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
December 6-7, 1993

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Introduction

SECOFASE

The research project *Development, improvement and standardization of test systems for assessing sublethal effects of chemicals on fauna in the soil ecosystem (SECOFASE)* receives funding from the Environmental Research Programme 1991-1994 of the European Commission, starting in January 1993. The First Workshop of the SECOFASE Project Group took place in Silkeborg, Denmark, on 18th and 19th January 1993 and resulted in *The Manual of SECOFASE* (Løkke & van Gestel, 1993). It describes the scope and work programme of SECOFASE, and contains guidelines and recommendations for the development of test methods. Issues of importance for culturing techniques, choice of test parameters, experimental design, analysis and interpretation of data, and Good Laboratory Practice were also dealt with.

Second SECOFASE Workshop

The present annual progress report was finalized at the 2nd SECOFASE Workshop which was held in Braunschweig (FRG) on 6th and 7th December 1993. The report presents the preliminary results of the project and outlines important features on the standardization following a common conceptual scheme. Further, the report presents modifications and adjustments of the research programme.

New partners in Eastern and Central Europe

In 1993 the SECOFASE project was invited by The European Commission to apply for an extension of the project with Eastern and Central European partners. The application was successful and resulted in grants for partners in Poland, the Czech Republic, and Hungary. Dr. R. Laskowski from the Institute of Environmental Biology, Jagiellonian University, Ingardena, Krakow, Poland, will join the SECOFASE project with a sub-project on the development of a test method for Chilopoda. Dr. K. Tajovsky, Institute of Soil Biology, Na Sadkach, Ceske Budejovice, Czech Republic, will join with a sub-project on the development of a test method for Diplopoda. Finally, Professor, Dr. E. Fisher, Department of Zoology, Janus Pannonius University, Ifjuság U., Pécs, Hungary, will join with a project on the development of a test procedure for Isopoda. The new partners took part in the 2nd Workshop in Braunschweig and their sub-projects are expected to start in January 1994.

Model test compounds

To allow for a comparison of test results among different laboratories three model test chemicals were chosen representing different chemical classes: pesticides, heavy metals and detergents. All three groups are relevant from the point of view of environmental protection. Furthermore, the compounds differ in environmental behaviour, which is of importance because the test methods developed within the project should be applicable to a wide range of classes of chemical substances found in the soil environment. The pesticide dimethoate was chosen because it is a widely used organophosphate insecticide that is accepted as a toxic standard in terrestrial ecotoxicology studies. It is being tested as a commercial formulation. The element copper was taken to repre-

sent heavy metals. It is being tested in the form of cupric chloride. A cationic surfactant, dihardened-ditallow dimethyl ammonium chloride (DHTDMAC) was chosen as a representative of detergents. However, initial experiments with this compound have shown that it is extremely difficult to handle in the laboratory. In addition, there is no simple chemical analytical method available, and the compound will not be on the market in the near future in some member countries of the European Union. Therefore, it was decided by the Co-ordination Committee of SECOFASE to replace DHTDMAC by Linear Alkylbenzene Sulfonate (LAS) which is a widely used anionic surfactant.

The general progress of all project participants is outlined in Chapter 3 in terms of milestones reached and resources used. In Chapter 4 summaries of the results obtained by each of the subprojects of SECOFASE are presented. These include:

Participant 1
Development of Single and Two-Species Sublethal Tests on *Hypoaspis aculeifer* (Acari: Gamasida) and *Folsomia fimetaria* (Collembola, Isotomidae).

Participant 2
Development and Validation of Sublethal Laboratory and Semi-Field Test Methods for Staphylinidae (Coleoptera).

Participant 3
Development and Standardization of Acute and Sublethal Laboratory Test methods for Different Earthworm Species.

Participant 4
Development of Sublethal Test Methods for *Platynothrus peltifer* (Acari, Oribatida).

Participant 5
Development of a Two-Species Nematode Toxicity Test.

Participant 6
Reproduction, Growth and Survival in the Enchytraeid *Cognettia sphagnorum* (Vejdovsky).

Participant 7
Development of Sublethal Test Methods for the Collembola *Isotoma viridis* (Bourlet) and *Folsomia candida* (Willem) (Collembola, Isotomidae).

Deviations from the work plan in 1993 and modifications to the work plan for 1994 are presented in Chapter 5. This is followed by a summary of the progress made in standardization of the test systems in Chapter 6. Standardized protocol forms for each test under development are given in Annex 1. In Chapter 7, attendance of meetings, presentations and scientist visits by SECOFASE project participants are listed.

Acknowledgement

Dr. Hartmut Barth from The EU Commission, DGXII, is thanked for valuable comments on th manuscript.

Summary

This report outlines the progress made by the SECOFASE project group in 1993. The report describes the general progress of the project and presents summaries of results, resources employed, deviations from and modifications to the work plan and progress with standardization and the development of test systems by each participant.

Overall, good progress was made by all seven participants in terms of the milestones reached, as described in the SECOFASE manual (Løkke and van Gestel, 1993), and all participants are on schedule to meet the proposed work plan. During 1993 most participants have established permanent laboratory cultures of their proposed test organism(s). Satisfactory culturing methods have been developed for several organisms, such as Collembola, mites and nematodes, however for others, such as beetles, Enchytraeids and earthworms, further work is required during 1994. Because of this, some of the tests described in this report were carried out with laboratory cultured animals and some with field collected animals.

All participants have begun development of their test system(s). Preliminary test protocols for each organism are presented as standardized forms in Annex 1 of this report. Several participants have already carried out preliminary acute and/or chronic tests with their organism, in either an artificial soil or LUFA Speyer soil 2.2, with one or more of the three reference chemicals selected for the SECOFASE project (i.e. dimethoate, copper and diharde-ned-ditallow dimethyl ammonium chloride (DHTDMAC)). DHTDMAC appeared to be relatively non-toxic to the test organisms and, as a consequence, such high amounts had to be added to the soils that the soil structure was severely damaged. In fact, at high concentrations the effects of a changed soil structure was tested rather than the effects of DHTDMAC. Therefore, the Co-ordination Committee of SECOFASE has decided to replace DHTDMAC with another detergent, the anionic surfactant linear alkylbenzene sulfonate (LAS). This detergent will now be used as the third reference compound in the SECOFASE project.

During 1993 participant No.s 2 and 3 have carried out residue analyses of dimethoate in the standard test soils. These results will be used to interpret the behaviour of dimethoate in the test systems. Residue analysis of copper in the standard test soils will be carried out during 1994.

Finally, the SECOFASE project group are pleased to welcome three new participants from Eastern and Central Europe in 1994 who will be developing test systems for diplopods, chilopods and isopods.

1 General Progress

Overall, good progress was made by all SECOFASE participants during 1993. The following sections outline details of the general progress of all sub-projects in terms of the common milestones reached, as described in the SECOFASE manual (Løkke and van Gestel, 1993), and the resources employed.

1.1 Summary of Progress

Two of the key milestones reached by most participants during the current year were 1) the establishment of laboratory cultures of the proposed test organisms and 2) the initial development of the test systems. For some organisms satisfactory culturing methods are now available (e.g. Collembola, mites and nematodes), however for others (e.g. beetles, Enchytraeids and earthworms) some additional work will be required in 1994. Therefore some of the tests described in this report (Chapter 4) were carried out with laboratory cultured animals while others were carried out with field collected animals. A large amount of new information has been gathered about the biology and life-histories of many of the test species whilst establishing the cultures which will prove extremely useful during the development of test protocols. All participants have begun to develop standardized test protocols for their test organisms (see Annex 1) and several participants have already carried out preliminary tests with one or more of the test chemicals.

1.2 Resources Employed

Participant No. 1
The resources employed include climate chambers, culturing and extraction facilities, and image analysis equipment. The technical staff were Z. Gavor, K.K. Jacobsen, E. Jørgensen and S. Ibrahimi. They were involved with breeding the test animals and carrying out the experiments. The scientific staff consisted of Dr. H. Løkke, MSc. P. Folker-Hansen, MSc. M. Holmstrup and MSc. P.H. Krogh. All technicians and scientists were employed on a part-time basis.

Participant No. 2
The resources employed include climatic chambers and chemical application facilities at the BBA. MSc. K. Metge was employed as a scientist on a half-time basis and A. Wehlisch was employed as a technician, also on a half-time basis. Assistance with residue analysis of dimethoate in the test soils and beetle culturing and testing was provided by colleagues at the BBA.

Participant No. 3
Standard laboratory equipment, climatic incubators and climatic chambers at the BBA were used for culturing earthworms and for incubating the chemical tests. MSc. H. Kula and a technical assistant C. Behrend were employed on a half-time basis.

Participant No. 4
The resources employed include climate rooms and arthropod culturing facilities, Tullgren extraction equipment and metal analysis facilities in the Department of Ecology and Ecotoxicology at Vrije Universiteit, Amsterdam. Miss A. Doornekamp was employed full-time as a technician. Assistance with metal analysis, the development of Tullgren extraction techniques and the identification of mites was obtained from colleagues in the same department.

