observed effects (NOEC) and lowest observed effect concentration (LOEC)
- description of obvious or pathological symptoms or changes in behaviour (e.g. low activity, escape behaviour) observed in the test organism per test container
- water content and pH of test substrate
- all operating details not specified in the standard, and any occurrences liable to have affected the results
- some specimens used in the test should be preserved for future reference

11 References

Abrahamsen, G. (1972): Ecological study of Enchytraeidae (Oligochaeta) in Norwegian coniferous forest soils. Pedobiologia 12:26-82.

Lundkvist, H. (1981): Enchytraeidae (Oligochaeta) in pine forest soils: Population dynamics and response to environmental changes. Acta Universitatis Upsaliensis. Abstracts of Uppsala Dissertations from the Faculty of Science 606.

Makulec, G. (1983): Enchytraeidae (Oligochaeta) of forest ecosystems: I. Density, biomass and production. Ekol. Polska 31:9-56.

O'Connor, F.B. (1967): The Enchytraeidae. In: Soil biology. A. Burges and F. Raw (eds). Academic Press, London and New York. pp. 212-257.

Standen, V. (1973): The production and respiration of an enchytraeid population in blanket bog. J. Anim. Ecol. 42:219-245.

Nielsen, C.O. & Christensen, B. (1959): The Enchytraeidae. Critical revision and taxonomy of European species. Natura Jutlandica 8-9:1-160.

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8 Sublethal toxicity test with the springtail *Isotoma viridis (Bourlet, 1839) and Folsomia candida* (Willem, 1902) (Collembola: Isotomidae)

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8.1 Objectives for the reporting period

- To improve culturing methods for *Isotoma viridis* (Bourlet, 1839) (Collembola: Isotomidae)
- To continue development of laboratory test systems to detect the sublethal effects of chemicals on the growth and reproduction of *I. viridis* and *Folsomia candida* (Willem, 1902) (Collembola: Isotomidae)
- To determine the impact of the three SECOFASE test compounds (dimethoate, copper and LAS) on *I. viridis* and *F. candida* in artificial soil (OECD)
- To select or develop appropriate population models which may be incorporated into risk assessment procedures
- To begin to gather life-history data to allow sensitivity analysis of fitness parameters for *I. viridis* and *F. candida*

8.2 Objectives for the next reporting period

- To finalise test system protocols for detecting sublethal effects of chemicals on the growth and reproduction of *I. viridis* and *F. candida*
- To complete runs of the test systems for *I. viridis* and *F. candida* exposed to dimethoate, copper and LAS in artificial soil (OECD) and LUFA 2.2 soil
- To investigate the impact of sublethal effects on the phenotypic fitness of the test organisms
- To investigate the population consequences of sublethal effects detected in the test systems and to determine how these may be used for risk assessment

8.3 Main results obtained

8.3.1 Introduction

Culturing

During the reporting year significant progress has been made towards improving

culturing methods for *I. viridis*. It is now possible to produce several hundred hatchlings per week. However culturing of this species is labour intensive and regular (4 monthly) introductions of field collected adults are required in order to maintain productive stock cultures. Populations of *I. viridis* in the stock cultures perform best when provided with yeast, green algae and a small amount of defaunated soil. A comparison of some life-history characteristics and the culturing performances of *I. viridis* and *F. candida* is given in Table 8.1.

Table 8.1: Comparison of some characteristics of the two collembolan test species.

Characteristic	Test Species	
	I. viridis	*F. candida*
Presence in European soils	Common	Uncommon
Edaphon	Hemiedaphic	Euedaphic
Reproduction	Sexual (iteroparous)	Parthenogenetic (iteroparous)
Ease of culturing	Labour intensive	Labour extensive
Sustainability of cultures	Relatively poor	Very good
Genetic variability in culture	High	Low
Maximum adult size (mm)	4.0	3.0
Size of hatchlings (mm)	0.55	0.30
Mean generation time (d)		
15 °C.	82	45
20 °C.	48	26

Taxonomy
While establishing the *Isotoma* cultures some taxonomic queries have come to light. Specimens from the stock cultures have been identified as *I. anglicana* using the most up-to-date European key for Collembola (Fjellberg, 1980). However according to the British list of species (Kloet & Hinks, 1964) *I. anglicana* is listed as a subspecies of *I. viridis*. Therefore until this matter is resolved further we propose to continue to call the test species *I. viridis*.

Test systems
Test systems to detect sublethal effects of chemicals on the growth and reproduction of *F. candida* and *I. viridis* have also been developed further during the year. Separate methodologies, in terms of replication and test duration, are required for each species because of their different life-history characteristics (see Table 8.1 and draft test protocols).

8.3.2 Results

Acute toxicity tests
Short-term (6 day exposure) acute toxicity tests were carried out with the three SECOFASE test chemicals, firstly to determine appropriate dose-rates for sublethal tests and secondly to compare the relative susceptibilities of the two test species to the three SECOFASE test chemicals. Juveniles (hatchlings) were tested because they were likely to be the most susceptible life-stage to chemicals. Table 8.2 summarises some of the results obtained.

Table 8.2: Susceptibility of juvenile Collembola in 6 day exposure bioassays at 15 °C. to the test compounds in artificial soil (OECD).

Species	LC_{50} for test compound (mg/kg d.w.)		
	Dimethoate	Copper	LAS
I. viridis	0.7	1150	590
F. candida	3.7	5090	2020

Development of test systems to detect sublethal effects of chemicals
Reproduction tests were carried out with *F. candida exposed to* dimethoate, copper and LAS in artificial soil (OECD) and copper in LUFA 2.2 soil. Example data from the copper bioassays are given in Figure 8.1. LOEC values for the mean numbers of juveniles produced were approximately 200 mg/kg d.w. in both soils. Tests with *I. viridis* exposed to dimethoate and copper in artificial soil (OECD) were also carried out, however, to date, adult survival in the test chambers over the 12 week exposure period has proved to be low, leading to poor reproductive success.

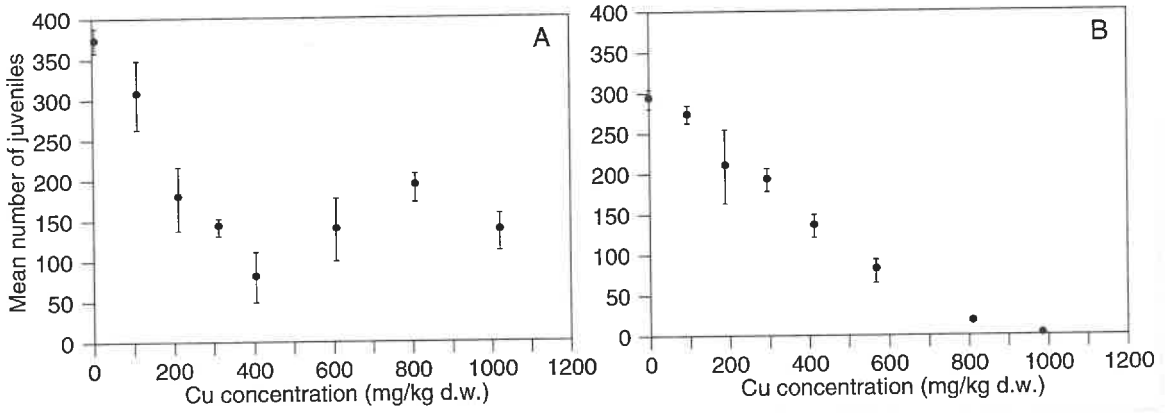

Figure 8.1: Example data from reproduction tests for *F. candida* exposed to copper in A) artificial soil (OECD) and B) LUFA 2.2 soil (high-low markers indicate S.E.'s).

Development of test systems to detect sublethal effects of chemicals on growth: Measurements of collembolan growth rates were made using computerised image-analysis software. Studies with both *F. candida* and *I. viridis* were carried out in artificial soil (OECD) contaminated with dimethoate, copper and LAS. Example growth curves for *F. candida* and *I. viridis* exposed to copper are given in Figure 8.2. Significant inhibition of growth was detected with *F. candida* exposed to nominal copper concentrations of 413 and 800 mg/kg d.w., however inhibition of growth was not detected at similar copper concentrations (398 and 794 mg/kg d.w.) with *I. viridis*.

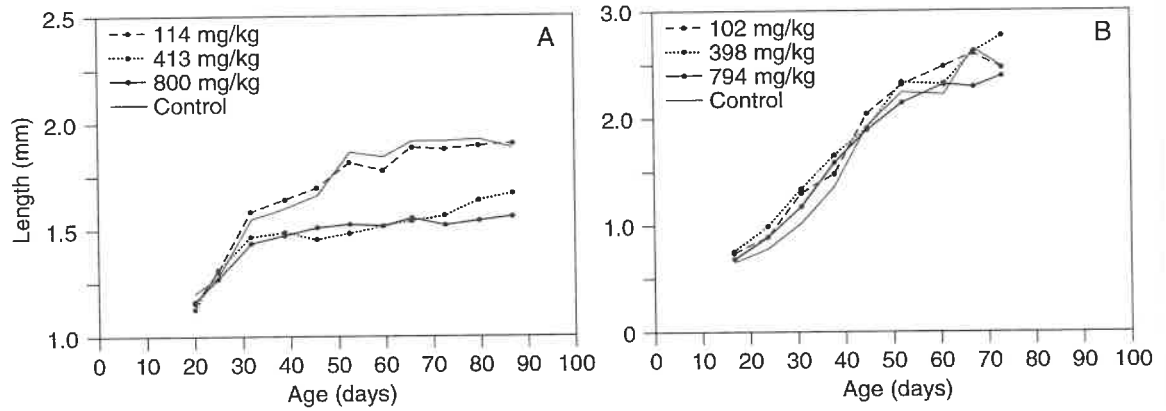

Figure 8.2: Example growth curves for A) *F. candida* and B) *I. viridis* exposed to copper (mg/kg d.w.) in artificial soil (OECD) at 15 °C.

8.3.3 Discussion

Results from the acute toxicity bioassays indicated that *I. viridis* juveniles were significantly more susceptible to all three test chemicals than *F. candida* juveniles.

The reasons for this difference remain unclear at present. One possible explanation may be that laboratory strains of *F. candida* are relatively tolerant to pollutants.

To date, *F. candida* has been found to perform well in both growth and reproduction test systems, however *I. viridis* has performed less well because of its lower survival rate. One possible reason for this is that the present food source (yeast) is sub-optimal. Additional food sources, such as suspensions of bacteria and algae, may be introduced into the test soil to attempt to alleviate this problem in future runs of the test system.

Once data sets are complete, the measurements of collembolan fecundity and survival rates in the test systems will be used to construct matrix models to estimate the influence of exposure to given concentrations of the test chemicals on the intrinsic rates of natural increase of the two test species. Long-term studies in which the Collembola are exposed to given test chemical concentrations for several generations are currently being conducted to allow comparisons of the predictions with observed population trends.

8.4 References

Fjellberg, A. (1980): Identification Keys to Norwegian Collembola. The Entomological Society of Norway. 151 pp.

Kloet, G.S. & Hinks, W.D., (1964): A Check list of British Insects (Second Edition). Part 1. Small Orders and Hemiptera. Handbooks for the Identification of British Insects, 11 (1), 1-119. Royal Entomological Society of London.

ANNEX 8.1 - Test System Standardization Form 7.1

Sublethal toxicity test with the springtail *Isotoma viridis* (Bourlet, 1839) and *Folsomia candida* (Willem, 1902) (Collembola: Isotomidae)

N.B. This is a single species test. Methodologies are the same for both species unless otherwise stated. The initial test protocol was based upon modifications of the draft ISO test for *F. candida* (Riepert, 1992).

General data about the test organism at the start of the experiment

Test organism

Taxonomic group:	Springtails (Collembola: Isotomidae)
Species:	*Isotoma viridis* (Bourlet, 1839) *Folsomia candida* (Willem, 1902)
Life stage:	Juvenile
Sex:	*I. viridis* (males and females - sexually reproducing) *F. candida* (probably only female - parthenogenetic)
Age:	10-12 days old (*I. viridis* and *F. candida*)
Gen. composition:	not known
Background:	*I. viridis* - Field collected and cultured in the laboratory *F. candida* - Laboratory culture

Type of surroundings and ambient climate

Surroundings	
Type:	Completely enclosed
Ambient climate	
Air temperature:	15 ± 1 °C.
Relative humidity:	70 - 90%
Light intensity:	10 - 100 Lux (depending upon the position in the incubator). Vessels placed on shelves in random order and rotated at approx. 7 day intervals.
Light regime:	light:dark 12:12

Type of test system and test conditions

Type:	120 cm^3 jar with screw top
Material:	Glass
Aeration:	Approx. every 7 days
Species/test system:	Single species test system
Individuals/test system:	20 *I. viridis* or 10 *F. candida*

Type of habitat

Soil/Litter:	Artificial soil (OECD) or natural soil (eg. LUFA 2.2 soil), homogeneously mixed
'Habitat characteristics'	
Soil charact.:	Artificial soil (OECD) - consisting of 10% sphagnum peat, 20% kaolin clay, 70% industrial quartz sand and some $CaCO_3$ added to adjust the pH to 6.0 ± 0.5 (expressed as dry weight). Natural soil - LUFA 2.2 soil
Hydrological charact.:	Approx. 50% water holding capacity
Food provided:	Yes, upon initial exposure and after 2 weeks of exposure
Food type:	Granulated Bakers yeast (dry)
Feeding rate:	4 granules/jar at the beginning and at biweekly intervals

Exposure characteristics

Exposure route:	contact with the test soil (possibly also by contaminated diet as the yeast absorbs soil moisture)
Composition of test soil:	test soil with distilled or deionised water, test substance and auxiliary agent (if used); all constituents well homogenised
Composition of control soil:	a) test soil and distilled or deionised water b) test soil, distilled or deionized water and auxiliary agents (max. recommended 100 mg/kg d.w. of test soil)

Duration of the experiment

Acclim. time:	2-5 days (if Collembola transferred from different culturing temp.)
Exposure time:	12 weeks for *I. viridis* 6 weeks for *F. candida*
Recovery period:	none

Exposure concentrations

Replicates:	4 for each concentration and a control
Concentrations:	range finding test usually 5 concentrations in a geometric series (eg. $\sqrt{10}$); A definitive range of doses may then be selected for final testing. The maximum recommended concentration is 1000 mg/kg d.w.
Ref. chemical:	work in progress

Exposure responses

Effect parameter:	reproduction: a) average number of juveniles (alive) per test vessel after the exposure period; b) average number of live juveniles/live adult per test vessel after the exposure period.
Other parameters:	Survival: average number of adults (alive) after the exposure period.
Measurement of variables:	water content and pH of soil before and after the test (1M KCl).
Toxic endpoints:	a) NOEC, i.e. the highest tested concentration of the test chemical showing no significant decrease in reproduction in comparison with the control b) LOEC i.e. the lowest concentration of the test chemical showing a significant decrease on reproduction in comparison with the control (based on a nominal concentration)
Unit:	mg test chemical/kg d.w. test soil
Assessment methods:	use of water floatation method to separate live adult and juvenile collembola from the test soil within each test vessel. Assessment by light microscope or photographic methods.
Analytical methods:	analysis of soil and animals to determine actual test concentrations and body burdens, is recommended.

Miscellaneous test information

Practicability:	the required apparatus (eg. incubators, insect culturing facilities and laboratory mixers) are easily obtainable. The test protocol can be performed by the technical personnel with a small amount of training.
Cost-effectiveness:	the test is not particularly labour intensive. The most time consuming procedures include a) preparation of the test soil, b) introduction of Collembola into the test vessels and c) assessment of the numbers of live adults and juveniles at the end of a test.
Sensitivity:	*F. candida* appears to be fairly insensitive to cadmium compared to other animals (e.g. earthworms) (Léon and van Gestel, 1993). *F. candida* also appears to be less sensitive to the three SECOFASE test chemicals than *I. viridis* in acute studies.

Reproducibility:	in an international ring test performed within the framework of ISO (Riepert, 1993), large variations in test results were found between laboratories. Therefore the current ISO test protocol may need further improvement, especially with respect to the description of the preparation of the artificial soil (pH and moisture content).
Validity of test:	a) statistical methods (analysis of homogeneity, ANOVA with Dunnett's test) b) parental mortality within the control group should be less than 20 per cent c) parental mortality within the treatment groups should not be significantly higher than parental mortality within the control group
Standardization:	work in progress
Ecological realism:	*F. candida* is a subterranean species and is not common in most soils. It is not known if this species differs notably in response/sensitivity to pollutants compared with other subterranean species. The above protocol will be adapted for a surface dwelling collembolan species *Isotoma viridis*, which is common in many European soils and has a markedly different life-history strategy compared to *F. candida*. The temperature regime chosen for the SECOFASE project was 15 °C. The light regime used was ambient. It is possible to use 16:8 Light: Dark regime to simulate a summer field photoperiod. A water content of approx. 50% of the water holding capacity is in agreement with a moist field soil.
Broad chemical responsiveness:	no restrictions are given with respect to the chemical substances that may be evaluated by this test.

References

Léon, C.D. & van Gestel, C.A.M. (1993): Selection of a set of standardized laboratory toxicity tests for the hazard assessment of chemical substances in terrestrial ecosystems. Report for the Netherlands Ministry of Housing, Physical Planning and the Environment, DO94004, Vrije Universiteit, Amsterdam.

Riepert, F. (1992): Effects of soil pollutants on Collembola: determination of the inhibition of reproduction. Paris, ISO/AFNOR. ISO document 92/51653, working draft.

Riepert, F. (1993): ISO Ring test of a method for determining the effects of soil contaminants on the reproduction of Collembola. ISO document, draft summary.

ANNEX 8.2 - Test System Standardization Form 7.2

Sublethal toxicity test with the springtail *Isotoma viridis* (Bourlet, 1839) and *Folsomia candida* (Willem, 1902) (Collembola: Isotomidae)

N.B. This is a single species test. Methodologies are the same for both species unless otherwise stated.

General data about the test organism at the start of the experiment

Test organism

Taxonomic group:	springtails (Collembola: Isotomidae)
Species:	*Isotoma viridis* (Bourlet, 1839) *Folsomia candida* (Willem, 1902)
Life stage:	juvenile
Sex:	*I. viridis* (males and females - sexually reproducing) *F. candida* (probably only female - parthenogenetic)
Age:	10-12 days old (*I. viridis* and *F. candida*)
Gen. composition:	not known
Background:	*I. viridis* - Field collected and cultured in the laboratory *F. candida* - Laboratory culture

Type of surroundings and ambient climate

Surroundings	
Type:	completely enclosed
Ambient climate	
Air temperature:	15 ± 1 °C.
Relative humidity:	70 - 90%
Light intensity:	10 - 100 Lux (depending upon the position in the incubator). Vessels placed on shelves in random order and rotated at approx. 7 day intervals.
Light regime:	light:dark 12:12

Type of test system and test conditions

Type:	tubes with stopper (2.5 cm diameter x 5 cm high)
Material:	glass
Aeration:	approx. every 7 days or at each growth assessment
Species/test system:	single species test system
Individuals/test system	10 *I. viridis* or 10 *F. candida*

Type of habitat

Soil/Litter:	artificial soil (OECD) or natural soil (eg. LUFA 2.2 soil), homogeneously mixed
'Habitat characteristics'	
Soil charact.:	artificial soil (OECD) - consisting of 10% sphagnum peat, 20% kaolin clay, 70% industrial quartz sand and some $CaCO_3$ added to adjust the pH to 6.0 ± 0.5 (expressed as dry weight). Natural soil - LUFA 2.2 soil
Hydrological charact.:	approx. 50% water holding capacity
Food provided:	yes, upon initial exposure and after 2 weeks of exposure
Food type:	granulated Bakers yeast (dry)
Feeding rate:	3 granules/tube at weekly intervals

Exposure characteristics

Exposure route:	contact with the test soil (possibly also by contaminated diet as the yeast absorbs soil moisture)
Composition of test soil:	test soil with distilled or deionised water, test substance and auxiliary agent (if used); all constituents well homogenised
Composition of control soil:	a) test soil and distilled or deionised water b) test soil, distilled or deionized water and auxiliary agents (max. recommended 100 mg/kg d.w. of test soil)

Duration of the experiment

Acclim. time:	2-5 days (if Collembola transferred from different culturing temp.)
Exposure time:	10-12 weeks for *I. viridis* or *F. candida* or until no further significant increase in body length
Recovery period:	none

Exposure concentrations

Replicates:	4 for each concentration and control for *I. viridis* 3 for each concentration and control for *F. candida*
Concentrations:	range finding test usually 5 concentrations in a geometric series (eg. $\sqrt{10}$); A definitive range of doses may then be selected for final testing. The maximum recommended concentration is 1000 mg/kg d.w.
Ref. chemical:	work in progress

Exposure responses

Effect parameters:	growth: measurement of body length at regular (weekly) intervals using image analysis computer software.
Other parameters:	survival: number of live adults at each growth assessment. Reproduction: number of juveniles produced per week (assessed by incubating soil once adults removed for growth assessment to allow eggs laid during that period to hatch).
Measurement of test variables:	water content and pH of soil before and after the test (1M KCl).
Toxic endpoints:	growth: time until no further significant increase in body length occurs
Unit: Body length (mm)	
Assessment methods:	image analysis computer software

Miscellaneous test information

Practicability:	the required apparatus (eg. incubators, insect culturing facilities, a laboratory mixer and a hand-held air aspirator) are easily obtainable. The test protocol can be performed by the technical personnel with a small amount of training. Computerised image analysis equipment required.
Cost-effectiveness:	the test is relatively labour intensive. The most time consuming procedures include a) removal of the Collembola from the test soils and b) measurement of individual body lengths c) assessment of the number of juveniles produced.
Sensitivity:	unknown
Validity of test:	50% of adults should survive until the end of the test
Standardization:	work in progress
Ecological realism:	*F. candida* is a subterranean species and is not common in most soils. It is not known if this species differs notably in response/sensitivity to pollutants compared with other subterranean species. The above protocol will be adapted for a surface

dwelling collembolan species *Isotoma viridis*, which is common in many European soils and has a markedly different life-history strategy compared to *F.candida*.
The temperature regime chosen for the SECOFASE project was 15 °C. The light regime used was ambient. It is possible to use 16:8 light:dark regime to simulate a summer field photoperiod. A water content of approx. 50% of the water holding capacity is in agreement with a moist field soil.

Broad chemical responsiveness:

no restrictions are given with respect to the chemical substances that may be evaluated by this test.

References

Léon, C.D. & van Gestel, C.A.M. (1993): Selection of a set of standardized laboratory toxicity tests for the hazard assessment of chemical substances in terrestrial ecosystems. Report for the Netherlands Ministry of Housing, Physical Planning and the Environment, DO94004, Vrije Universiteit, Amsterdam.

Riepert, F. (1992): Effects of soil pollutants on Collembola: determination of the inhibition of reproduction. Paris, ISO/AFNOR. ISO document 92/51653, working draft.

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ANNEX 8.3 - Draft test protocol 7.1

Sublethal toxicity test with the springtails *Isotoma viridis* (Bourlet, 1839) and *Folsomia candida* (Willem, 1902) (Collembola: Isotomidae)

Introduction

This standard protocol is a preliminary draft of a test system to detect the sublethal effects of chemicals on the reproduction of two collembolan species *I. viridis* (Bourlet, 1839) and *F. candida* (Willem, 1902). The test is a single species test, however because modifications of the same methodology are used when testing either species, both tests are described in this protocol. Where differences occur in methodologies for the two species this will be stated in the protocol. The test may be used to compare the effects of different chemicals or different soil types on Collembola reproduction.

A reproduction test is currently being developed for *F. candida* (ISO draft document 92/51653, Riepert 1992). The test protocol reported here is broadly based upon that guideline. Two of the main differences are a lower test temperature (15 °C.) in this protocol, as agreed by all SECOFASE participants (Løkke and van Gestel, 1993), and consequently a longer duration of exposure (6 weeks for *F. candida*). *I. viridis* has been selected as a new and complementary test species, because it is more common and widespread in many European soil ecosystems than *F. candida*. *I. viridis* also has a different life-history strategy from *F. candida*, being both sexually reproductive and surface active whereas *F. candida* reproduces parthenogenetically and is subterranean.

1 Scope

This guideline describes a method for determining the effects of chemicals on the reproduction of *I. viridis* and *F. candida* exposed to chemicals mixed homogeneously in artificial or natural soils.

2 Definitions

2.1 Survival

Mean number of adults surviving at the end of the test period.

2.2 Reproduction

Mean number of live juveniles present at the end of the test period and also mean numbers of live juveniles per surviving adult at the end of the test.

2.3 NOEC (No Observed Effect Concentration)
The highest tested concentration of a chemical at which no lethal or other effect is observed, expressed as mass of test chemical per dry mass of the test substrate.

2.4 LOEC (Lowest Observed Effect Concentration)
The lowest tested concentration of a chemical at which a lethal or other effect is observed, expressed as mass of test chemical per dry mass of the test substrate.

3 Principle

Batches of Collembola (*F. candida* or *I. viridis*) 10 to 12 days old are placed in vessels containing a known mass of test soil treated with a series of concentrations of the test chemical. The vessels are incubated at 15 ± 1 °C. until juveniles (F_1) emerge from eggs laid by mature adults. The number of offspring and surviving adults are determined after a 6 week period for *F. candida* and a 12 week period for *I. viridis*.

4 Reagents

4.1 Sampling and culturing
I. viridis is common in grassy habitats throughout Europe and may be collected from field sites by suction sampling. Suction samples are sorted in a white tray in the laboratory and Collembola may be removed using a hand-held air aspirator. The Collembola are tentatively identified and the *I. viridis* are transferred into new breeding boxes containing a layer of moist plaster of Paris (prepared with powdered charcoal). Bakers' yeast, green algae, and a layer of defaunated field soil is provided as the food source. Samples of individuals are taken from new cultures for further taxonomic verification.

Collembola specimens may be identified using Fjellberg (1980).

F. candida is not common in the field and may be most easily obtained from established laboratory cultures. New cultures may then be established in boxes containing moist plaster of Paris (prepared with powdered charcoal). Bakers' yeast is provided as food.

4.2 Biological material
Synchronised cultures of *F. candida* and *I. viridis* should be established to obtain 10-12 days old juveniles. Synchronisation of *F. candida* is achieved by transferring adults from stock cultures into boxes containing a freshly prepared layer of plaster of Paris (black plaster of Paris is preferable to facilitate observations). Adults are removed after 24-48 hours and the boxes incubated until hatchlings emerge. Synchronisation of *I. viridis* is achieved by transferring egg batches from stock cultures into small tubes containing a layer of white plaster of Paris. Egg batches are observed daily and upon hatching

juveniles are transferred to screw top jars containing a layer of plaster of Paris. Food is provided in the form of Bakers' yeast.