Participant No. 5
The facilities used included nematode culturing facilities and chemical application facilities in the Department of Nematology at Wageningen Agricultural University. Mr. J.A.G. Riksen was employed as a technician on a full-time basis. Also taxonomic expertise was obtained from colleagues in the Nematology Department.

Participant No. 6
The resources employed include culturing facilities in the Department of Ecology, University of Lund. MSc. A. Augustsson was employed as a scientist.

Participant No. 7
The resources employed include the use of insect culturing facilities, chemical application facilities and photographic and image analysis facilities in the Department of Biology, University of Southampton. Dr. J.A. Wiles was employed full time and a temporary trainee technician was employed on a part-time basis for a ten week period. Computer hardware and software were purchased for the project and the Department of Biology obtained high quality incubators to be used specifically for this project.

2 Summary of Results

2.1 Participant No. 1

Development of Single and Two-Species Sublethal Tests on *Hypoaspis aculeifer* (Acari: Gamasida) and *Folsomia fimetaria* (Collembola: Isotomidae)

Dr. H. Løkke, MSc. P.H. Krogh, MSc. P. Folker-Hansen, MSc. M. Holmstrup. Department of Terrestrial Ecology, The National Environmental Research Institute (NERI), Denmark.

Introduction

The primary biological parameter (endpoint) in this project is reproductive output of microarthropods in soil test systems. In order to improve the knowledge of the test species in soil microcosms investigations of population variables were undertaken.

During 1993 the influence of test chemicals on the following biological variables were investigated:

a) growth of *Hypoaspis aculeifer*, *Folsomia fimetaria* and *Hypogastrura succinea*

b) consumption and asexual egg-production of *H. aculeifer*

c) sexual reproduction of single species populations of *H. aculeifer* and *F. fimetaria*

A test system for *H. succinea* (Collembola: Poduridae) as a single species reproductive test has been formulated which is similar to test systems developed for *F. fimetaria* (Krogh, in press) and *H. aculeifer* (Krogh, in press). Thus, suggestions for single species test systems have been made for all three species which are necessary prerequisites for the planned predator/prey test system. Preliminary studies with a predator/prey system have been carried out and these results will be described in the third technical report.

Tests with dimethoate were carried out with individuals of all species. Digital image processing (DIP) was successfully used for the determination of body size parameters. Effects of dimethoate were investigated for all species in duplicate tests: one pilot study to establish optimum test conditions and concentrations and a subsequent main study with increased replication to enable discrimination of small sublethal effects.

Studies at the individual level: the effect of dimethoate on growth, egg-production and consumption

The growth rates of individual *F. fimetaria* and *H. aculeifer* were determined. Results from studies of allometric growth revealed good correlations between weight and length for all three species (Krogh and Holmstrup in prep.). Studies of growth as a sublethal effect parameter were based on measurements of length. Different ways of expressing growth were used, including the growth para-

meters of von Bertalanffy. Different models were used to describe dose-response curves in order to estimate effects on growth, for example growth differences of 10% from the controls (EC_{10}) were estimated. Results were obtained from tests with *F. fimetaria* and *H. aculeifer* exposed to dimethoate. Records of the prey consumption and asexual egg-production were made simultaneously with the growth studies for the predatory mites.

Methods

The experimental setup consisted of small containers (circular holes in multi-dishes) each holding 2.65 g soil. The soil was subsequently compressed by help of a pestle to avoid cracks in which the animals could hide. One neonate larva was added to each container along with food; dried bakers yeast for the Collembola and *F. fimetaria*-prey for the predatory mite. Each hole was closed with a rubber plug. Growth was recorded twice weekly for both species as well as reproduction and prey consumption of the predatory mite. A highest concentration of 0.3 mg dimethoate/kg soil was used for *F. fimetaria* and 0.7 mg/kg soil for *H. aculeifer*. Twenty-four neonate larvae were used per concentration, however because the sex ratio in the cultures was not fixed, a predetermined number of replicates for each sex could not be ensured.

Evaporation was compensated by adding deionized water once a week. Collembola were provided with bakers yeast every two weeks. The predatory mites were offered a surplus of prey by adding up to 40 prey-items twice weekly. Juvenile mites were offered juvenile prey (0 to 7 days old), and adult mites were offered a mixture of juvenile and adult prey (0 to 20 days old). In order to count unconsumed prey, the animals were anaesthetized by a weak CO_2-stream. Dead prey was discarded and excluded from the consumption data. Tests were terminated as growth became asymptotic (i.e. after 4.5 to 5 weeks).

Results

a) Growth

Length was compared between doses for each record-day (Duncans multiple range test) and the course of individual growth was compared (MANOVA-test); levels of significance: $P<0.05$. For each individual the growth-parameters of von Bertalanffy, k and L_∞, were estimated by least squares iteration. To fit data showing hormesis, a linear logistic model, proposed by van Ewijk and Hoekstra (1993), was adopted. The model was slightly modified for the estimation of EC_{10}. Otherwise a standard logistic model (van Ewijk and Hoekstra, 1993) was used if possible.

H. aculeifer

Results for females were based on 14 to 19 individuals. There were significant differences in length between concentrations for all record-days. The rates of growth of individuals exposed to the different concentrations also differed significantly. The growth rate constant k of von Bertalanffy was significantly different from the control for the lowest and highest test-concentration - the lowest concentration showing a greater k-value. The values of L_∞, the asymptotic length, did not differ significantly for individuals exposed to different concentrations. The linear logistic model gave a better fit to the k-values than a standard logistic model not allowing for hormesis.

F. fimetaria

Results for females were obtained from 5 to 14 individuals in the concentration range of 0 to 0.17 mg dimethoate/kg soil and 3 individuals at 0.3 mg/kg soil. Significant differences in length between individuals exposed to different concentrations were found from day 7 to 20. No overall difference in the course of growth was discovered. The values for L_∞ were significantly higher at the two highest concentrations compared to the control. The growth rate parameter k was significantly lower at 0.3 mg/kg compared to the control. A standard logistic model fitted the k-values. Estimates of the effect concentrations are given in Table 1.

Results for males were based on 4 to 12 individuals at the lowest concentrations, and 2 individuals at the highest concentration. The only significant difference in length was between the lowest concentration and the control on day 13. No differences in growth rates, k and L_∞ were found between concentrations.

b) Consumption

Prey consumption by *H. aculeifer* females decreased at the lowest concentrations tested. At the highest concentration, 0.7 mg dimethoate/kg, the consumption rate was similar to the controls, except in the case of the 2 day old nymphs where consumption was stimulated. The consumption rate was reduced by 30% at the lowest test concentration for nymphs.

c) Egg-production

Asexual egg-production by *H. aculeifer* followed the linear logistic model, with a significant stimulation at a dose of 0.3 mg dimethoate/kg. The EC_{10} and EC_{50} values increased slightly with age indicating that effects were compensated for during the test period.

d) Single species reproduction

Toxicity data for all three species are given in Table 1. The test procedures are presented as standardized test protocols in Annex 1.

Discussion

The asymptotic length seemed to be unaffected or only slightly affected (*F. fimetaria* ♀♀) by dimethoate. Thus, any effect on individual length was reduced as time progressed. Growth rates, however, were affected as indicated by differences in the von Bertalanffy constant k. Individual parameters and the population reproduction in soil microcosms were equally sensitive in terms of EC_X and NOEC/LOEC values.

Table 1. Effect of dimethoate on: growth, mortality, consumption, egg-production, reproduction and survival of *H. aculeifer*, *F. fimetaria* and *H. succinea*.

Test system	**Parameter**	**Test species**	**Time (days)**	**Sex**	**Effect concentration with 95% confidence interval (mg/kg dry soil)**		
Studies of individuals on compressed soil	Growth: k*	*H. aculeifer*	34	♀♀	[a]EC_{+10+}	0.395	[0.219;0.571]
				♀♀	[b]EC_{-10}	0.571	[0.450;0.692]
Age at start: 0-2 days (larvae)				♀♀	[c]NOEC	<0.230	
				♂♂	NOEC	0.230	
				♂♂	[d]LOEC	0.333	
		F. fimetaria	38	♀♀	EC_{10}	0.100	[0.009;0.194]
				♀♀	NOEC	0.167	
				♀♀	LOEC	0.30	
				♂♂	[e]$LOEC_H$	0.30	
	Consumption	*H. aculeifer*		♀	[f]$NOEC_H$	<0.23	
				♀	$LOEC_H$	0.23	
				♀	[g]NOAEC	0.48	
				♀	[h]LOAEC	0.70	
	Egg-production (asexual)	*H. aculeifer*	19	♀	EC_{10}	0.55	[0.49;0.62]
				♀	EC_{50}	0.65	[0.58;0.73]
				♀	NOEC	0.23	
				♀	LOEC	0.33	
	Mortality	*F. fimetaria*	16	♂♀	LC_{50}	0.300	
			27-38	♂♀	LC_{50}	0.240	
Single species test system in soil (micro-cosm)	Reproduction	*H. aculeifer*	21	♀♂	EC_{10}	0.7	[0.6;0.8]
				♀♂	EC_{50}	0.9	[0.8;1.0]
				♀♂	NOAEC	0.7	
				♀♂	LOAEC	1.3	
		F. fimetaria	28	♂♀	EC_{10}	0.1	[0.01;0.2]
					EC_{50}	0.3	[0.1;0.5]
				♂♀	NOAEC	<0.1	
				♂♀	LOAEC	0.1	
		H. succinea	21	♂♀	EC_{50}	-	-
	Mortality	*H. aculeifer*		♀♀	LC_{50}	0.8	[0.7;1.0]
		F. fimetaria	28	♂♀	LC_{50}	0.2	[0.2;0.3]

* k: von Bertalanffy's growth-rate constant
[a]EC_{+10+}: second 10%-stimulatory effect concentration
[b]EC_{10}: 10% inhibitory effect concentration
[c]NOEC: no observed effect concentration
[d]$LOEC_H$: lowest observed hormesis effect concentration
[e]$LOEC_H$: lowest observed hormesis effect concentration
[f]$NOEC_H$: no observed hormesis effect concentration
[g]NOAEC: no observed adverse (=reduction) effect concentration
[h]LOAEC: lowest observed adverse (=reduction) effect concentration

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2.2 Participant No. 2

Development and Validation of Sublethal Laboratory and Semi-Field Test Methods for Staphylinidae (Coleoptera)

MSc. K. Metge and Dr. U. Heimbach
Federal Biological Research Centre for Agriculture and Forestry (BBA), Institute for Plant Protection of Field Crops and Grassland, Braunschweig, Germany.

Introduction

One of the aims of this project was to establish and improve breeding methods for the common staphylinid beetles *Philonthus fuscipennis* (Mannh.), *Lathrobium fulvipenne* (Grav.) and *Tachyporus hypnorum* (L.). Once developed these cultures will provide staphylinid beetles of different life-stages that may be used to developand improve laboratory and semi-field tests methods. At the beginning of this project little information was available concerning the rearing of *P. fuscipennis* (Eghtedar, 1970) and *T. hypnorum* (Lipkow, 1966) and no information concerning *L. fulvipenne*. Therefore, research during the current year has concentrated on developing rearing methods.

Methods

In spring 1993 individuals of the three staphylinid species were collected in fields around Braunschweig (FRG). Different methods were tested to rear the beetles. Also preliminary laboratory and semi-field tests were carried out.

Rearing method for P. fuscipennis

The rearing method adopted for the staphylinid beetle *P. fuscipennis* was based on a technique for mass rearing the carabid beetle *Poecilus cupreus* (L.), which was developed in the Institute for Plant Protection of Field Crops and Grassland at the Biological Research Centre for Agriculture and Forestry (BBA) in Braunschweig (Heimbach, 1989). It seems that this culturing method, adapted for *P. fuscipennis*, is capable of meeting the culturing requirements for testing species of the SECOFASE project (i.e. it can produce individuals of known history, age and physiological condition, and in large numbers for testing).

For egg laying, 1 to 3 pairs of the staphylinid beetles were kept in plastic boxes (18.3 x 13.6 x 6 cm) filled with moistened clay granules with gauze at the bottom and a gauze lid. They were cultured at 20°C for a long day period (16 h light/8 h dark). Three times a week the eggs (2 mm long) were washed out with water and collected with a pair of tweezers. They were then placed separately on damp filter paper in 24-well tissue culture plates. After hatching, the larvae were transferred separately into 19 ml glasstubes filled with moistened, ground peat. The larvae were found to be incapable of entering the peat themselves and therefore a hole (3 cm long) was prepared in the peat for each larva. The eggs and larvae were cultured at 20°C in the dark. Several days before the pupae hatched the tubes were closed with lids to prevent the newly emerged adults from escaping. The immature beetles were separated according to sex and were kept in batches of 10 in plastic boxes (19 x 19 x 5 cm) filled three quarters with wet peat and a quarter with dried peat. For 3 to 4 weeks the beetles were kept under long day conditions at 20°C followed by a short day period (for more than 8 weeks) at 5 or 8°C. All stages were fed with cut *Calliphora spp.* pupae.

The period of development, mortality during development and hatching weight of the beetles were measured at different temperatures. Egg production and the duration of the egg stage was also recorded.

The preliminary results indicate that it is possible to rear *P. fuscipennis* in large numbers in the laboratory with this method (Table 1).

Table 1. Results from the first breeding period of 188 eggs. The mortality rate for complete development was 30%. L = Larval stage, PP = prepupa stage.

Development at 20°C	**Egg**	**LI**	**LII**	**LIII**	**PP**	**Pupa**	**Imago**
Mean (Days)	6.7	5.96	5.82	5.23	4.38	12.94	40.72
Min. - Max. (Days)	2 - 10	4 - 11	3 - 16	2 - 12	2 - 8	6 - 18	28 - 51
Dead %	12.8	14.0	3.5	3.7	1.5	3.1	0.8
Number	188	164	141	136	130	128	124

Rearing method for L. fulvipenne

The rearing method for the staphylinid beetle *L. fulvipenne* was similar to *P. fuscipennis*. Moistened soil and expanded clay granules were used to provide egg laying conditions. It was not necessary to prepare a hole in the peat for these larvae. At present only relatively few eggs from a few females have been obtained for this species. The reared F1-generation produced eggs and larvae but the numbers of females of this generation was too low to start a mass rearing up till now.

Rearing method for T. hypnorum

For this species, 1 to 2 pairs of beetles were cultured in small boxes filled with plaster of Paris and activated coal (5:1). A wet-

ted woolen thread was placed in each box for egg laying. The females deposited the eggs onto the thread. Different methods for culturing larval stages were tested. At present none of the methods tested appear good enough to produce high numbers of beetles for tests.

Methods for testing staphylinid larvae

A preliminary testing method for *P. fuscipennis* larvae was evaluated. Prior to the experiment the dried test soil was stored at 5°C. One week before the test started the soil was moistened up to 20% of its water holding capacity (WHC). One day before the larvae were introduced into the soil, the chemical and an amount of water that corresponded to 10 - 20% of the WHC were mixed into the soil for a 30 minutes period. Afterwards the soil was wetted up to 50% of the WHC and stored at 5°C. On the day of the test 20 g samples of dried soil were placed into 19 ml glass tubes. A larvae (1 to 2 days old) was placed into each tube. One total of twenty larvae were used. Observations took place at 1, 2, 3, 4, 5, 7, 10, 14, 21 and 28 days after introduction. Also the period of development, hatching weight and sex were observed. The full test procedure is given in Annex 1.

The preliminary results indicated that this seemed to be a good method for testing *P. fuscipennis* larvae. The LC_{50}-value for complete development of larvae was approximately 1 mg a.i. dimethoate/kg dried soil. A similar method will be used for *L. fulvipenne*.

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2.3 Participant No. 3

Development and Standardization of Acute and Sublethal Laboratory Test Methods with Different Earthworm Species

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Introduction

The aim of the first year was to establish laboratory cultures of

the indigenous earthworm species *Aporrectodea caliginosa* and *Allolobophora chlorotica* as well as with the reference species *Eisenia fetida*. Furthermore, the acute and sublethal effects of the test chemicals were investigated in order to select suitable test parameters for the development of new test systems.

Methods and Results

Establishment of laboratory cultures

A laboratory culture of *E. fetida* was established and currently yields about 400 adult individuals per month. Juveniles of defined age were obtained by incubating hand sorted cocoons in artificial soil for two weeks.

A. caliginosa and *A. chlorotica* were cultured in the laboratory for some time, but cocoon production was rather poor. Due to the unfavourable weather conditions in spring 1993 it was very difficult to obtain sufficient numbers of animals. Most of these hand collected animals went into a resting phase when transferred to the culture medium in the laboratory. Only 80 cocoons of *A. caliginosa* collected in arable land were obtained from the cultures started in spring. Animals collected in grassland produced only a small amount of cocoons. Juveniles hatched after 3 to 4 months and were used in the experiments described below. In autumn 1993 the main activity period of earthworms again was very short. Several hundred animals of *A. caliginosa* and *A. chlorotica* were collected from a grassland site and an orchard. Artificial soil, LUFA 2.2 soil and a natural orchard soil (silty 10am, 4.2% organic matter, pH 7.5) were used as culture media. Soil moisture was adjusted to approximately 50% of the water holding capacity (WHC). The animals were fed with finely ground cattle manure. The cultures were initially kept at 15°C in order to allow optimal cocoon production. Because of the observed inactivity of the animals the temperature was lowered to 10°C.