4.3 Test substrate
The test substrate consists of the test soil, the test chemical and distilled or de-ionised water. A defined artificial soil is commonly used as the test substrate, as described in the OECD guideline 207, "Earthworm, Acute Toxicity Tests" and in Part C of Annex V of the EEC-Directive 67/548.

4.3.1 Artificial soil substrate:

10%	Sphagnum Peat (air dried, finely ground and sieved (mesh width 1mm))
70%	Sand (dominant fine sand with more than 50% of particle size 0.05-0.2 mm)
20%	Kaolin Clay (kaolinite content preferably above 30%)

Add sufficient Calcium Carbonate (approx. 0.5%), pulverized, recognised analytical grade to bring the pH to 6.0 ± 0.5

4.3.2 Artificial soil (OECD):
The dry constituents of the soil substrate are blended in the correct proportions and mixed thoroughly in a laboratory mixer. A small volume of the total volume of distilled or de-ionised water may be added during mixing. It is advisable to add the calcium carbonate shortly before introducing the test chemical.

4.4 Food
Granules of Bakers' yeast (4 per test chamber).

5 Apparatus

Standard laboratory equipment and:

5.1 Glass containers with screw top lids (120 cm^3 capacity). Containers require aeration at weekly intervals and additional food at biweekly intervals.

5.2 Equipment for experimental assessment, including Kentmere black photographic dye to enhance contrast (for *F. candida* only) and photographic facilities.

6 Test environment

Containers are incubated at 15 ± 1 °C. under an ambient light regime (mean light intensity approximately 70 lux). However the test species appear to be influenced little by changes in light regime and intensity. Vessels are placed on shelves in random order and rotated at approximately 7 day intervals.

7 Procedure

7.1 Preliminary test

Acute tests should be carried out to determine a suitable range of concentrations for sublethal tests. These may use the same methodology and equipment as the sublethal tests but may be of shorter duration. An initial dose range should consist of at least five concentrations in a geometric series e.g. using a factor of $\sqrt{10}$.

7.2 Final test

In the sublethal tests at least five concentrations should be selected with none exceeding 1000 mg/kg d.w. of the test chemical. Ideally the highest test concentration should not result in significantly higher mortality of the parental Collembola than the control. Four replicates for each concentration and control are recommended for *F. candida* and *I. viridis*. An additional replicate for each concentration and control is recommended to allow measurements of soil pH, moisture content and actual concentrations of the test chemical (where analytical techniques are available).

7.2.1 Incorporation of the test chemical into soil:

a) Water soluble substances

Determine the volume of water to be added to the test soil (40-60% WHC). The overall water content of the test and control substrate should be adjusted to give the substrate a crumbly structure to enable Collembola to penetrate the cavities. Prepare test concentrations in the appropriate volume of distilled or de-ionised water (allowing for any water added during soil preparation). Add prepared solutions (suspensions) of the test chemical to the correct mass of soil and blend in the laboratory mixer for a standard period of time until homogeneous mixing has been achieved.

b) 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). Add it to a small portion of quartz sand or dry soil substrate, mix thoroughly and evaporate the solvent by placing it under a fume hood for 1 h. Then add the remainder of the soil substrate (4.3.1) and the correct volume of water to achieve 50% WHC and mix thoroughly.

c) 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 4.3.1) or 10 g of the dry soil substrate and the quantity of the test substance required to obtain the desired concentration. Place the mixture, the remainder of the soil substrate (4.3.1) and the water into a beaker an mix thoroughly before introducing it into the test container (5.1).

Weigh out 30 ± 0.5 g (wet weight) of test soil into each test container. Determine the pH and moisture content of each test soil treatment at start and end of the test.

NOTE - ultrasonic dispersion, organic solvents, emulsifiers or dispersants may be used to disperse substances with low aqueous solubility. When such auxiliary substances are used, all test concentrations and an additional con

trol should contain the same minimum of auxiliary substance. To allow solvents to volatilize, the test containers should remain open for a period of 1 h.

7.2.2 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 7.2.1) use additional control containers without the test substance. Treat these containers in the same way as those without the test substance. Include an additional two containers for the control and each treatment for pH and soil moisture determination at the end of the test.

7.2.3 Addition of biological material:
Ten *F. candida* and 20 *I. viridis* of 10-12 days old are introduced per test container. Higher numbers of *I. viridis* are required as this species reproduces sexually, whereas *F. candida* is parthenogenetic.

7.2.4 Evaluation of the test:
After the appropriate period of exposure (i.e 6 weeks for *F. candida* and 12 weeks for *I. viridis*) empty the test soil into larger containers (e.g. 300 cm^3 capacity) and add 150 ml of distilled water. Stir thoroughly with a fine brush and allow to settle. Count adult and juvenile Collembola on the water surface. A grid may be used if numbers are high. Alternatively photograph the surface and count the individuals on the print either manually or using computer image analysis software.

N.B. Counts of *F. candida* may be facilitated by adding Kentmere black photographic dye into the water to enhance the contrast between the Collembola and water.

7.3 Reference substance
Work in progress.

8 Calculation and expression of results

8.1 Calculation
For each concentration determine the percentage mortality of adults and number of juveniles produced. Analyze the homogeneity of replicate results for each concentration using a Chi-square test for normality. If homogeneity is proved use an appropriate statistical analysis to indicate significant differences between the control and test concentration e.g. Dunnett's Test. If data is heterogeneous non-parametric methods such as the Mann Whitney U-Test may be used (Sokal and Rohlf, 1981).

8.2 Expression of results
The following results should be expressed in mg/kg d.w. soil

- the highest concentration tested showing no significant difference to the control in mortality and reproduction rates (NOEC)
- the lowest concentration tested with significant difference to the control (LOEC)

9 Validity of the test

9.1 Mortality

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

10 Test report

The test report shall refer to this standard and shall provide the following information:

- complete description of the biological material used (species, age, culturing conditions, and source)
- method of preparation of test substrate and any auxiliary substances used for a low-/non-water soluble substance
- results obtained with the reference substance, if performed
- detailed conditions of the test environment
- a table giving the percent mortality of adults obtained for each replicate for each concentration and for the control
- the number of offspring per test container at the end of the test
- the highest concentration causing no observed effects (NOEC)
- the lowest concentration causing observed effects (LOEC)
- a record of the body weight or body size of adult Collembola at the end of the test period
- a description of obvious or pathological symptoms or distinct changes in behaviour observed in test organisms per test container
- the water content and pH of artificial soil (in 1M KCl) at start and at end of the test
- all operating details not specified in the standard, and any occurrences liable to have affected the results
- some specimens used in the test should be preserved for future reference

11 References

Løkke, H. & van Gestel, C.A.M. (eds.) (1993): Manual of SECOFASE. 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, January 18-19, 1993. National Environmental Research Institute. 41 pp.

Riepert, F. (1992): Effects of soil pollutants on Collembola: determination of the inhibition of reproduction. Paris, ISO/AFNOR. ISO document 92/51653, working draft.

Sokal, R.R. & Rohlf, F.J. (1981): Biometry. 2nd Edition, Freeman, New York, 859 pp.

Fjellberg, A. (1980): Identification Keys to Norwegian Collembola. The Entomological Society of Norway. 151 pp.

ANNEX 8.4 - Draft test protocol 7.2

Sublethal toxicity tests with the springtails *Isotoma viridis* (Bourlet, 1839) and *Folsomia candida* (Willem, 1902) (Collembola: Isotomidae)

Introduction

This standard protocol is a preliminary draft of a test system to detect the sublethal effects of chemicals on the growth of two collembolan species, *I. viridis* (Bourlet, 1839) and *F. candida* (Willem, 1902). Essentially the same methodology may be used when testing either species, however where differences occur these will be stated in the protocol. The test may be used to compare the effects of different chemicals or different soil types. The methodology may also be adapted to assess sublethal effects on reproduction by determining the egg production of individuals between each growth measurement. Individuals may be removed from test soils using a hand-held air aspirator. After adult removal the soil may then be incubated for a suitable period (e.g. 2-3 weeks at 20 °C.) after which the number of juveniles can be assessed using a floatation technique.

1 Scope

This guideline describes a method for determining the effects of chemicals on the growth of *I. viridis* and *F. candida* exposed to chemicals mixed homogeneously in artificial or natural soils. The test may also be adapted to assess sublethal effects on reproduction by determining the egg production of individuals between each growth measurement.

2 Definitions

2.1 Survival
Number of individuals surviving at each measurement of growth.

2.2 Growth
Measurement of body length (head to tip of abdomen) at regular intervals until the end of the test period.

2.3 Reproduction
Measurement of numbers of juveniles produced between each growth measurement.

3 Principle

Batches of Collembola (*I. viridis* or *F. candida*) 4 to 6 days old are placed into glass tubes containing a known mass of test soil and defined concentrations

of the test chemical. The tubes are incubated at 15 ± 1 °C. and the Collembola are extracted weekly, by disrupting the soil in the tube and collecting individuals with a hand-held air aspirator, so that measurements of individual body lengths can be made using computerised image-analysis software. After measurement, individuals are re-introduced into tubes containing the same appropriate test concentration as the tube they were removed from. In addition, the soil from which individuals have been removed may be incubated and the number of juveniles produced may be assessed by floatation after a suitable period. Test measurements are carried out for a period of 10 to 12 weeks or until asymptotic body length has been reached.

4 Reagents

4.1 Sampling and culturing

I. viridis is common in grassy habitats throughout Europe and may be collected from field sites by suction sampling. Suction samples are sorted in a white tray in the laboratory and Collembola may be removed using a hand-held air aspirator. The Collembola are tentatively identified and the *I. viridis* are transferred into new breeding boxes containing a layer of moist plaster of Paris (prepared with powdered charcoal). Bakers' yeast, green algae, and a layer of defaunated field soil is provided as the food source. Samples of individuals are taken from new cultures for further taxonomic verification. Collembolan specimens may be identified using Fjellberg (1980).

F. candida is not common in the field and may be most easily obtained from established laboratory cultures. New cultures may then be established in boxes containing moist plaster of Paris (prepared with powdered charcoal). Bakers' yeast is provided as food.

4.2 Biological material

Synchronised cultures of *F. candida* and *I. viridis* are established to obtain 4-6 day old juveniles. Synchronisation of *F. candida* is achieved by transferring adults from stock cultures into boxes containing a new layer of black plaster of Paris. Adults are removed after 24-48 h and the boxes incubated until hatchlings emerge.

Synchronisation of *I. viridis* is achieved by transferring egg batches from stock cultures into small tubes containing a layer of white plaster of Paris. Egg batches are observed daily and upon hatching juveniles are transferred to screw top jars containing a layer of plaster of Paris. Food is provided in the form of Bakers' yeast.

4.3 Test substrate

The test substrate consists of the test soil, the test chemical and distilled or de-ionised water. A defined artificial soil is used as a soil substrate, as described in the OECD guideline 207, "Earthworm, Acute Toxicity Tests" and in Part C of Annex V of the EEC-Directive 67/548.

4.3.1 Artificial soil substrate:

10%	Sphagnum Peat (air dried, finely ground and sieved (mesh width 1mm)
70%	Sand (dominant fine sand with more than 50% of particle size 0.05-0.2mm)
20%	Kaolin Clay (kaolinite content preferably above 30%)

Add suficient (approx. 0.5%) Calcium Carbonate, pulverized, recognised analytical grade to bring the pH to 6.0 ± 0.5)

4.3.2 Artificial soil (OECD):
The dry constituents of the soil substrate are blended in the correct proportions and mixed thoroughly in a laboratory mixer. It is advisable to add the calcium carbonate shortly before introducing the test chemical. A small volume of the total volume of distilled or de-ionised water may be added during mixing.

4.4 Food
Granules of Bakers' yeast (3 per test glass tube).

5 Apparatus

Standard laboratory equipment and:

5.1 Screw top bioassay jars (120 cm^3 capacity) for incubation of test soils.

5.2 Glass tubes (2.5 cm diameter x 5 cm high) with tight fitting lids, used for exposing Collembola to test soils.

5.3 Glass tubes (2.5 cm diameter x 5 cm high) with tight fitting lids, containing a layer (1 cm) of plaster of Paris, used as transferral tubes during growth assessments.

5.4 Computerised image-analysis software calibrated to measure lengths between 0.5 and 3.5 mm.

6 Test environment

Containers are incubated at 15 ± 1 °C. under an ambient light regime (mean light intensity approximately 70 lux). However the test species appear to be influenced little by changes in light regime and intensity. Vessels are placed on shelves in random order and rotated at approximately 7 day intervals.

7 Procedure

7.1 Preliminary test

Acute tests should be carried out to determine a suitable range of concentrations for the sublethal tests. These may use the same methodology and equipment as the sublethal tests but may be of shorter duration. An initial dose range should consist of at least five concentrations in a geometric series e.g. a factor of $\sqrt{10}$.

7.2 Final test

In the sublethal tests at least three concentrations should be selected with none exceeding 1000 mg/kg d.w. of the test chemical. Ideally the highest test concentration should not result in significantly higher mortality of the Collembola than the control.

Ten replicates for each concentration and control are recommended for *F. candida* and *I. viridis* allowing for sufficient soil for 10 or more weeks of assessment. An additional replicate for each concentration and control is recommended to allow measurements of soil pH, moisture content and actual concentrations of the test chemical (where analytical techniques are available).