At the moment reproduction of the indigenous species is not satisfactory. In the future modified culture media and other food sources will be examined.

Acute toxicity tests

The acute toxicity of dimethoate and copper to earthworms was investigated in order to determine adequate concentration levels for the testing of sublethal effects. Because the insecticide dimethoate and the heavy metal copper are known to have effects on earthworms all experiments in 1993 were conducted using these test chemicals only.

The acute toxicity tests were conducted according to OECD-Guideline no. 207. In addition, body weight development, occurrence of external injuries and general behaviour, such as burrowing, were recorded. In tests with the indigenous worm species, a reduced number of test animals were used per replicate.

The most sensitive species was the epigeic species *L. castaneus*. In general it was difficult to estimate the number of dead animals in tests with dimethoate as this chemical paralysed the test animals at very low concentrations. Copper appeared to have a strong repellent effect on *E. fetida* at concentrations higher than 320 mg/kg.

Because most of the results with the indigenous species so far are derived from preliminary range finding tests with reduced numbers of test animals, further interpretation of the results is difficult. More detailed tests are planned for next year in order to establish data on the specific sensitivity of earthworm species belonging to different ecological categories.

Table 1. LC_{50} [mg a.i./kg dry weight test substrate].

	Dimethoate		**Copper**	
	Art. Soil	**LUFA 2.2**	**Art. Soil**	**LUFA 2.2**
E. fetida				
- adult	206.7	97.7	1002.0	452.7
- juvenile	142.1	89.7[a]	>320 - <1000[a]	>320 - 1000[a]
A. califinosa				
- adult	54.1[a]	15.2[a]	>100 - <1000[a]	>100 - <1000[a]
- juvenile	10.0[a]	31.6[a]	748.0[a]	240.0[a]
A. chlorotica				
- adult	191.4[a]	316.2[a]	>100 - <1000[a]	>100 - <1000[a]
A. rosea				
- adult	89.1[a]	11.7[a]	>100 - <1000[a]	>100 - <1000[a]
L. castaneus				
- adult	2.5[a]	15.2[a]	>10 - <100[a]	>100 - <1000[a]

[a] preliminary results from range finding tests

Sublethal toxicity tests

Sublethal effects were investigated in reproduction toxicity tests. A 500 g sample of dry soil was mixed homogeneously with the test substance and then transferred into plastic boxes. These were sealed with transparant plastic lids punctured with small holes to allow exchange of air. Usually 10 test animals were introduced into the soil substrate. Finely ground cattle manure was spread on the soil surface as food source. The feeding activity of earthworms was checked visually at weekly intervals by semi-quantitative estimation of the food remains. Fresh food was added afterwards. After 28 days of exposure, mortality, body weight development, cocoon production and eventually numbers of hatched juveniles were determined. The cocoons were transferred to Petri dishes containing moist filter paper. The total number of hatching juveniles and the number of infertile cocoons were evaluated during the following weeks.

Soil from the reproduction toxicity test with *E. fetida* and dimethoate was analysed to determine the levels of chemical residues (in cooperation with participant no. 2 and the BBA - see section 6.2). The initial concentration of dimethoate was 26.9 mg a.i./kg dry weight of soil. Test animals were introduced 1, 4 and 8 days after incorporation of the chemical into the soil substrate. Suble-

thal effects on *E. fetida* introduced on three different dates are summarized in Table 2. A detailed description of these results will be published elsewhere.

Table 2. Acute and sublethal effects of Dimethoate on *Eisenia fetida* in two different soils.

	Mortality [%]	Body weight development [%]	Feeding rate	Total number of cocoons	Infertile cocoons [%]	Total number of juveniles
Art. soil						
Control	0	120.7± 7.0	2.8±0.3	51.4±6.6	2.3	187.8±24.7
Di (day 1)	4.0	105.0±14.9	1.6±0.8	1.2±2.7 *	50.0	2.4±5.4 *
Di (day 4)	0	113.5± 1.3	2.0±0.6	6.8±4.9 *	7.4	19.0±19.8 *
Di (day 8)	2.5	99.2±16.4	2.4±0.3	7.3±4.3 *	3.4	25.0±15.3 *
LUFA 2.2						
Control	2.0	124.1± 8.6	2.9±0.2	23.4±7.2	27.4	54.0±24.6
Di (day 1)	14.0	93.1±16.3 *	1.9±0.6	0 *	-	0 *
Di (day 4)	2.5	116.8±12.4	2.3±0.6	2.0±2.4 *	50.0	4.3± 8.5 *
Di (day 8)	12.5	95.0±13.7 *	2.2±0.9	3.0±0.8 *	8.3	6.3± 1.3 *

Di = treated with 26.9 mg/kg Dimethoate at day 0 (test animals introduced at day 1, 4 and 8)
Feeding rate: semiquantitative rating 0: absent, 1: low, 2: medium, 3: high feeding activity
* significant difference, p≤0.05, multiple t-test by TUKEY

A residue analysis experiment was conducted with a rather high dimethoate concentration of 26.9 mg a.i./kg to ensure that effects occurred. This explains the mortality observed in some treatments (see Table 1). No cocoons were produced in either soil when test animals were introduced one day after chemical incorporation and cocoon production was reduced by approximately 90% for test animals introduced on 4 and 8 days after chemical incorporation. The control with LUFA 2.2 soil showed by its lower cocoon production, its high percentage of infertile cocoons, and its low number of hatched juveniles, that conditions for *E. fetida* are suboptimal in this soil. Similar trends were observed in other experiments for *A. caliginosa*. It seems that a soil moisture content of 50% of the water holding capacity is too low for earthworms in this soil. Currently experiments are being conducted to optimize these conditions.

A more detailed investigation of the sublethal effects of dimethoate and copper on *E. fetida* was conducted in an artificial soil.

Table 3. Acute and sublethal effects of Dimethoate and copper on *Eisenia fetida* after 28 days of exposure in artificial soil (10 animals/treatment, 4 replicates/treatment).

	Mortality	Body weight development [%]	Feeding rate	Total number of cocoons	Number of juveniles [1]
Control	0	81.9± 2.9	3.0±0	57.8± 4.8	47.0± 9.9
Dimethoate					
1 mg/kg	0	84.0± 5.6	3.0±0	66.3± 6.1	53.0±10.6
5 mg/kg	0	92.5± 5.3	2.9±0.1	33.3±11.5 *	55.5±14.2
10 mg/kg	2.5	91.7± 6.1	2.2±0.6	13.0± 5.7 *	42.3±15.2
50 mg/kg	30.0	44.4± 5.0 *	1.1±1.0	0.5± 0.6 *	4.0± 3.3 *
Copper					
200 mg/kg	0	96.4± 4.2	2.9±0.3	43.3± 8.0 *	2.5±2.6 *
400 mg/kg	0	99.1± 3.9 *	1.5±1.0	5.8± 1.3 *	0 *
600 mg/kg	10.0	80.9±10.9	0.7±0.4	0 *	0 *
800 mg/kg	52.5	71.5±10.5	0.6±0.1	0 *	0 *

[1] juveniles already hatched at day 28
Feeding rate: semiquantitative rating 0: absent, 1: low, 2: medium, 3: high feeding activity.
* significant difference, $p \leq 0.05$, multiple t-test by TUKEY

In contrast to the residue analysis experiment, juveniles in this test had already hatched during the 28 days of exposure. No effects were found with 1 mg/kg dimethoate although there seemed to be a slight (but statistically insignificant) increase in cocoon production. With 5 mg/kg and 10 mg/kg cocoon production was significantly reduced by 45% and 75% respectively. No differences were found in the numbers of juveniles that had hatched after 28 days of exposure between the control, 1, 5 and 10 mg/kg treatments. These juveniles must have hatched from cocoons produced during the first 10 days of the experiment. This might indicate a delayed effect of dimethoate on cocoon production. The total number of juveniles hatching from all cocoons is currently being investigated.

Treatment with copper resulted in repellent effects when using concentrations of 400 mg/kg or higher. Test animals tried to escape from the test boxes and avoided entering the soil. They did not feed during the first two weeks. Cocoon production was significantly reduced with all concentrations tested.

Discussion

Dimethoate and copper are suitable model test chemicals. Dimethoate shows a considerable acute toxicity especially to the indigenous earthworm species while sublethal effects occur at low concentrations. Copper, in contrast, has a moderate acute toxicity, but sublethal effects can also be observed at low concentration levels. In addition copper shows strong repellent effects on earthworms.

The acute and sublethal effects of the third test chemical remains to be investigated. Studies of species-specific sensitivity of adult

and juvenile life stages of earthworm species belonging to different ecological categories will be continued in 1994. Laboratory cultures with the indigenous species *A. caliginosa* and *A. chlorotica* will be established by using modified culture media and food sources. Optimum conditions for using the LUFA 2.2 soil will be investigated in order to ensure that the quality criteria for the SECOFASE project are met.