7.2.1 Incorporation of the test chemical into soil:

a) Water soluble substances

Determine the volume of water to be added to the test soil (40-60% WHC). The overall water content of the test and control substrate should be adjusted to give the substrate a crumbly structure to enable Collembola to penetrate the cavities. Prepare test concentrations in the appropriate volume of distilled or de-ionised water (allowing for any water added during soil preparation). Add prepared solutions/suspensions of the test chemical to the correct mass of soil and blend in the laboratory mixer for a standard period of time until homogeneous mixing has been achieved. Weigh out 35 ± 0.5 g (wet weight) samples of test soil into each bioassay jar and incubate jars at 15 °C. until required.

b) 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). Add it to a small portion of quartz sand or dry soil substrate, mix thoroughly and evaporate the solvent by placing it under a fume hood for 1 h. Then add the remainder of the soil substrate (4.3.1) and the correct volume of water to achieve 50% WHC and mix thoroughly.

c) 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 4.3.1) or 10 g of the dry soil substrate and the quantity of the test substance required to obtain the desired concentration. Place the mixture, the remainder of the soil substrate (4.3.1) and the water into a beaker an mix thoroughly before introducing it into the test container (5.1).

NOTE - ultrasonic dispersion, organic solvents, emulsifiers or dispersants may be used to disperse substances with low aqueous solubility. When such auxiliary substances are used, all test concentrations and an additional control should contain the same minimum of auxiliary substance. To allow solvents to volatilize, the test containers should remain open for a period of 1 h.

Weigh out 5 ± 0.1 g samples of the test soil into each replicate glass tubes (four for *I. viridis* and three for *F. candida*). Determine the pH and soil moisture content of each soil treatment at the beginning and end of the test.

7.2.2 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 7.2.1) use additional control containers without the test substance. Treat these containers in the same way as those without the test substance. Include an additional two containers for the control and each treatment for pH and soil moisture determination at the end of the test.

7.2.3 Addition of biological material:
Ten 4-6 day old *F. candida* or *I. viridis* are introduced per replicate test container.

7.2.4 Evaluation of the test:
Weekly measurements of individuals from batches of Collembola (*F. candida* or *I. viridis*) are made by emptying the soil from the glass tubes into a tray. The Collembola from each batch are placed into labelled glass tubes containing a layer of plaster of Paris. After being measured the Collembola are reintroduced into test soil containing the appropriate concentration of test chemical. Three granules of Bakers' yeast are placed in each tube as a food source. N.B. For *I. viridis*, determine the sex of surviving individuals at the end of the test. The numbers of juveniles produced by individuals between each growth assessment may be assessed using floatation and photographic techniques after a suitable incubation period.

7.3 Reference substance
Work in progress.

8 Calculation and expression of results

8.1 Calculations
Calculate the mean percentage survival of individuals in the different treatments at each growth assesment.

Calculate the mean body sizes of individuals exposed to each test concentration.

Growth curves may be analysed using repeated measures ANOVA or using a von Bertalanffy growth model.

Body lengths of individuals exposed to different concentrations may be compared on a date-by-date basis using one-way ANOVA providing the

data meets the normal requirements (i.e. normally distributed and of equal variance).

Where more than 3 test concentrations have been used it may be possible to calculate EC_{50} values for growth inhibition.

Comparisons of the numbers of juveniles produced may be made using non parametric tests, eg. contingency Chi-squared tests.

8.2 Expression of results

- present ANOVA tables and results from multiple range tests
- EC_{50} values should be expressed in mg/kg d.w. soil

9 Validity of the test

More than 50% of the Collembola should survive for the 10 weeks of assessment or until asymptotic growth has been reached.

10 Test report

The test report shall refer to this standard and shall provide the following information:

- complete description of the biological material used (species, age, culturing conditions, and source)
- method of preparation of test substrate and any 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 percent mortality of Collembola obtained for each concentration and for the control during the experiment
- results from ANOVA and/or probit analysis
- description of obvious or pathological symptoms or distinct changes in behaviour observed in test organisms per test container
- water content and pH of artificial soil (in 1M KCl) at start and at end of the test
- all operating details not specified in the standard, and any occurrences liable to have affected the results
- some specimens used in the test should be preserved for future reference

References

Fjellberg, A. (1980): Identification Keys to Norwegian Collembola. The Entomological Society of Norway. 151 pp.

9 Sublethal toxicity test with Centipedes

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9.1 Objectives for the reporting period

- To develop methods of maintaining field-captured centipedes and isopods in laboratory populations
- To establish laboratory populations of centipedes and isopods
- To begin the development of a laboratory test system to detect sublethal effects of chemicals on population parameters of predaceaous centipedes
- To gather life-history data to allow sensitivity analysis of fitness parameters for centipedes

9.2 Objectives for the next period

- To develop a laboratory test system for detecting sublethal effects of chemicals on population parameters and individual characteristics of centipedes
- To complete investigations of sublethal toxicity effects of copper, dimethoate and LAS on centipedes
- To investigate the population consequences of sublethal effects of copper, dimethoate and LAS on centipedes
- To refine and standardize the test system

9.3 Main results obtained

9.3.1 Methodology

Field captured predaceous centipedes (*Lithobius mutabilis* Koch., Chilopoda) and their prey animals (*Porcellio scaber* Latr., Isopoda) have been maintained in the laboratory since spring 1994. The animals were held in 30 × 50 × 20 cm containers, approximately 200-300 individuals in each, under constant laboratory conditions at a temperature of 15 ± 1 °C., a relative humidity ≥ 80%, and a light:dark cycle of 16:8 h. The bottoms of the tanks were covered with a layer of a moist natural soil 2-3 cm thick with some leaf-litter on the surface.

During 1994 preliminary studies on the development of toxicity tests for copper have been started. Adults of 15.0-45.0 mg fresh mass (10-15 mm length) were used in the experiment. Individual animals were kept in plastic containers of 11 × 7.5 × 4.5 cm, and for population studies two males and two females were kept in boxes

of 16 × 11 × 6 cm. The boxes were filled with a 1-cm layer of the standard artificial soil (OECD; moisture content = 50% Water Holding Capacity) and covered with loose transparent plastic lids. The centipedes were fed twice a week with a half of a deep-frozen *P. scaber* per individual. Two sets of experimental designs were used: one with copper contaminated soil and uncontaminated food (isopods) and the other with contaminated soil and food. The concentrations of copper in soil and food offered to *P. scaber* were: 0 (control), 10, 40, 160 and 640 mg/kg dry weight soil/food. To get contaminated food for centipedes, *P. scaber* were fed an artificial food (oak-hornbeam leaf litter + rabbit chow + water) contaminated with copper at nominal concentrations as above.

The variables measured were: mortality, number of eggs, consumption rate, growth rate, respiration rate, locomotor activity level and pattern (work in progress, Digital Image Processing methods), copper concentration in *P. scaber* and *L. mutabilis* (Atomic Absorption Spectrometer) and expression of heat shock proteins (hsp70) in tissues (work in progress). The experiment was terminated after 127 days.

9.3.2 Results

Isopods fed on a copper-contaminated diet, and used as prey animals for centipedes in the second experimental series, showed a linear increase in total body concentrations of copper with increasing copper concentration in food (Fig. 9.1). Copper concentrations in centipedes fed copper-contaminated isopods revealed a clear increase as compared to control. However, no differences (ANOVA, $p>0.05$) in copper concentrations were found in centipedes kept on contaminated soil but fed uncontaminated food.

The respiration rate was measured 8, 26, 43, 72 and 99 days after the start of the experiment. A significant decrease (regression analysis) in the respiration rates in copper-treated centipedes was found on day 26 in animals fed with copper-contaminated food and exposed to copper-treated soil ($p<0.0005$), and on day 43 animals exposed to copper-treated soil ($p<0.005$) (Fig. 9.2). However, later the respiration rates recovered to their initial levels.

No effect of copper treatment was found on consumption, growth or mortality rates during the 127 day-long experiment. Due to a long life cycle of *L. mutabilis* and egg-laying spread almost evenly throughout a year, no estimates of the effect of treatment on fertility are available yet.

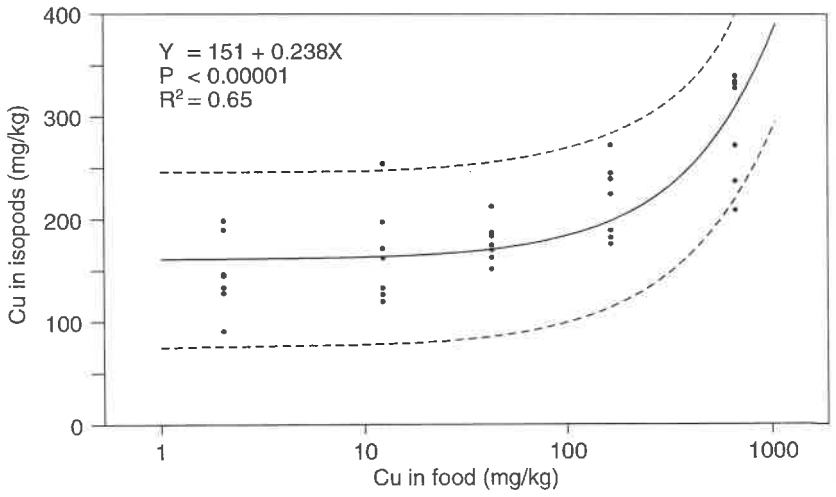

Figure 9.1: Copper concentration in isopods fed Cu-contaminated diet.

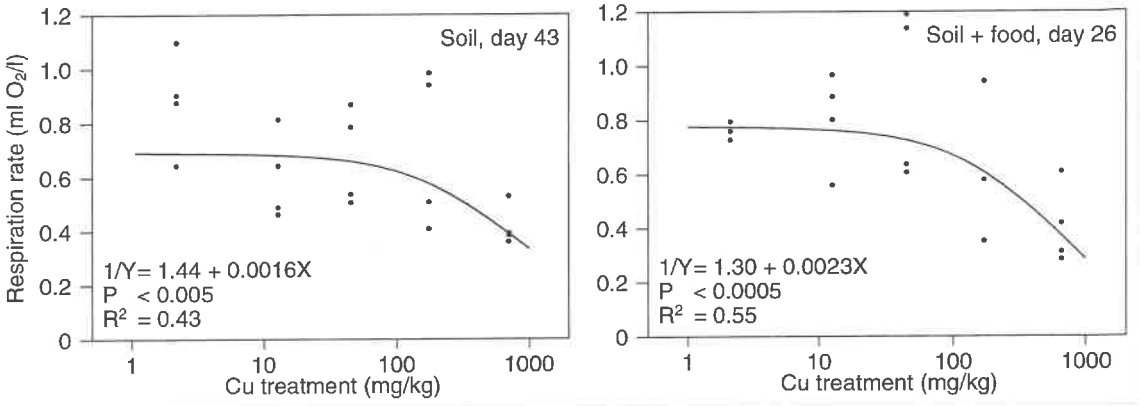

Figure 9.2: Respiration rates in centipedes held on Cu-contaminated soil and fed uncontaminated diet (left) and held on Cu-contaminated soil and fed Cu-contaminated diet (right); calc. per 1 g of fresh mass.

9.3.3 Discussion

Preliminary results of copper toxicity on centipedes indicated that among the variables considered, measurements of the respiration rate may be a promising tool for monitoring sublethal effects of pollution. However, the respiration rate seems to be affected for a relatively short period. Thus, the measurements have to be carried out at intervals not longer than those used in this study, i.e. at least approximately every 20 days. The decrease in the respiration rate in copper treated animals probably indicates physiological stress caused by an increased copper level in the environment but the animals are able to recover their initial respiration rates 3-5 weeks after the start of the experiment, depending on the exposure route.

Although at the highest treatments (160 and 640 µg/g) the concentrations of copper in centipedes fed contaminated isopods were lower than those in their diet, at the lower treatments (10 and 40 µg/g) they were almost identical to those found in isopods. These results indicate that centipedes may regulate copper concentration when exposed to a highly contaminated food.

Because of the long and complicated life-cycle of centipedes, some population parameters, especially fertility, are extremely difficult to estimate even under laboratory conditions. *L. mutabilis* lay single eggs throughout the year, covering each egg with soil and humus particles. To overcome these problems, other methods for indirect estimation of pollution effect on fertility are considered. Preliminary studies indicate that one of the possibilities is dissecting females at the end of the experiment and counting eggs found in their body.

For the future standardization of the centipede test it is suggested that housefly pupae should be used as a prey animal instead of isopods. This would make it easier to maintain large laboratory populations of centipedes, and the time of treatment for prey animals could be easily standardized and set to the whole larval period. Although some initial tests have been done already, this will be tested in detail during the next stage of the project.

9.3.4 Deviations and modifications to the work plan

For the sake of standardization, both the carnivore and its prey species have been changed. It was originally planned to use *L. forficatus* as a carnivore and the isopod *Oniscus asellus* as its prey. However, both *L. mutabilis* and *P. scaber* are more widespread in the forests of continental Europe, being thus more suitable for standard test animals. In the next stage of the project it is planned to test the suitability of the housefly pupae as a standard food for toxicity tests on centipedes.