2.4 Participant No. 4

Development of Sublethal Test Methods for *Platynothrus peltifer* (Acari: Oribatida)

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Introduction

Studies during 1993 have concentrated on the establishment and development of culturing methods for *Platynothrus peltifer* (Acari, Oribatida). In addition, soil extraction methods and preliminary test protocols have been developed. Tests have been carried out with copper and dimethoate.

Methods and Results

Development of a culture method for P. peltifer

The life table of *P. peltifer* is given in Table 1, based on literature data. The data indicates that *P. peltifer* has a considerably long generation time. This is confirmed by data from a field study (Vera, 1993), indicating that the life cycle of *P. peltifer* is about one year. In nature, most eggs are produced in autumn, and the animals overwinter mainly as nymph stages II and III.

Table 1. Different life-stages of *P. peltifer* taken from Weigmann (1975).

Stage	Duration of this stage (days)	Temperature (°C)	Size/identification
Eggs	19-24	15	-
Larvae	21	15	3 pairs of legs; nymphs
Nymphe I	24-29	15	330-462 µm
Nymphe II	37	11	486-618 µm
Nymphe III	33-37	15	636-792 µm
Adult	Several months		0.9-1.0 mm
	Young adults can be recognized from having a light brown colour, while older adults have a dark brown colour		

Siepel (1990) described the differences in food choice between different oribatid mite species, and concluded that *P. peltifer* prefers plant material as a food source. Decayed oak leaves seemed to be most preferred, while the mites also feed on green algae of the species *Chlorococcum sp.* Vera (1993) demonstrated that *P. pelti-*

fer from an oak forest produced fewer eggs when fed green algae instead of oak leaves; egg production of mites from a coniferous forest did not show such a dependency on food source.

To start a culture, adult mites were extracted from needle litter which was collected in February 1993 in a coniferous forest near Roggebotszand, a reference area of the Department of Ecology and Ecotoxicology located in the centre of the Netherlands. The mites were kept on moist plaster of Paris and fed with green algae, growing on small pieces of tree bark. The culture was kept in a climate room at 18°C, at a day/night cycle of 12/12 hours.

Soon after incubation, the mites started to produce eggs, and after approximately 5 months the first juveniles hatched from these eggs had reached nymph stage III. From this it may be concluded that it will probably take about 6 months to get adult mites in the culture. It is however, uncertain whether the mites will reach the adult stage while being held at a constant temperature. As indicated above, *P. peltifer* mainly overwinters in the nymph stages II or III. This might imply that a cold period is essential for reaching the adult stage.

Therefore overall good progress has been made towards developing a culture method for *P. peltifer*. Already after 4 to 5 months large numbers of juveniles have been produced.

Development of extraction methods

As indicated in Table 1, mites of the species *P. peltifer* are very small and are therefore not easy to recover from soil by hand sorting. Extraction methods need to be developed to allow recovery of the mites and their juveniles from soil. Tullgren extraction appears to be useful for extracting *P. peltifer* from soil and litter material. This method was therefore chosen as a starting point for the development of extraction methods.

Test containers were designed to allow extraction of the mites from the test soil at the end of the experiment without necessitating transfer of the soil to other containers. The test containers consisted of a perspex ring (diameter 5 cm), on the bottom end closed with gauze (mesh size 1 mm). The containers were filled with a layer of artificial soil and the bottom was sealed with a piece of parafilm. At the end of the test, the parafilm was removed and the containers were placed in the Tullgren apparatus to extract the mites from the soil.

The recovery of mites from the soil appeared to depend on the amount of soil used (i.e. the thickness of the soil layer), the moisture content of the soil and the conditions in the Tullgren apparatus. Several experiments were performed to determine optimal conditions for recovery. For these experiments, 10 adult mites were introduced into the soil and incubated for 2 to 3 days, after which the test container was placed in the Tullgren apparatus. The temperature in the Tullgren apparatus was set at 25 to 30°C in the upper part and 5°C in the lower part.

To begin with, extraction methods were optimized for the OECD artificial soil. When this soil was prepared with sieved peat and brought up to a moisture content of 50% (based on dry weight), recovery was optimal when 20 g wet soil was used. When 10 to 25 g of wet soil was used per container, 90% of the mites were recovered from soil. Recovery was 0% when 30 g wet soil was used, apparently because the mites had not reached the bottom of the container before the soil had dried out.

In a second experiment using soil with a moisture content of 67%, 5 and 10 g of wet soil per container gave maximal recovery. In further experiments using soil with a moisture content of 100%, 20 g of soil gave maximal recovery. Similar experiments were carried out with the LUFA 2.2 soil. In these experiments, 15 g of soil with a moisture content of 23% gave maximal recovery. At lower moisture contents, the recovery of mites appeared to be reduced. This problem appears to have been solved by using lids with a hole covered with cheese cloth. In this case the soil has to be moistened at regular intervals to prevent it from drying out.

These experiments have shown that optimum combinations of soil moisture content and air humidity have to be found for the extraction process to be efficient. Due to insufficient numbers, extraction experiments have not been performed with juveniles yet. Results of experiments in soil (see next section) demonstrate however, that extraction of juveniles (larvae and nymph I) is possible with the Tullgren method.

Tests in soil

It was decided to use fairly dry soil for the soil tests because of the problems with mites climbing out of the soil at high moisture contents, and the experience reported in the literature that *P. peltifer* prefers a dry rather than a wet environment. A moisture content of about 33% was considered most suitable for the artificial soil. It should be noted, however, that the optimal moisture content of the OECD artificial soil will strongly depend on the way the soil is prepared. The procedure for preparing the peat will greatly influence the moisture retention characteristics of the artificial soil. Preferably, the peat should not be dried or ground before use; peat sieved through a 1 to 2 mm sieve seems to be most suitable for this purpose.

Table 2 shows the results of a preliminary test with copper in OECD artificial soil. For this experiment, test containers were filled with 20 g wet soil at a moisture content of 67%. The experiment was carried out at 20°C.

The results indicate that after 2 weeks, recovery in the control soil had dropped by as much as 50%. This was very low and at the present the reason for this is unclear. One possible explanation was the growth of fungi on the soil. The mites may have become trapped in the fungal hyphae which prevented them from escaping the soil in the Tullgren apparatus. When a food source (green algae) was added to the artificial soil, even more fungal growth was observed. In later experiments, juveniles and adults were

however, recovered from soil showing fungal growth. Apparently, fungi were not the only reason for the low recovery.

Table 2. Effect of copper on the recovery of *Platynothrus peltifer* from OECD artificial soil.

Nominal concentrations µg Cu/g dw.	Recovery (% of introduced animals) after 1 week	Recovery (% of introduced animals) after 2 weeks
0	80	50
16.7	80	70
167	70	100
1667	100	20
16667	0	0

Further experiments with copper and dimethoate in artificial soil have failed. In these experiments the soil was supplemented with 1% green algae as a food source. This however caused an enormous growth of fungi on the soil surface which may have been the reason for the failure of the tests. Only a few animals were recovered from the soil, and recovery was not dose-related.

A long-term experiment with artificial soil was initiated to study the mites; to investigate their food requirements in the soil and to determine possible ways to overcome the excessive growth of fungi. In this study, soil was supplemented with 1 or 2% finely ground oak leaves or 1% (freshly sampled) green algae as a food source. The food was mixed homogeneously into the soil and the amount of peat added was adjusted to ensure that the organic matter content of the soil remained at the prescribed level (10%). For each treatment test containers were filled with the given soil preparation and 20 or 40 adult mites were introduced. The soil moisture content was kept at 25%. After 1, 2, 3, 4, 6 and 10 weeks, one container from each treatment was sacrificed. Results up to week 6 are plotted in Figure 1.

Although the recovery of adult mites shows a gradual decrease with time, after 6 weeks the first juveniles (mainly larvae) appeared. From this it can be concluded that *P. peltifer* is able to reproduce in the OECD artificial soil; the extraction technique used allows for the recovery of juveniles; green algae were the best food source for reproduction studies.

Additional experiments

To gain experience with *P. peltifer* as a test organism and to obtain further knowledge about its sensitivity to copper, a feeding study was performed applying the method described by Denneman & van Straalen (1991). In these studies, adult mites caught from leaf and needle litter, were exposed to copper added to green algae. The mites were kept on moist plaster of Paris, 10 animals per test container. Food was renewed weekly and the numbers of dead animals, eggs and faecal pellets were counted.

The test concentrations were as follows (nominal values): 0, 63, 200, 630, 2000, 6300 µg Cu/g (the actual, measured concentrations were 26, 77, 237, 718 , 1949, 5055 µg Cu/g dry food). Table 3 shows the results of this experiment.

Table 3. Effect of copper on the survival, egg production, faeces production (as a measure of consumption) and growth of *Platynothrus peltifer*, when exposed via the food (algae).

Parameter	Time (weeks)	NOEC (µg Cu/g dry food)	EC_{50}
Survival	9	-	>6300*
	12	-	1600* (654 -3816)
Egg production	9	630	-
	12	200	603 (164 - 2223)
Faeces production	9	200	946 (654 - 1359)
	12	200	800 (787 - 806)
Growth	12	630	>6300

* LC_{50}

From Table 3, it can be concluded that faecal production is of similar sensitivity as egg production. Studies by Janssen (1991) indicated that *P. peltifer* is a slow accumulator of cadmium, taking a very long time to reach an equilibrium between internal (body) and external concentrations. This may also be the case for copper, thus explaining the decrease in EC_{50} still continuing after 12 weeks.

Analysis of the residues in the surviving animals after 12 weeks showed that the internal copper concentration increased with increasing food concentration (Figure 2), except at the lowest concentration. In general, the results of this study correspond well with those obtained by Denneman & van Straalen (1991). In a 3 week feeding experiment no effects on survival were noticed at dimethoate concentrations up to 100 µg/g dry food.

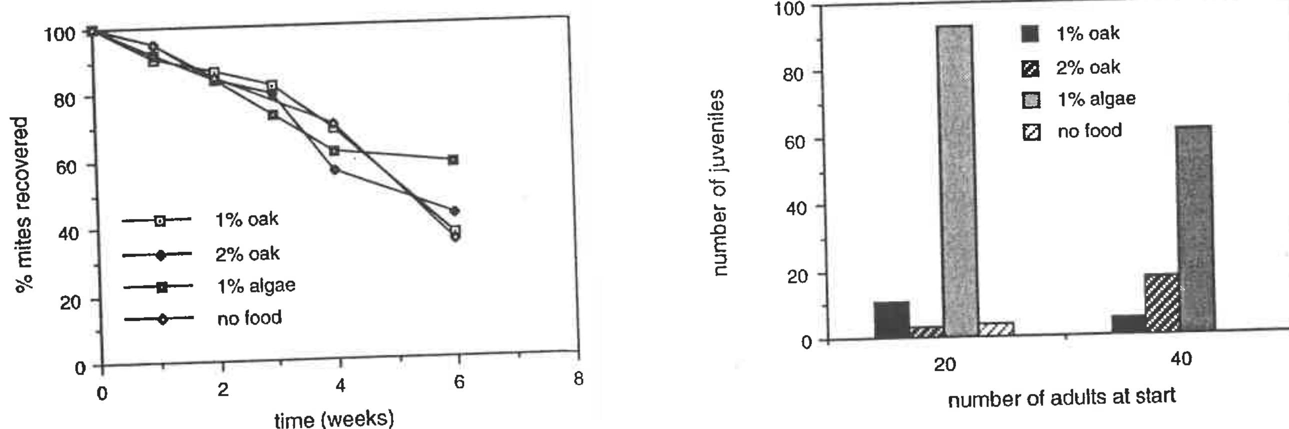

Figure 1. Recovery of *Platynothrus peltifer* from OECD artificial soil, unamended or amended with 1 or 2% oak leaves or 1% green algae as a food source (left; average of two replicates with either 20 or 40 adult mites introduced at the start), and number of juveniles recovered after 6 weeks from containers with 20 or 40 adult mites at the start (right).

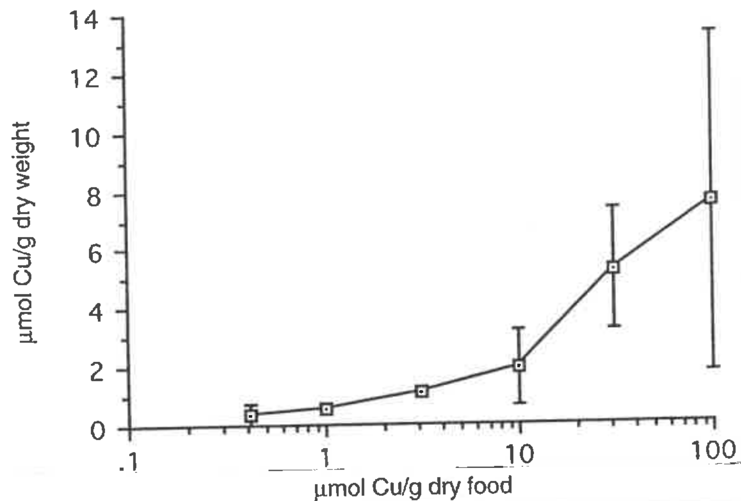

Figure 2. Average copper concentrations in *Platynothrus peltifer* after 12 weeks exposure to copper in the food (green algae).

2.5 Participant No. 5

Development of a Two-Species Nematode Toxicity Test

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Introduction

The work carried out during 1993 has focussed on determining life-cycle parameters for the nematodes *Plectus acuminatus* and *Heterocephalobus pauciannulatus*, and carrying out preliminary experiments with the SECOFASE reference compounds in order to develop the test system.

Methods

Culturing of the nematodes in agar and soil

Two bacterivorous nematode species, *P. acuminatus* and *H. pauci annulatus,* were extracted from soil and are presently being maintained at the Department of Nematology. Both species are parthenogenetic and can be reared in the laboratory without major difficulties on the soil bacterium *Acinetobacter johnsonion.* Stock cultures were kept in agar in small Petri dishes at 15 or 20°C in the dark and maintenance was not labour intensive. Furthermore, cultures of *P. acuminatus* can be stored at 4°C in order to preserve initial genetic variability. In addition it was possible to rear *P. acuminatus* in standard OECD soil (75% moisture content d.w., pH 5.5) at 20°C.

Life cycle experiments

Complete life cycle experiments were conducted with both species in agar with added bacteria. Individually kept females were observed during the entire life cycle, from birth to death. Juvenile and adult variables were recorded (Table 1). Both species have different life-history strategies and it appears that *P. acuminatus* has a longer life cycle than *H. pauciannulatus*.

Table 1. Life cycle variables of *P. acuminatus* and *H. pauciannulatus* in agar which were fed on *A. johnsonion* (2.10^8 cells/ml) at 20°C.
t_j: juvenile period (days), s_j: juvenile survival, n_t: daily reproduction (day^{-1}), t_a: reproductive period (days), s_a: survival over the reproductive period. (i.p.=in progress)

	t_j	s_j	n_t	t_a	s_a
P. acuminatus	18	0.74	9.5	59	0.9
H. pauciannulatus	8	i.p.	20	i.p.	i.p.

Toxicity experiments

To gain insight into the relative toxicity of various compounds to the nematode species, acute toxicity experiments were conducted in water. The LC_{50} values were estimated using the trimmed Spearmann-Kärber method (Hamilton et al., 1977; 1978) and are shown in Table 2. It appeared that *P. acuminatus* was more sensitive to all of the compounds tested than *H. pauciannulatus*. The results were used to estimate the concentration range required for sublethal toxicity experiments.

Table 2. Acute toxicity (LC_{50}, 72 h) of copper (Cu), cadmium (Cd), dimethoate (DM) and DHTDMAC (DHTM) in mg/L water for *P. acuminatus* and *H. pauciannulatus* at 20°C and pH = 6.

	Cu	Cd	DM	DHTM
P. acuminatus	14	107	1432	719
H. pauciannulatus	67	674	>2000	>1500

Chronic life cycle experiments

Currently, complete life cycle studies are underway to investigate the influence of copper and dimethoate on different life-history components of *P. acuminatus* and *H. pauciannulatus* in agar.

Discussion

Recently, the genus *Plectus* has been revised by nematode taxonomists. The species which was selected, *P. parietinus*, appears to be *P. acuminatus*. Also a second species of nematode *Heterocephalobus pauciannulatus* has been reared instead of *Cephalobus persegnis*. These modifications do not influence the development of the test or the ecological realism. Moreover, both species can be reared very well in the laboratory and have different life-history strategies which is very interesting from an ecological point of view.

It is planned to continue the toxicity experiments on life cycle performance of *H. pauciannulatus* in agar for various toxicants. Additionally, studies will be undertaken to gain insight into the competition process between the two species in agar and in soil.

References

Hamilton, M.A., Russo R.C. & Thurston, R.V. (1977; 1978): Trimmed Spearmann-Kärber method for estimating median lethal concentrations in toxicity bioassays. Environ. Sci. Technol. **11**: 714-719. Correction. Environ. Sci. Technol. **12**: 417.

2.6 Participant No. 6

Reproduction, Growth and Survival in the Enchytraeid *Cognettia sphagnetorum* (Vejdovsky)

MSc. A. Augustsson and Dr. S. Rundgren
Division of Animal Ecology, Department of Ecology, University of Lund, Sweden.