ANNEX 9.1 - Test System Standardization Form 8

Sublethal toxicity test with Centipedes

General data about the test organism at the start of the experiment

Test organism

Taxonomic group:	Centipedes (Myriapoda: Chilopoda)
Species:	*Lithobius mutabilis* L. (Koch, 1852)
Life stage:	adult
Sex:	males and females
Age/weigh/size:	adult age: at least 2 years weight: 15.0 - 45.0 mg (fresh mass) size: 10 - 15 mm
Gen. composition:	natural population
Background:	laboratory maintained field captured animals

Type of surroundings and ambient climate

Surroundings	
Type:	climate chamber
Ambient climate	
Air temperature:	15 ± 1 °C.
Relative humidity:	≥ 80%
Light wave length:	artificial light (Tungsten tubes)
Light intensity:	not measured
Light regime:	light:dark cycle 16:8 h

Type of test system and test conditions

Type:	containers: for individuals 11 × 7.5 × 4.5 cm for population studies 16 × 11 × 6 cm
Material:	plastic
Openness:	continuously covered with loose transparent lids
Spec./test system:	single species test system (*L. mutabilis* fed on deep frozen *Porcellio scaber* Latr.)
Ind./test system:	1 for studies of individual parameters 4 for studies of population parameters

Type of habitat

Soil/litter:	A) Centipedes: artificial soil (OECD); B) Isopods: artificial food based on natural one-year-old oak-hornbeam litter (defaunated by deep freezing and heating) + rabbit chow + water
'Habitat characteristics'	
Soil:	10% peat, 20% caolin clay, 70% quartz sand, plus some $CaCO_3$ (dry weight), soil pH 6.94 (in water), 6.03 (in KCl)
Hydrol. charact.:	50% WHC
Food supply:	during the complete test
Feeding rate:	twice a week
Type of food:	1/2 deep frozen *Porcellio scaber*

Exposure characteristics

Exposure route:	contaminated soil or contaminated soil + contaminated food
Composition of test habitat:	soil substrate mixed homogeneously with an aqueous solution (in deionized water) of a test chemical: 2 days before the test begins, the soil is moistened with a solution up to 50%WHC and stored at 5 °C. One day before the test starts, closed containers

	with 50 g d.w. of treated soil for individual studies and 100 g d.w. soil for population studies are put for acclimatization at 15 °C. At the day the test starts, Centipedes are put into the test containers.
Composition of control habitat:	see above: the same procedure without a chemical
Duration of experiment	
Acclimat. time:	one month
Exposure time:	work in progress
Recovery period:	-
Exposure concentration	
Replicates:	4 per concentration and control
Concentrations:	at least 4 concentrations
Series:	1
Ref. chemical	work in progress
Exposure responses	
Effect parameters:	survival, growth rate, respiration rate, activity pattern, potential fertility
Other parameters:	none
Measurement of variables:	A) number of individuals, number of eggs, number of juveniles: observation after 1, 2, 3, 4, 5, 7, 10, 14, 21, 28 days and weekly further on; B) growth rate: weekly measurements; C) respiration rate after 1st, 2nd, 4th and 8th week D) activity: after the end of the experiment within one week E) fertility: number of eggs in ovaries after the end of the experiment
Toxic endpoint:	LD_{50}, EC_{50} (based on actual concentrations)
Unit:	mg test substance/kg d.w. test soil
Working methods:	counting survivals, and eggs; weighing; respirometry (closed system); DIP for activity measurements
Analytical methods:	AAS for measuring Cu concentrations
Miscellaneous test information	
Practicability:	the required materials are easily obtainable from commercial agents, DIP is a commercially available software; test animals must be cultured or collected in the field and acclimatized to laboratory conditions; the test protocol can be performed without complicated training by technical staff.
Cost-effectiveness:	time for culturing the Centipedes and Isopods; no expensive materials
Sensitivity:	work in progress
Reproducibility:	work in progress
Validity of test:	A) standard statistical methods; Regression analysis B) mortality in the control group should not exceed 10%
Standardization:	soil type (artificial soil), pollutant application (mixing with soil, food preparation), food type, pollutant application
Ecological realism:	Centipedes are surface-living invertebrates, thus, the breeding methods do not deviate much from their natural habitat
Broad chemical responsiveness:	no restrictions with respect to the chemicals that may be evaluated with this test

References

Hopkin, S.P. (1989): Ecophysiology of Metals in Terrestrial Invertebrates. Elsevier Appl.Sci., London.

Lewis, J.G.E. (1981): The biology of centipedes. Cambridge University Press.

Camatini M. (1979): Myriapod Biology. Academic Press Inc. (London) Ltd.

ANNEX 9.2 - Draft test protocol 8

Sublehtal toxicity test with Centipedes (*Lithobius mutabilis* L.)

1 Scope

This test protocol describes a method for determining the effects of substrate exposure and alimentary uptake of chemical substances on mortality, growth, fertility, respiration rate and locomotor activity of a carnivorous centipede *Lithobius mutabilis L.* (Koch, 1852), using a defined artificial soil substrate, standardized food and defined concentration of the tested substance in the substrate. The transfer of the test chemical to carnivore species (*L. mutabilis*) from its food (food-chain effect) is also estimated.

2 Definitions

2.1 Growth
Increase in biomass, i.e. the fresh mass of the organism at the end of test expressed as a percentage of its fresh mass at the start of the test.

2.2 Fertility
Mean number of eggs found in the body of the organism at the end of the test.

2.3 EC_{20} & EC_{50}
Estimated concentrations causing 20% and 50% effect respectively on the characteristic measured.

2.4 Biomagnification factor
Slope of the regression line (if significant regression can be found) describing the relation between total-body concentration of the test chemical in food (prey animal) to its concentration in centipedes. Biomagnification factor (slope) greater than 1.0 suggests a possibility of increase in concentrations of the test chemical along the food chains.

3 Principle

The percentage mortality, effects on growth, fertility, respiration rate and locomotor activity of the test species (*L. mutabilis*) are determined using a defined artificial soil substrate, standardized food and defined concentration of the tested substance in the substrate. The test substance is added in a single step. The effects on mortality and respiration rates are determined every 3rd week. The effects on fertility and locomotor activity are measured for 8 days after 12 weeks of experiment. Centipedes are analyzed for the total-body concentration of the test chemical at the end of the experiment, if possible.

For substances without known toxicity it is useful to conduct the test in two steps:

- a preliminary short-term acute toxicity test, using a wide range of test concentrations set at a geometric series with base 10, for determining the concentrations to be tested in the final test
- the final test on mortality and sublethal effects to determine the EC_{20} and EC_{50} of a test chemical on all characteristics measured

4 Reagents

4.1 Biological material

The biological material consists of adult centipedes of the species *Lithobius mutabilis* (approximately 15.0 - 45.0 mg fresh weight; 10 - 15 mm in length). Centipedes to be used for the test are selected from the laboratory maintained populations of field-captured animals acclimatized for at least two weeks to the laboratory conditions. Centipedes to be used for the test should be selected to form a population with a relatively homogeneous age, size and mass.

NOTE - *Lithobius mutabilis* exists as two subspecies: *L. mutabilis mutabilis* and *L. mutabilis sudeticus*. Either species may be used for this test, although *L. mutabilis sudeticus* as the endemic species will have minor importance. (Kaczmarski J, 1979).

4.2 Test substrate

4.2.1 Soil substrate:
The soil substrate should composed of (on dry weight basis):

10%	Sphagnum peat (oven-dried in 60 °C.) as finely ground as practicable and with no visible plant remains
20%	Kaolinite clay (oven-dried in 60 °C.)
70%	Quartz sand (oven-dried in 60 °C.) (predominately fine sand with more than 50% of particle size 0.05 mm - 0.2 mm)

Calcium carbonate ($CaCO_3$) analytical grade - sufficient amount to bring the pH (measured in 1 mol/l KCl solution) to 6.0 ± 0.5.

The mass of the soil prepared should be sufficient for use of 100 g d.w. soil substrate per test container.

4.2.2 Artificial soil (OECD):
Artificial soil is prepared by wetting the soil substrate (4.2.1.) with deionized/distilled water or solution of the test chemical to reach a water content of 50% of total water holding capacity (WHC).

4.3 Food

Deep frozen isopods (*Porcellio scaber* or *Oniscus asellus*) or pupae of the housefly (*Musca domestica*) (work in progress) may be used as a standard food. To estimate the effects of an alimentary uptake of the test chemical, the

housefly larvae or isopods are fed standard artificial medium mixed with the test chemical (test concentrations established as in 3.).

4.3.1 Artificial diet for isopods:
220 g Oak-hornbeam leaf litter (oven dried in 60 °C.) + rabbit chow (10:1)
780 g Deionized/distilled water or solution of the test chemical

4.3.2 Artificial diet for housefly:
490 g Rabbit chow
5 g Milk powder
5 g Sugar
500 g Deionized/distilled water or solution of test chemical

5 Apparatus

Standard laboratory equipment and:

5.1 Containers

Plastic containers with a surface area of about 100 cm^2 - 200 cm^2, covered with loose transparent plastic lids. The lids should permit gaseous exchange between the box and the atmosphere but should not be excessively perforated to avoid fast drying of the soil.

5.2 Respirometer

Any standard respirometer can be used. For simultaneous measurements of the respiration rates in a number of single individuals, small closed-system (Warburg- or Drastich-type) respirometers are the most suitable. Measurements should be done in a constant temperature (water bath), the same as used during the whole experiment (15 °C.).

5.3 Locomotor activity

Any suitable Digital Image Processing (DIP) apparatus and software can be used (e.g. Noldus, Oxalis).

6 Test environment

6.1 Temperature

Constant temperature room or a climatic chamber controlable to a temperature of 15 ± 1 °C. is a minimum requirement. High humidity (r.h. ≥ 80%) should be secured, e.g. with the use of a room humidifier.

6.2 Light conditions

Fluorescent tubes delivering an illumination of approximately 300 - 600 lux at a controlled light : dark cycle of 16:8 h.

7 Procedure

7.1 Preliminary test

If it is necessary to determine the range of concentrations for use in the final test, perform a preliminary acute toxicity test using the same conditions as described above. There has to be at least four experimental concentrations in a geometric series with base 10 (e.g. 1, 10, 100, 1000 mg/kg dry weight) plus control ("0"). Use twenty animals (ten males and ten females) per treatment. All concentrations are expressed in milligrams of the test substance per kilogram of dried soil substrate. In estimating the effects of the alimentary uptake, all concentrations are expressed in mg of the test substance per kilogram of dried food (prey animal).

Conduct the preliminary test for 30 days. Only concentrations below LC_{50} will be used in the final test.

7.2 Final test

7.2.1 Introduction of the test substances:
Use either method a), b), og c) as appropriate:

a) Water soluble substances
Two days before starting the test, dissolve the quantity of the test substance in the water required for the replicates of one concentration (or that of it necessary to wet the soil substrate in order to meet the requirements of 4.2.2) and mix it thoroughly with the test substrate. Leave the substrate mixed with the test chemical at 5 °C. for 24 h. One day before starting the experiment fill the test containers with artificial soil (50 g - 100 g d.w.), close the lids and leave for 24 h in a climate chamber for acclimatization.

b) Substances insoluble in water but soluble in organic solvents
Dissolve the quantity of the test substance required to obtain the desired concentration in a volatile solvent (such as acetone or hexane). Add it to test containers (5.1) with soil substrate (4.2.1). Mix thoroughly and evaporate the solvent by placing the container under a fume hood for 1 h. Then add the water in accordance with 4.2.2 and mix thoroughly.

c) 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 (4.2.1) and the quantity of the test substance required to obtain the desired concentration. Place the mixture, the reminder of the soil substrate (4.2.1) and the water into the test container (5.1) and mix thoroughly.

NOTE - Ultrasonic dispersion, organic solvents, emulsifiers or dispersants may be used to disperse substances with low aqueous solubility. When such auxiliary substances are used, all test concentrations and an additional control should contain the same minimum amount of the auxiliary substance. To allow solvents to volatilize, the test containers should remain open for a period of one hour.

The concentrations selected to estimate the EC_{20} and EC_{50} are based on the results of the preliminary test.

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).

Proceed simultaneously with at least four replicates per concentration and a control test without the test substance.

7.2.2 Control containers:
Prepare control containers in the same way as test containers without the test substance. If the preparation of the test requires the use of auxiliary substances (see 7.2) use additional control containers similar to test containers without the test substance. Treat these containers in the same way as those without the test substance. Include an additional control container for pH and soil moisture determination at the end of the test.

7.2.3 Addition of the biological material:
For each container (four replicates per treatment) select two male and two female centipedes (4.1) and weigh them. Place the centipedes on the soil surface and provide each container with a hiding place for the test animals (e.g two pieces of a ceramic bowl, approx. 2 cm × 2 cm, per container). Cover the containers as described in 5.1.

Place the containers in a climate chamber.

7.2.4 Determination:
Following 7.2.3, feed the centipedes twice a week with one housefly pupae or half of an adult *P. scaber* per individual placing the food on the soil surface.

Maintain the water content of the soil substrate in the test containers during the test period by re-weighing the test containers periodically and if necessary replenishing lost water (water content should not drop below 40% WHC).

Keep the centipedes in the test containers for a period of 12 weeks. Every third week record the number of living animals, weigh them and measure the respiration rate of the animals (NOTE - The respiration rate should be measured for each individual separately to avoid any effect of inter-individual interactions during the measurements).

After 12 weeks take at least one individual from each test container for the locomotor activity measurements. Measure the total level of activity and activity pattern for at least 5 days.

After finishing the measurements kill all centipedes by deep freezing and dissect females. Count number of eggs for each female.

Use males for chemical analyses of the total-body concentration of the test chemical, if possible.

Use at least five replicate prey animals per treatment for chemical analysis of the total-body concentrations (if possible).

7.3 Reference substance

Work in progress.

8 Calculation and expression of the result

8.1 Calculation

For each concentration determine the per cent mortality and per cent loss/increase in biomass of the centipedes and number of eggs produced in the final test.

Use standard regression analysis procedures (best fit) for the dose-response relationship. Significant ($p<0.05$) regressions will be used to calculate EC_{20} and EC_{50} of the test chemical.