Introduction

The enchytraeid *Cognettia sphagnetorum* (Vejdovsky) is commonly found in most Nordic coniferous forest soils, where it mainly is confined to the uppermost organic layer, the mor horizon (Lundkvist, 1977). The species reproduces parthenogenetically by fragmentation, though occasionally it may develop eggs. Whether or not the eggs ever hatch is unknown. Fragmentation takes place when the worm has reached a certain size. Information concerning size at fragmentation is scarce and inconsistent (Standen, 1973, Makulec, 1983). When fragmenting, the worm mostly splits in three parts: a head, a middle and a tail fragment, each consisting of 5-25 segments.

During 1993 investigations concerning the fragmentiation of *C. sphagnetorum* have been undertaken. The studies include the determination of:

a) the size of the worm at fragmentation
b) the number of fragments resulting from fragmentation
c) the fragmentation rate, i.e. the rate of fragmentation per day
d) the growth of different fragments
e) the survival of different fragments

Methods

Specimens of *C. sphagnetorum* were extracted in April/May from the mor of a coniferous forest soil using the wet-funnel method (O'Connor, 1967). The number of segments per individual was determined using light microscope. Only specimens considered to be adults, defined here as having reached the size of ≥ 35 segments, were used in the experiments. The worms were separated in different size categories: 35, 37, 39, 42, ≥ 42 segments.

Mor soil was sampled at the site where the worms were collected. It was sieved (mesh size 1.0 mm) to remove large particles and to obtain a fairly homogeneous experimental soil. It was frozen (-18°C) and thawed three times to reduce the indigenous soil fauna. The treatment seems to have only minor negative effects on the amount of mycelium, which are believed to be one of the main food sources of enchytraeids (O'Connor, 1967). Distilled water was added to obtain a soil humidity of 60 to 70%.

A 2.5 ml sample of soil was placed in a 5 ml glass vial. A healthy looking, undamaged and active worm was transferred into the vial and placed on the soil surface to check that it was able to burrow into the soil. The vial was then sealed. A total of 15 to 23 glass vials were used for worms of each size category. The vials were stored in darkness at 15°C.

Once a week the vials were checked for fragmentation. To recover the worms, the soil was flushed with tap water into a Petri dish. The numbers of fragments and the number of segments per fragment were determined. Each fragment was transferred to a separate vial and the survival and growth of the regenerating worm was followed weekly over a period of 10 weeks. Each week the adult worms and regenerating fragments were transferred to fresh soil.

To determine the fragmentation rate, a formula modified from that proposed by Makulec (1983) was used:

Fragmentation rate: $N_p/(N_z \cdot d)$

N_p = the number of head fragments
Nz = the number of worms at start of the experiment
d = the number of days

Results

Fragmentation occurred during the first week of the experiment. Mostly two fragments were found (49%of observations), but up to six fragments were recorded as well (3% of observations). Only fragments consisting of ≥ 5 segments were collected for further studies, since smaller ones were inactive and dying. By rearing the worms in separate vials it was possible to calculate the size of the adult at the time of fragmentation. This was estimated to be 46.0±6.4 segments (range 35 to 68).

The fragmentation rate varied between 7.5 x 10^{-3} and 11.7 x 10^{-3} fragments per day. Standen (1973) operated with a fragmentation rate of 8.3 - 33.1 x 10^{-3} at 15°C; the lower rate occurred during winter time and the higher one during summer time. There was no significant difference between the fragmentation rate and the number of segments of the worms at start. Evidently worms that have reached a size of ≥ 35 segments are disposed to fragmentation, whereas non-fragmenting adults (≥ 35 segments) continued to grow more slowly (1 to 3 segments per week). The growth of the regenerating fragments was faster. The weekly increase of head fragments was 2 to 3 segments per week and tail fragments 6 to 8 segments during the first week when the head was regene-rated. After that the increase was similar to that of the head fragment. Thus the growth of head and tail fragments did not differ significantly over the period of study. The growth of the middle fragments was, however, slightly higher especially during the first week, (10 to 12 segments), when both head and tail was regenerated. It was hypothesized that the growth of the head fragment should be more rapid than any of the other fragments, since the development of head segments is supposed to be energy-demanding. The rapid development of the middle fragment is interesting. Whether or not it is associated with the allocation of nutrients of the worm under fragmentation is not known.

The survival rate of the adult worms during the experimental period was high as only approximately 4% died. The mortality of fragments was slightly higher (9%).

Discussion

In an earlier experiment C. *sphagnetorum* was reared in a series of metal polluted soil. The fragmentation rate and the number of

resulting fragments were recorded during two weeks. The results obtained indicate that both fragmentation rate and the growth of the regenerating fragments of *C. sphagnetorum* are useful variables to study enchtytraeid worms exposed to environmental stresses. One obvious drawback, however, with using *Cognettia spp.* is that it is a fragile species and must be handled with care.

An experiment is currently in progress in which *C. sphagnetorum* is being reared in standard soil (LUFA soil 2.2) contaminated with copper. The experiment is being monitored weekly using the methods described above. This experiment will provide additional data on the fitness of *C. sphagnetorum* when exposed to stress. At the beginning of the experiment, it was noted that the mean size of the adults, which had been extracted in October, was approximately 60 segments. This raised the question of whether fragmentation is seasonal under natural conditions. It seems that fragmentation may not only depend upon external factors such as drought and temperature, as suggested in most papers concerning asexual reproduction of *Cognettia spp.*, but also on internal factors. Standen's data (1973) on fragmentation rates may also indicate seasonality of fragmentation. This therefore requires further investigation before *Cognettia spp.* can be adopted as a test organism in laboratory tests.

References

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

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2.7 Participant No. 7

Development of Sublethal Test Methods for the Collembola *Isotoma viridis* Bourlet and *Folsomia candida* (Willem) (Collembola: Isotomidae)

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Introduction

During 1993 progress was made towards establishing laboratory cultures of Collembola and developing test protocols. In addition, a second Collembolan species, *F. candida*, has been introduced into the studies (see deviations from the work plan during the reporting year).

The main areas of progress to date include:

a) collection of *I. viridis* from field sites in Hampshire, UK
b) establishment of laboratory cultures of *I. viridis* and *F. candida*
c) development of standard operating procedures (SOP's), including Collembola culturing techniques, measurement of growth parameters using image analysis computer software, preparation of test soils, measurement of soil moisture and pH, chemical incorporation into test soils, flotation techniques for Collembola extraction from soils
d) life-history studies of *I. viridis*, including growth rates, survival rates and dietary performance under laboratory conditions of 15°C and 20°C
e) testing of the three chemicals (dimethoate, copper and DHTDMAC) against *F. candida*, including chronic tests concerning effects on reproduction and acute toxicity tests concerning substrate-mediated toxicity

Results

Measurement of life-history parameters of I. viridis

Studies were carried out at two different temperatures, 15°C and 20°C, to determine the survivorship, dietary performance and growth rates of *I. viridis* under laboratory conditions. Survivorship and dietary performance experiments were carried out in glass vessels containing a layer of plaster of Paris and charcoal. Single egg batches were placed into each vessel and the number of eggs per batch was recorded. The vessels were transferred to incubators at 15°C and 20°C and the time to hatching and the percentage hatching success of each batch were recorded. After hatching each batch of juveniles was provided with one of three food types; yeast (granulated dried Bakers yeast, *Pleurococcus spp.* (green algae) or Tetramin (fish food); and their survival rates were monitored until death.

Table 1. Some life-history parameters of *I. viridis* at 15°C and 20°C.

Temperature (°C)	Mean no. of eggs laid per batch (±SE)	Mean hatching success (%) (±SE)	Mean days to hatching (±SE)
20	33.5 ± 4.6	70.6 ± 10.4	13.5 ± 1.2
15	36.1 ± 5.3	68.7 ± 13.7	17.7 ± 0.6

A summary of the mean numbers of eggs laid per batch, percentage hatching success and time taken for the eggs to hatch are given in Table 1. No significant differences were found between the mean numbers of eggs laid per batch or the percentage hatching success of *I. viridis* ($P>0.05$) at 15°C and 20°C. The mean number of days to hatching was however significantly longer ($P<0.01$) at 15°C than 20°C (Table 1).

Similar trends were evident in the dietary performance experiments at both temperatures. The percentage survival of *I. viridis* was greatest in the batches fed with yeast, lowest in batches fed with Tetramin and intermediate in batches fed with *Pleurococcus*. Egg batches were laid after 56 and 22 days at 15°C and 20°C respectively by individuals fed on yeast. Egg batches were produced after 27 days at 20°C by individiduals fed on Tetramin, however none were produced at 15°C. No egg batches were produced at either 15°C or 20°C for the individuals fed on *Pleurococcus*. It was also noted that these individuals tended to remain small in size, however no quantitative growth measurements were made. To date yeast appears to be the best food source, however other food types or blends of food may be tested in the future.

The growth rates of individual *I. viridis* at 15°C and 20°C were measured using image analysis computer software. Hatchlings of known age were placed individually in glass vessels similar to those used in the survivorship studies. All individuals were provided with yeast as food and measurements of growth parameters, including total body length, length for thorax to the tip of the abdomen, dorsal surface area and head capsule width, were made weekly for each individual until death. Example growth curves are given in Figure 1. The curves indicate considerable variability in growth rate between individuals, however growth rates were greater at 20°C than 15°C. These data will be analysed further using the von Bertalanffy growth model.