Use the regression analysis to calculate the biomagnification factor (slope concentration in prey/concentration in predator).

8.2 Expression of results

Indicate, in mg/kg d.w. of soil/food of prey, the estimated EC_{20} and EC_{50} (2; 8.1) on mortality, fertility, growth rate, respiration rate and total locomotor activity level.

9 Validity of the test

Work in progress.

10 Test protocol

The test report should refer to this standard and should provide the following information:

- complete description of the biological material employed (species, body size and mass ranges, breeding conditions, supplier or sampling area)
- preparation method of test substrate and any auxiliary substances used for low-/non-water soluble substance
- results obtained with the reference substance, if performed
- detailed conditions of the test environment
- table giving the percentage mortality obtained for each container for each concentration and for the control
- mass of adult centipedes alive at the beginning of the test and after a period of 12 weeks
- number of eggs (fertility) per female at the end of the test

- results of the regression analysis of the data (p, R^2 and regression equation if $p<0.05$)
- calculated EC_{20} and EC_{50} with their confidence intervals (95%) on mortality, fertility, growth and respiration rates, and total activity level, if $p<0.05$
- description of the obvious pathological symptoms or distinct changes in behaviour (e.g. registered changes in the locomotor activity pattern) observed in the test organisms per test container
- water content and pH of artificial soil at the start and after 12 weeks
- all operating details not specified in the standard and any occurrences liable to have affected the results
- some specimens used in the test should be preserved for future reference

References

Kaczmarski J. (1979): Pareczniki (Chilopoda) Polski. Wydawnictwa UAM, Ser. Zool. IX. Poznan (in Polish).

10 Sublethal toxicity test with the woodlouse *Porcellio scaber* (Latr., 1814) (Isopoda: Porcellionidae)

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Detailed report of the contractor/subcontractor

10.1 Objectives for the reporting period

- To improve an intensive culturing method for *Porcellio scaber* (Latr., 1814) (Isopoda: Porcellionidae)
- To optimize laboratory conditions and food composition
- To determine life-history parameters under laboratory conditions
- To determine the impact of copper on *P. scaber* in artificial soil (OECD)
- To carry out toxicity tests using copper introduced via food
- To determine - as endpoints - the mortality, food consumption, growth rate and induced oosorption

10.2 Objectives for the next period

- To repeat experiments using LUFA 2.2 soil
- To complete standardization using LAS and dimethoate as toxicants (both via food and soil), based on the developed laboratory methods
- To evaluate results statistically using the ANOVA approach

10.3 Main results obtained

10.3.1 Methodology

Culturing methods:
Plastic boxes were used (diameter of 8 cm) with plaster of Paris in the bottom. They were placed on wet plaster trays in order to obtain a nearly 100% relative humidity in the boxes. The laboratory conditions were: 23-25 °C. air temperature

and 16:8 hours light/dark cycle. The food consisted of leaf litter, potatoes and rabbit food. Food availability: ad libitum. 100 gravid females of *P. scaber* (females with marsupium) were kept individually in breeding boxes. After giving birth females were removed and put into new boxes. The offspring was counted to state the clutch size and weighed weekly. The following life history parameters were examined: rate of survival, weight increase of juveniles for both females and males, age of sexual maturation, age of females at the first reproduction, time interval between births, the number of reproductive periods within 6 months, average clutch size per birth. Among reproductive parameters it was attempted to follow ovarial development, the overall number of oocytes and that of normal and disrupted ones (i.e. visible oosorption) using dissection technique (Hornung and Warburg,1994).

Toxicity experiments:
For toxicity experiments Petri dishes with an artificial soil (OECD) in the bottom were used. They were put into wet boxes. Five or ten isopods were introduced in each dish, in five replicates, depending on the aim of the experiment. Cupric chloride ($CuCl_2$ $2H_20$) was used for toxicity tests. Eight copper concentrations were used between 100 and 10.000 mg/kg beside controls. Copper was introduced via soil and food in different tests. Food pills consist of 50% leaf litter powder, 40% powdered rabbit food and 10% potato powder. The studied endpoints were: survival, food consumption, weight increase of juveniles, and visible oosorption.

10.3.2 Results
Results of the laboratory culturing methods:
The food consumption of *P. scaber* proved to be 27.5 mg d.w./100 mg live weight/week. The natural copper-content of the food was 27.2 mg/kg. The offspring reached an average body weight of 60 mg within the first five months of their life.

Some of the females born in the laboratory became pregnant at the age of 3.5 months. When gravid females were separated after their manca (larvae) release and kept in breeding boxes without males, they could repeat breeding monthly up to five times. The average clutch size was 28 (Table 10.1).

Results of the toxicity experiments:
A significant mortality appeared within 2 weeks in the case of artificial soil (OECD) containing about 4000 mg/kg copper. Isopods tolerated the same copper content in their food for a longer time resulting in a significant mortality only after the fourth week of the treatment.

1000 mg/kg or more copper in the food caused a significant decrease in the consumption, and growth was heavily retarded. 100 mg/kg copper content in the food may result in a moderate stimulation of juvenile growth (hormesis).(Data in Table 10.2.)

Based on our preliminary data on reproduction, significant differences could be found in the visible oosorption between control isopods and copper treated ones (Fig. 10.1). The lowest copper content in the soil used resulting in stimulated oocyte disruption was 250 mg/kg.

Table 10.1: Life history parameters of *P. scaber* (*x: the number of weeks)

Data under laboratory conditions (20 °C., 80% air humidity, 16:8 light-dark cycle, standard food)

	mean	data by relevant literature
longevity	-	30-48 months
time interval between two consecutive births	35,56 days	marsupial stage 27-46 days
lenght of juvenile stage until first birth	94.6 days	till sexual maturity 8-12 months
number of broods/year	2 - 4	1 - 3
clutch size	28	29 min: 5; max:100
survival rate of juveniles	until 6th w.: 85% until 12th w.: 74.5% until 18th w.: 71.5%	-
breeding period	continuous (**)	Febr. - Nov.
sex rate (male:female)	about 50 : 50 %	40 : 60
weight at birth	0.42 mg	-
rate of weight increase	until the 8th week: * 0.357x1.62 R2-0.96 from the 8th week: * 2.91x-5.48 R2=0.99	-
rate of food consumption	3.93 mg/100mg live weight/day	-

**: Preliminary.

Table 10.2: Acute and sublethal effects of copper on *P. scaber*

Single species test system	Parameter	Time (days)	Sex	Effect concentration (mg/kg d.w.)
Treated artificial soil	Mortality	14	males+	LC_{50}4360
		28	females	LC_{50}3950
Treated food	Mortality	42	males+ females	LC_{50} 730
	Food consumption	28	males	$EC\text{-}_{10}$ 580
				$EC\text{-}_{50}$ 902
			females	$EC\text{-}_{10}$ 153
				$EC\text{-}_{50}$ 760
			males+	$EC\text{-}_{10}$ 573
			females	$EC\text{-}_{50}$ 877
	Body mass gain	28	males	$EC\text{-}_{10}$ 216
				$EC\text{-}_{50}$1942
			females	$EC\text{-}_{10}$ 867
				$EC\text{-}_{50}$2685
			males+	$EC\text{-}_{10}$ 350
			females	$EC\text{-}_{50}$2421

10.3.3 Discussion

The worldwide distribution and the easy laboratory culturing of *P. scaber* makes this woodlouse an ideal candidate for use in standardized toxicology tests. Our results proved that under the laboratory conditions described above a faster growth and more frequent reproduction can be reached at *P. scaber* compared with literature.

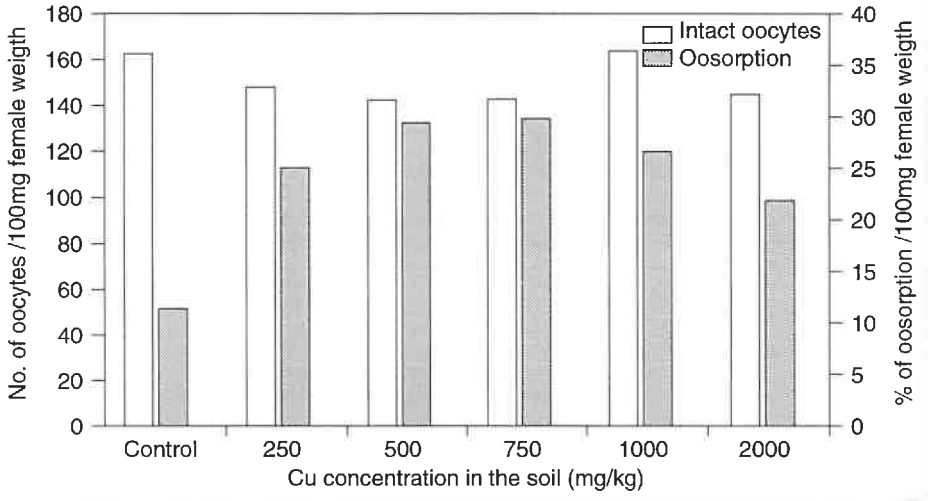

Figure 10.1: The effect of Cu on the development and oosorption of oocytes in *P. scaber.*

P. scaber proved to be more susceptible to copper in the artificial soil (OECD) than in the food. The reasons may be the lower copper binding capacity of artificial soil (OECD), and the refusal of food containing high copper concentration by the isopods. The stimulated oosorption seems to be a sensitive life history parameter using sublethal copper concentrations.

10.4 References

Hornung, E. & Warburg, M. R. (1990): Oosorption and oocyte loss in terrestrial isopod under stressful conditions. Tissue and Cell 26, 277-284.

Annex 10.1 - Test System Standardization Form 9.1

Sublethal toxicity test with the woodlouse *Porcellio scaber* (Latr., 1814) (Isopoda: Porcellionidae) using growth as endpoint

General data about the test organisms at the start of the experiment

Test organism

Taxonomic group:	woodlouse (Isopoda: Porcellionidae)
Species:	*Porcellio scaber* (Latr. 1814)
Life stage:	juveniles
Sex:	males and females
Age/weight/:	10-16 weeks / 20-40 mg
Genetic comp.:	not known
Source:	parental generation was collected from uncontaminated sites, the others are laboratory cultured

Type of surroundings and ambient climate

Surroundings

Type: wet boxes

Ambient climate

Temperature (°C.):	20 ± 1 °C.
Rel. humidity:	>80%
Light wave length:	natural and neon light
Light intensity:	not relevant
Light regime:	light:dark cycle 18:6 h

Type of test system and test conditions

Type:	Petri dishes
Material:	glass
Openness:	closed, opened weekly
Species/test syst:	single species
Ind./test system:	10 males + 10 females

Type of habitat

Soil:	artificial soil (OECD)
Medium:	artificial culture medium
'Habitat characteristics'	
Soil charact.:	artificial soil (OECD) consisting of Sphagnum peat (10%), kaolin clay (20%), sand (70%), some $CaC0_3$
Hydrol. charact:	40 - 60 % of water holding capacity
Food supply:	yes
Feeding rate:	ad libitum
Type of food:	complex food containing grained soil litter (50%; collected from an oak wood), grained rabbit food (40 %), dried and grained potato powder (10%), all mixed and compressed into pills.

Exposure characteristics

Exposure route:	via soil
Composition of test soil:	artificial soil (OECD), deionized water, test substance and auxiliary agent (if used), all constituents well homogenized.
Composition of control soil:	a) artificial soil (OECD), deionized water b) artificial soil (OECD), deionized water and auxiliary agent.

Duration of the experiment

Acclim. time:	1 week
Exposure time:	8 weeks
Recovery period:	none

Exposure concentrations

Replicates:	5
Concentrations:	5
Series:	1
Ref. chemical:	work in progress

Exposure responses

Effect parameter:	survival weight increase food consumption
Toxic endpoint:	LC_{50}, EC_{10}, EC_{50},
Unit:	mg test substance/kg d.w. test soil
Analytical methods:	not relevant

Exposure characteristics

Exposure route:	via food
Composition of test soil:	artificial soil, deionized water, test substance and auxiliary agent (if used), alle constituents well homogenized.
Composition of control soil:	a) artificial soil (OECD), deionized water b) artificial soil (OECD), deionized water and auxiliary agent.

Duration of experiment

Acclim. time:	1 week
Exposure time:	8 weeks
Recovery period:	none

Exposure concentrations

Replicates:	5
Concentrations:	5
Ref. chemical:	work in progress

Exposure responses

Effect parameter:	survival weight increase food consumption
Toxic endpoint:	LC_{50}, EC_{10}, EC_{50},
Unit:	mg test substance/kg d.w. of food
Analytical methods:	no

Miscellaneous test information

Practicability:	*P. scaber* can be easily cultured from populations obtained from the field. All the steps of the test can be performed without specific training of technical staff
Cost effectiveness:	culture and test are laborious
Sensitivity:	work in progress
Reproducibility:	work in progress
Validity:	the mortality of the control should be less than 20 %
Standardization:	suggested size of animals should be 20 - 40 mg
Ecological realism:	the woodlouse *Porcellio scaber* is a widely distributed, cosmopolitan species in European ecosystems.
Broad chemical responsiveness:	the test system is in principle able to test any chemical that can be incorporated into soil or food

References

Hornung, E. & Warburg, M. R. (1990): Oosorption and oocyte loss in terrestrial isopod under stressful conditions. Tissue and Cell 26, 277-284.

Annex 10.2 - Test System Standardization Forms 9.2

Sublethal toxicity test with the woodlouse *Porcellio scaber* (Latr., 1814) (Isopoda: Porcellionidae) using oosorption as endpoint

General data about the test organism at the start of the experiment

Test organism

Taxonomic group:	Woodlouse (Isopoda: Porcellionidae)
Species:	*Porcellio scaber* (Latr., 1814)
Life stage:	sexually matured animals
Sex:	males and females
Age/weight:	about 10-16 weeks / 20-40 mg
Genetical composition:	not known
Background:	parental generation was collected from uncontaminated sites, the test ones are laboratory cultured

Type of surroundings and ambient climate

Surroundings	
Type:	wet boxes
Ambient climate	
Air temperature:	20 ±1 °C.
Rel. humidity:	>80%
Light wave length:	natural and neon light
Light intensity:	not relevant
Light regime:	light:dark cycle l8:6 h

Type of test system and test conditions

Type:	test container
Material:	plastic (diameter about 12 cm; height about 8 cm)
Openness:	closed, opened weekly
Spec./test system:	single species test system
Ind./test system:	12 females + 6 males

Type of habitat

Soil/litter:	artificial soil (OECD)
'Habitat characteristics'	
Soil charact.:	artificial soil (OECD) consisting of Sphagnum peat (10%), kaolin clay (20%), sand (70%), some $CaCO_3$
Hydrol. charact.:	40 - 60 % of water holding capacity
Food supply:	yes
Feeding rate:	ad libitum
Type of food:	complex food containing grained soil litter (50%; collected from an oak wood), grained rabbit food (40 %), dried and grained potato powder (10 %), all mixed and compressed into pills

Exposure characteristics

Exposure route:	via soil
Composition of test soil:	artificial soil (OECD), deionized water, test substance and auxiliary agent (if used), all constituents well homogenized
Composition of control soil:	a: artificial soil (OECD, deionized water b: artificial soil (OECD), deionized water and auxiliary agent

Duration of experiment

Acclim. time:	not relevant
Exposure time:	10 weeks
Recovery period:	not relevant

Exposure concentrations

Replicates:	5
Concentrations:	5
Series: 1	
Ref. chemical:	work in progress

Exposure responses

Effect parameter:	reproduction, (% of oosorption. No. of eggs/embryos/mancas/juveniles)
Toxic endpoint:	EC_{50}, NOEC
Unit:	mg/kg test substance/kg d.w. test soil
Analytical methods:	not relevant yet

Exposure characteristics

Exposure route:	via food
Composition of test soil:	artificial soil (OECD), deionized water, test substance and auxiliary agent (if used), all constituents well homogenized
Composition of control soil:	a) artificial soil (OECD), deionized water b) artificial soil (OECD), deionized water and auxiliary agent

Duration of the experiment

Acclim. time:	not relevant
Exposure time:	10 weeks
Recovery period:	not relevant

Exposure concentrations

Replicates:	5
Concentrations:	5
Series:	1
Ref. chemical:	work in progress

Exposure responses

Effect parameter:	reproduction
Toxic endpoint:	EC_{50}, % of oosorption. No. of eggs /embryos/ mancas/juveniles
Unit:	mg test substance/kg d.w. food
Analytical methods:	not relevant yet

Miscellaneous test information

Practicability:	*P. scaber* can be easily cultured from populations obtained from field. Test containers don't need any construction.
Eost effectiveness:	culture and test are laborious. Dissection technique requires some training
Sensitivity:	work in progress
Reproducibility:	work in progress
Validity:	at least 10 females must be dissected in each ovarial/marsupial stage
Standardization:	suggested size of animals should be 20 - 40 mg
Ecological realism:	the woodlouse *Porcellio scaber* is a widely distributed, cosmopolitan species in European ecosystems.
Broad chemical responsiveness:	the test system is in principle able to test any chemical that can be incorporated into the soil or food.

References

Hornung, E. & Warburg, M. R. (1990): Oosorption and oocyte loss in terrestrial isopod under stressful conditions. Tissue and Cell 26, 277-284.

Annex 10.3 - Draft test protocol 9.1

Sublethal toxicity test with the woodlouse *Porcellio scaber* (Latr., 1814) (Isopoda: Porcellionidae) in artificial soil using growth as endpoint

1 Scope

This guideline describes a method for determining the effects of chemicals on survival, food consumption and growth of *Porcellio scaber* by dermal and alimentary uptake using defined food and soil substances.

2 Definitions

2.1 Survival
Mean number of adults surviving until the end of the test period (4-8 weeks).

2.2 Food consumption
Mean consumed food/week/100 mg live weight.

2.3 Growth
Mean fresh mass gain, expressed as a percentage of the fresh mass of animals at the beginning of the experiments.

3 Principle

The woodlice are placed in a defined artificial soil substrate and fed with defined food containing the test substance in different concentrations. The percentage survival, the food consumption and the mass gain of woodlice are determined. The mortality, the effect on food consumption and on mass gain are determined weekly. The results are expressed as 4 weeks' survival, and as average of 8 weeks' food consumption and body mass gain.

4 Reagents

4.1 Sampling of animals
The woodlouse *Porcellio scaber* may be collected from uncontaminated fields. They may easily be cultured in laboratory. The animals used for the test are selected from a laboratory culture with relatively homogenous age, size and body mass.

4.2 Culturing of animals
Woodlice are cultured in wet boxes with plaster of Paris on the bottom.

Relative humidity: >80%. Temperature: 20 °C. (or 22-24 °C.). Light:dark cycle 18:6 hours. Woodlice are fed with soil litter (collected from an oak wood and hot [80 °C.] treated for one day, commercial rabbit food, and potato. Feeding rate: ad libitum. Gravid females with developed marsupium are kept in distinct culturing boxes. After the birth the adult female is placed in another box, while the offspring remain in the original ones.

4.3 Test substrate
4.3.1 Soil substrate:
The soil substrate should be composed of (on dry weight basis):

10% Sphagnum peat (air dry)
20% kaolin clay
70% industrial quartz sand (particle size 0.05-0.2 mm)

Add calcium carbonate to bring pH to 6.5-7.0. Artificial soil is prepared by wetting the soil substrate with deionized water to reach a water content of 50% of the total water holding capacity.

4.3.2 Food:
Complex food contains grained soil litter (50%) collected from an oak wood and hot treated (80 °C., one day) before use, grained commercial rabbit food (40%), dried and grained potato powder 10%) mixed and compressed into pills.

5 Apparatus

Standard laboratory equipment, and:

5.1 The exposure of the woodlice takes place in Petri dishes kept in plastic wet boxes.

5.2 Apparatus capable of measuring the dry mass of the substrate.

5.3 For adding the chemicals and mixing the soil and food glass containers with lid can be used.

6 Test environment

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

6.2 Light source capable of delivering a constant illumination at a light:dark cycle of 18:6 h.

7 Procedure

7.1 Preliminary test
If it is necessary to determine the range of concentrations for use in the final test, perform a preliminary acute test for five concentrations of the test sub

stance and a control. The concentrations being expressed in milligrams of the test substance per kilogram of the dried soil or food substrate. This test can be done with 1 woodlouse per concentration. The final test will be carried out with concentrations below the LC_{50}-value.

7.2 Final test

7.2.1 Introduction of the test substances:
Use either method a), b) or c), as appropriate.

a) 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 in water and mix it thoroughly with the test substrate before introducing it into a test container.

b) 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). Add it to a small portion of quartz sand or dry soil substrate, mix thoroughly and evaporate the solvent by placing it under a fume hood for 1 h. Then add the remainder of the soil substrate and the water and mix thoroughly.

c) Substances insoluble in water or organic solvents
For a substance insoluble in a volatile solvent, prepare a mixture of 10g of finely ground industrial quartz sand or 10g of the dry soil substrate and the quantity of the test substance required to obtain the desired concentration. Place the mixture, the remainder of the soil substrate and the water into a beaker and mix thoroughly before introducing it into the test container. Mix the test substance into the artificial soil before the woodlice are introduced.

The concentrations selected to provide the NOEC are based on the results of the preliminary test.

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

Proceed simultaneously with at least five replicates per concentration and a control test without the test substance and if necessary with an additional one and placing the containers in the test environment 6. Prepare such a series of test and control containers for each determination time.

7.2.2 Control container:
Prepare control containers in the same way as the test containers without the test substance. Treat these conditions in the same way as those without the test substance. Include 2 additional containers for the control and each treatment for pH and soil moisture determination at the end of the test.

7.2.3 Addition of the biological material:
For each concentration 5-10 woodlice are placed in one Petri dish and 5 replicates are made. The weight of animals should be 20-40 mg.

7.2.4 Determination:
Maintain the water content of the soil substrate in the test containers during the test period by reweighing the test containers periodically (once a week) and if it is necessary replenishing the lost water. At the end of the test the water content should not differ by more than 10% from the beginning of the test.

Replace the food particles twice a week. The food particles that have been removed should be dried and weighed in order to determine the food consumption.

Weigh animals weekly.

7.3 Reference substance
To be determined.

8 Calculation and expression of results

8.1 Calculation
For each concentration determine the percent mortality, the food consumption and the weight gain in the final test.

Compare means by suitable statistical methods, e.g. Dunnet's or William's, Student's test and test for significance (a = 0.05) of difference from control(s).

Calculate LC_{50} for survival and EC_{50} for effects on food consumption and weight gain.

8.2 Expression of results
Indicate, in mg/kg dry weight soil or food substrate, the concentration resulting in significant changes of the observed parameters in 10 or 50 percent of exposed woodlice (EC_{10}, EC_{50}).

9 Validity of the test

Work in progress.

10 Test report

The test report shall refer to this standard and, in addition to the results expressed as in 7.2, shall provide the following information:

- complete description of the biological material employed (species, source, etc.)
- method of preparation of the test substrate, and any 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 percentage mortality obtained for each container for each concentration and for the control
- number of offspring per test container at the end of the test (not if the test is restricted to an exposure period of 2 weeks)
- the LC_{50} for the effect on survival after 2 weeks (in case of an acute toxicity test) and if possible also after 6 and 10 weeks
- the EC_{50} for the effect on the number of juveniles produced after 6 and 10 weeks of exposure
- the highest concentration causing no observed effects (NOEC) and LOEC
- water content and pH of the artificial soil at start and at the end of the test
- all operating details not specified in the standard, and any occurrences liable to have affected the results: Some specimens used in the text should be preserved for future reference

11 References

Hornung, E. & Warburg, M. R. (1990): Oosorption and oocyte loss in terrestrial isopod under stressful conditions. Tissue and Cell 26, 277-284.

ANNEX 10.4 - Draft test protocol 9.2

Sublethal toxicity test with the woodlouse *Porcellio scaber* (Latr., 1814) (Isopoda: Porcellionidae) in artificial soil using oosorption as endpoint

1 Scope

This guideline describes a method for determining the effects of chemicals on the number of intact and destructing oocytes (oosorption) and the number of viable offspring of *Porcellio scaber* by dermal and alimentary uptake using defined food and soil substances.

2 Definitions

2.1 Survival
Mean number of adults surviving until the end of the test period (maximum 10 weeks).

2.2 Oosorption
Mean number of oocytes destroyed in the ovaries during the oogenesis.

2.3 Reproduction
Mean numbers of offspring produced during the test period (maximum 10 weeks).

3 Principle

The woodlice are placed in a defined artificial soil substrate and fed with defined food containing the test substance in different concentrations. The percentage survival, oosorption and the reproduction of woodlice are determined. Surviving adults is counted weekly. The percentage of oosorption is determined and the number of offspring is counted after the exposure period. Duration of the exposure period: work in progress.

4 Reagents

4.1 Sampling of animals
The woodlouse *Porcellio scaber* may be collected from uncontaminated fields. They may easily be cultured in laboratory. The animals used for the test are selected from laboratory culture with relatively homogenous age, size and body mass.

4.2 Culturing of animals
Woodlice are cultured in wet boxes with plaster of Paris on the bottom. Relative humidity: >80%. Temperature: 20 °C. (or 22-24 °C.). Light-dark cycle

18-6 hours. Woodlice are fed with soil litter (collected from an oak wood and hot [80 °C.] treated for one day, commercial rabbit food, and potato. Feeding rate: ad libitum. Gravid females with developed marsupium are kept in distinct culturing boxes. After the birth the adult female is replaced in another box, while the offspring remain in the original ones.

4.3 Test substrate
4.3.1 Soil substrate:
The soil substrate should be composed of (on dry weight basis):

10% Sphagnum peat (air dry)
20% kaolin clay 20%
70% industrial quartz sand (particle size 0.05-0.2 mm)

Add calcium carbonate to bring pH to 6.5-7.0. Artificial soil is prepared by wetting the soil substrate with deionized water to reach a water content of 50% of the total water holding capacity.

4.3.2 Food:
Complex food contains grained soil litter (50%) collected from an oak wood and hot treated (80 °C., one day) before use, grained commercial rabbit food (40%), dried and grained potato powder 10%) mixed and compressed into pills.

5 Apparatus

Standard laboratory equipment, and:

5.1 The exposure of the woodlice takes place in plastic dishes kept in wet boxes.

5.2 Apparatus capable of measuring the dry mass of the substrate.

5.3 For adding the chemicals and mixing the soil and food glass containers with lid can be used.

6 Test environment

6.1 Enclosure, controllable to temperature of 20 ± 1 °C.

6.2 Light source capable of delivering a constant illumination at a light:dark cycle of l8:6 h.

7 Procedure

7.1 Preliminary test
If it is necessary to determine the range of concentrations for use in the final test, perform a preliminary acute test for five concentrations of the test sub

stance and a control. The concentrations being expressed in milligrams of the test substance per kilogram of the dried soil or food substrate. This test can be done with 10 woodlice per concentration. The final test will be carried out with concentrations below the LC_{50}-value.

7.2 Final test

7.2.1 Introduction of the test substances:
Use either method a), b) or c), as appropriate.

a) 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 in water and mix it thoroughly with the test substrate before introducing it into a test container.
b) 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).
Add it to a small portion of quartz sand or dry soil substrate, mix thoroughly and evaporate the solvent by placing it under a fume hood for 1 h. Then add the remainder of the soil substrate and the water and mix thoroughly.

c) 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 or 10 g of the dry soil substrate and the quantity of the test substance required to obtain the desired concentration. Place the mixture, the remainder of the soil substrate and the water into a beaker and mix thoroughly before introducing it into the test container. Mix the test substance into the artificial soil before the woodlice are introduced.

The concentrations selected to provide the NOEC are based on the results of the preliminary test.

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

Proceed simultaneously with at least five replicates per concentration and a control test without the test substance and if necessary with an additional one and placing the containers in the test environment 6. Prepare such a series of test and control containers for each determination time.

7.2.2 Control container:
Prepare control containers in the same way as the test containers without the test substance. Treat these conditions in the same way as those without the test substance. Include 2 additional containers for the control and each treatment for pH and soil moisture determination at the end of the test.

7.2.3 Addition of the biological material:
For each concentration 12 females and 6 males are placed in one plastic box. 5 replicates are made both for determining the percentage oosorption and counting the number of offspring. Suggested weight of introduced animals should be 20-40 mg.

7.2.4 Determination:
Maintain the water content of the soil substrate in the test containers during the test period by reweighing the test containers periodically (once a week) and if necessary replenishing the lost water. At the end of the test the water content should not differ by more than 10% from the beginning of the test.

Replace the food particles twice a week.

7.3 Reference substance
To be determined.

8 Calculation and expression of results

8.1 Calculation
For each concentration, determine the percent mortality, the number of oosorption and the number of offspring.

Compare means by suitable statistical methods, e.g. Dunnet's, William's or Student's test and test for significance (a = 0.05) of difference from control(s).

Calculate LC_{50} for survival and EC_{50} for effects on oosorption and on the number of offspring.

8.2 Expression of results
Indicate, in mg/kg dry weight soil or food substrate, the concentration resulting in significant changes of the observed parameters in 10 or 50 percent of exposed woodlice (EC_{10}, EC_{50}).

9 Validity of the test

Work in progress.

10 Test report

The test report shall refer to this standard and, in addition to the results expressed as in 7.2, shall provide the following information:

- complete description of the biological material employed (species, source, etc.)
- method of preparation of the test substrate, and any 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 obtained for each container for each concentration and for the control
- number of offspring per test container at the end of the test (not if the test is restricted to an exposure period of 2 weeks)

- the LC_{50} for the effect on survival after 2 weeks (in case of an acute toxicity test) and if possible also after 6 and 10 weeks
- the EC_{50} for the effect on the number of juveniles produced after 6 and 10 weeks of exposure
- the highest concentration causing no observed effects (NOEC) and LOEC
- water content and pH of the artificial soil at start and at the end of the test
- all operating details not specified in the standard, and any occurrences liable to have affected the results: Some specimens used in the text should be preserved for future reference

11 References

Hornung, E. & Warburg, M. R. (1990): Oosorption and oocyte loss in terrestrial isopod under stressful conditions. Tissue and Cell 26, 277-284.

11 Sublethal toxicity test with the millipode *Polydesmus superus* (Latzel, 1884), (Diplopoda: Polydesmidae)

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11.1 Objectives for the reporting period

- To screen millipede species for laboratory culturing
- To establish laboratory cultures
- To study population responses to environmental conditions
- To study postembryonic development of selected species
- To determine suitable endpoints for testing sublethal effects

11.2 Objectives for the next period

- To improve the laboratory culturing of field collected animals
- To study population responses to environmental conditions
- To study and evaluate different sublethal parameters
- To formulate and standardize the test system

11.3 Main results obtained

The aim of the first year was to search for millipede species suitable for laboratory culturing and subsequent utilization in test procedures. The millipede, *Polydesmus superus* (Latzel, 1884) (cited also as *Brachydesmus superus*) was chosen as a potential test species. It has a life cycle of one year. The laying of eggs into a nest built up by the female on the soil or litter surface is perceptible. Postembryonic stadia are well distinguishable in live individuals. Semiquantitative evaluation of feeding acivity is possible by observation of dark gut contents visible through the body tissues.

In spring 1994 millipedes were collected in a deciduous forest near Ceske Budejovice. Hand sorting of individuals and/or heat extraction from leaf litter by a suitable apparatus is possible. Animals (1 female and 1 male) were placed in plastic boxes (8 to 10 to 4 cm) with moistened soil. Moist leaf litter from the same biotope was used as a food source.

The temperature preference of the millipede was determined by means of a temperature gradient constructed according to Haacker (1968). *Polydesmus superus* is

an eurythermic species with an activity range from 2 to 26 °C and a temperature optimum between 18 - 24 °C.

The main parameters of postembryonic development are given in Table 10.1.

The postembryonic development and life cycle of *Polydesmus superus* was studied in the field by soil sampling. Results were compared with previous data collected the same way during 1992-93 (complete 12-month sampling), and with data from a laboratory culture in a thermostated incubator continually simulating outdoor soil temperature.

Table 10.1: Characteristics of separate postembryonic stadia of the millipede *Polydesmus superus*.

Stadium	I	II	III	IV	V	VI	VII
rings	7	9	12	15	17	18	19
podous	4	6	8	11	14	16	17
apodous	2	2	3	3	2	1	1
leg pairs	3	6	11	17	23	27	29female
	3	6	11	16	22	26	28male
mean body weight	-	0.11	0.21	0.48	0.86	1.88	3.8female
(mg)	-	-	-	0.40	0.70	1.35	3.07male
mean length	0.8	1.4	2.2	3.0	4.3	5.5	8.3female
(mm)	-	-	-	3.3	4.6	5.9	9.0male

Notes: Rings = number of podous rings (with legs) + number of apodous rings (without legs) + telson. Body weight of individuals from the laboratory cultures. Mean body length according to Stephenson (1960).

Polydesmus superus was cultured at constant temperatures of 5, 10, 15, and 20 °C. By October 1994, the first adults (stadium VII) could be observed at 15 and 20 °C, whereas at 5 °C only stadia I and II, and at 10 °C stadium IV in maximum were observed. Nevertheless in most cultures up to this time the development was not finished. Although the determined optimum temperature was higher than 15 °C, the performance of cultures at this temperature differed little from those of 20 °C and were also closely related to cultivation at soil temperature simulation. It is therefore possibly to carry out final tests at 15 °C to ensure the interspecies comparability of toxicity tests.

The application of reproduction parameters and parameters of postembryonic development requires long-term test methods. Nest opening and egg counting is accompanied by the risk of dessication and damaging the eggs. The time overlap and presence of several stadia in the same period during the development, moulting periods in chambers in soil, and a relatively high mortality up to the stadium III (about 60 %) make the observation difficult. The completion of cultures by further collection of adult millipedes from the field for testing proved to be necessary.

Unciger foetidus (C.L.Koch, 1838) was considered as a second potential test species utilizing a more quantitative evaluation of feeding activity. The use of reproduction parameters is more limited here. Females lay eggs into chambers burrowed deep into the soil layers, only individuals of the third stadium leave chambers and move on the surface. The complete life cycle takes several years.

During the reporting period *Unciger foetidus* was cultivated in the same way as the previous species with the aim to describe separate stadia and postembryonic development. In autumn 1994 stadia VI and VII were observed in the cultures.

11.4 References

Haacker U. (1968): Deskriptive, experimentelle und vergleichende Untersuchungen zur Autökologie rhein-mainischer Diplopoden. Oecologia (Berl.), 1: 87 - 129.

Stephenson J.W. (1960): The biology of Brachydesmus superus (Latz.) Diplopoda. Annals and Magazine of Natural History, 13th Series 3: 311 - 319.

ANNEX 11.1 - Test System Standardization Form 10

Toxicity test with the millipede *Polydesmus superus* (Latzel, 1884), (Diplopoda: Polydesmidae)

General data about the test organism at the start of the experiment

Test organism

Taxonomic group:	Millipedes (Diplopoda: Polydesmida)
Species:	*Polydesmus superus* (Latzel, 1884)
Life stage:	adult
Sex:	female and male
Age/weight/size:	8-10 mm
Genetical comp.:	not known
Source:	field collected population / laboratory maintained animals

Type of surroundings and ambient climate

Surroundings	
Type:	climate chamber (thermostated incubator)
Ambient climate	
Air temperature:	15±1 °C
Relative humidity:	high (≥80 %)
Light wave length:	-
Light intensity:	-
Light regime:	darkness

Type of test system and test conditions

Type:	culture boxes of about 250 cm^3
Material:	plastic
Openness:	closed
Spec./test system:	single species
Ind./test system:	1 female and 1 male

Type of habitat

Soil/litter:	artificial soil (OECD), or LUFA 2.2 soil
'Habitat characteristics'	
Soil characteristics:	artificial soil (OECD) consists of sphagnum peat (10%), kaolin clay (20%), sand (70%), or LUFA 2.2 soil; work in progress on the influence of soil characteristics.
Hydrol. charact.:	work in progress
Food supply:	yes
Feeding rate:	not applicable
Type of food:	leaf litter collected in the same biotope as animals

Exposure characteristics

Exposure route:	via soil or food
Composition of test soil:	A) soil, deionized water, chemical, and contaminated food B) soil, deionized water, chemical, and uncontaminated food (work in progress)
Composition of control soil:	soil, deionized water, uncontaminated food (work in progress)

Duration of the experiment

Acclimat. time:	not relevant

Exposure time:	work in progress
Recovery period:	work in progress

Exposure concentrations

Replicates:	work in progress
Concentrations:	work in progress
Series:	work in progress

Exposure responses

Effect parameters:	survival of adult test animals, number of nests built by female, number of eggs and number of offspring of the first stadium per nest, time of offspring hatching,
Other parameters:	general behaviour, mobility, presence and amount of food in gut; postembryonic development
Measurement of variables:	work in progress
Toxic endpoint:	LC_{50}, NOEC, LOEC, EC_X
Unit:	mg test substance/kg d.w. test soil mg test substance/kg d.w. food
Working methods:	counting survivals, nests, eggs and/or offspring
Analytical methods:	not relevant

Miscellaneous test information

Practicability:	due to the one year life cycle (adults only in autumn-spring) and a relatively high mortality during postembryonic development, addition of animals from field populations is necessary. Collection of animals by hand sorting as well as by extraction from leaf litter sampled in the field by modified Tullgren apparatus is possible. Work on a culture method is in progress. Identification of sex, nest building, and different juvenile stages requires some training.
Cost-effectiveness:	work in progress
Sensitivity:	work in progress
Reproducibility:	work in progress
Validity of the test:	work in progress
Standardization:	work in progress
Ecological realism:	The species is spread throughout most of Europe, it lives in moist leaf litter and upper layers of woodland and garden soil, sometimes also in arable fields.
Broad chemical responsiveness	work in progress

References

Blower, J.G. (1985): Millipedes. Linnean Society Synopses of the British Fauna (New Series), Number 35. E.J.Brill/Dr W.Blackhuys, London, 1-242.

Schubart, O. (1934): Diplopoda. Tierwelt Deutschlands, 28, 1-318.

Sublethal toxicity test with the millipede *Polydesmus superus* (Latzel, 1884), (Diplopoda: Polydesmidae)

1 Scope

This test guideline describes a method for determining the effects on survival, behaviour, mobility, feeding parameters, reproduction and development of *Polydesmus superus* (Latzel, 1884) by superficial and alimentary uptake of a substance using a defined soil substrate (and food) treated with a defined amount of that substance.

2 Definitions

Work in progress.

3 Principle

Effects on survival, behaviour, reproduction of adult millipedes and on postembryonic development of offspring placed in a defined soil substrate containing the test substance in different concentrations will be determined. Test substance will be added in a single step.

Work in progress.

4 Reagents

4.1 Biological material

The biological material consists of adult millipedes of the species *Polydesmus superus* (Latzel, 1884) (= *Brachydesmus superus* Latzel, 1884) (Schubart 1934, Blower 1985). Because of culturing difficulties due to the high natural mortality during postembryonic development, collection of adult millipedes (females and males) from the field is necessary.

Work in progress.

4.2 Test substrate

4.2.1 Soil substrate:
Standardized natural soil (LUFA 2.2 soil).
Use of artificial soil (OECD) (peat, kaolinite clay, quartz sand). Work in progress.

4.3 Food

Leaf litter partly decomposed by soil microorganisms collected in the same biotope as animals.

5 Apparatus

Standard laboratory equipment, and:

5.1 Plastic culture boxes of about 250 cm^3 covered with lid, with about 60 - 80g dry mass of the soil substrate.
Work in progress.

6 Test environment

Thermostated incubators for maintenance of constant temperature (15 ±1 °C).
Work in progress.

7 Procedure

7.1 Preliminary test
Work in progress.

7.2 Final test
Work in progress.

7.3 Reference substance
Work in progress.

8 Calculation and expression of results

Work in progress.

9 Validity of the test

Work in progress.

10 Test report

Work in progress.

11 References

Blower, J.G. (1985): Millipedes. Linnean Society Synopses of the British Fauna (New Series), Number 35. E.J.Brill/Dr W.Blackhuys, London, 1-242.

Schubart, O. (1934): Diplopoda. Tierwelt Deutschlands, 28, 1-318.