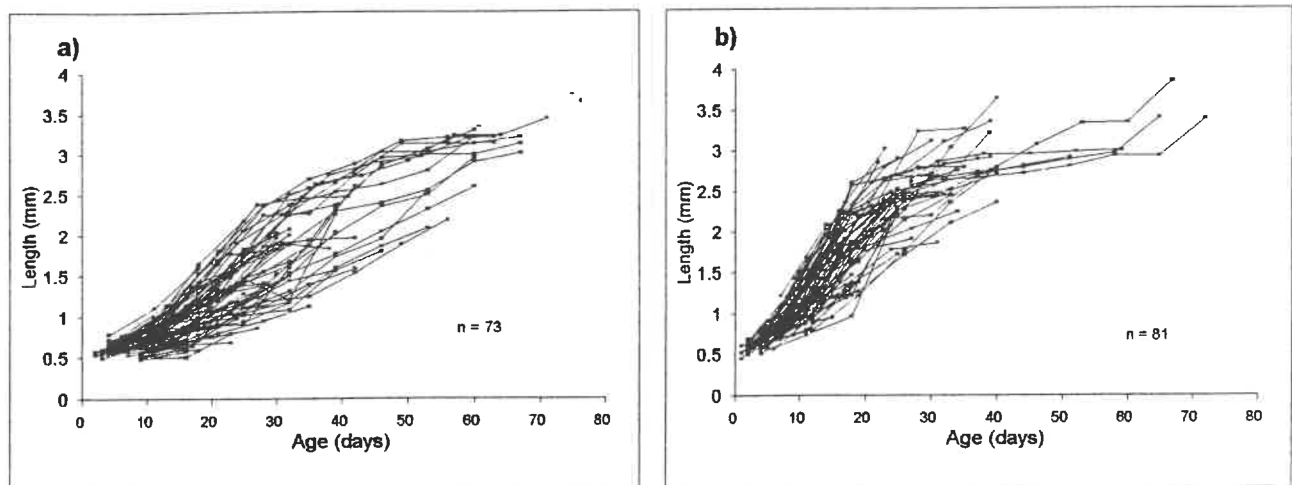

Figure 1. Example growth curves of *I. viridis* at a) 15° C and b) 20° C.

Effects of chemicals on reproduction

Tests were carried out to determine the effects of dimethoate, copper and DHTDMAC on the reproduction of *F. candida*. Two soil types were tested for each chemical, an artificial soil (OECD), consisting of 10% sphagnum peat, 20% kaolin clay, 1% $CaCO_3$ and 69% industrial quartz sand as dry weight, and a natural LUFA Speyer soil (LUFA 2.2). The test methods used are given in the standardization form (Annex 1 - Participant 7). Example results for dimethoate and copper are given in Figures 2 and 3. In general the toxic effects of dimethoate and copper were greater in the LUFA soil than in the artificial (OECD) soil. In the preliminary studies so far there was little evidence of sublethal effects with dimethoate but some evidence of effects with copper.

Results for DHTDMAC are not presented here. Difficulties were encountered when handling and incorporating DHTDMAC into the test soils. The formulation (5.3% activity) was very viscous and made precise incorporation into the soils problematical. The viscous nature of the detergent also affected the soil texture and it is likely that this would dramatically reduce the ability of the Collembola to move through the soil.

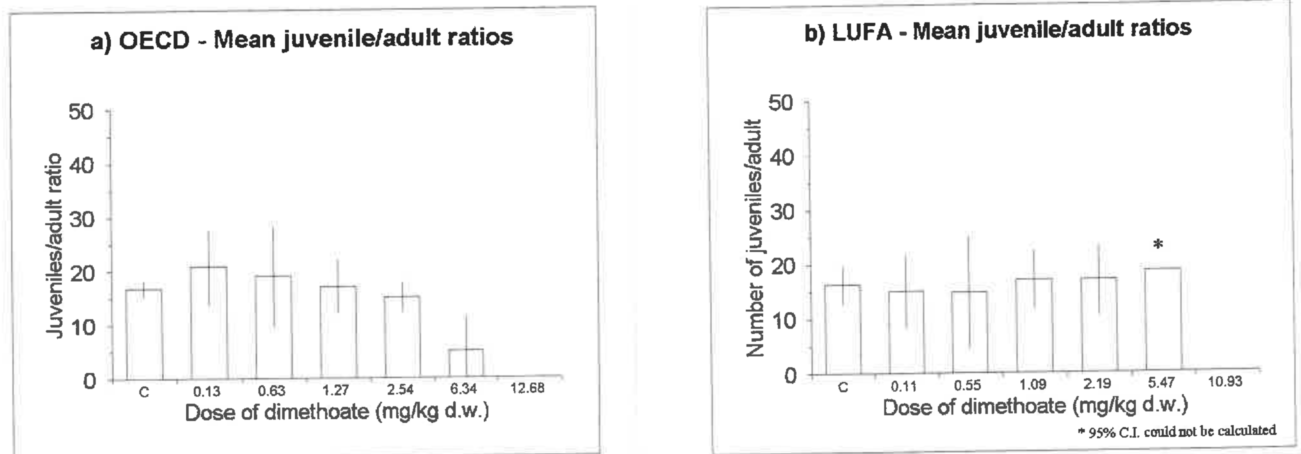

Figure 2. Results from reproduction tests with dimethoate and *F. candida* in OECD and LUFA soils at 15° C.

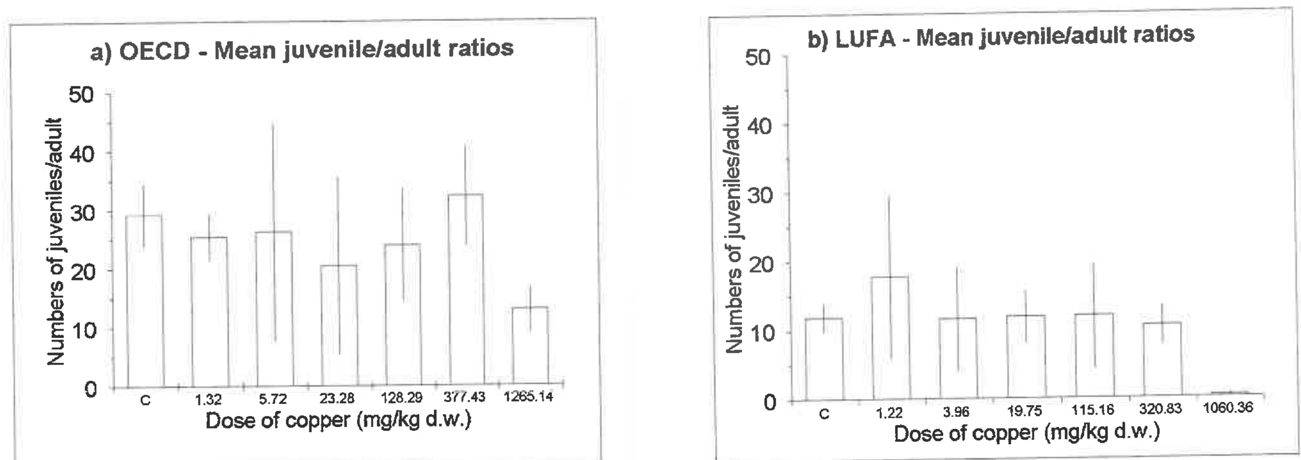

Figure 3. Results from reproduction tests with copper and *F. candida* in OECD and LUFA soils at 15°C.

Effects of soil type on the toxicity of the pollutants to Collembola

Preliminary studies were carried out to investigate the effect of soil type on the toxicity of dimethoate, deltamethrin (a synthetic pyrethroid insecticide), copper, and DHTDMAC to *F. candida*. A short-duration (6 days) exposure bioassay was carried out to determine the influence of soil mineral and organic matter composition on the toxicity of the given chemicals to *F. candida*. Six artificial soils were used in the experiments, including pure sand and soils consisting of 1%, 3.2%, 10%, 32.3% and 100% peat. In general, the toxicity of the chemicals decreased as the soil organic matter content increased.

3 Deviations from and Modifications to the Work Plan

The work plan adopted for the SECOFASE project was outlined in the manual of SECOFASE (Løkke and van Gestel, 1993). In general, the participants have successfully followed the schedule during 1993. Deviations from the work schedule by each participant are outlined below together with modifications for the work plan for 1994. All of the deviations have arisen from minor technical problems and the necessary changes can easily be accomodated in the work plan for 1994.

3.1 Deviations from the Work Plan in 1993

Participant No. 1
Single individual tests with the detergent DHTDMAC have not been made. Investigations with copper are currently underway.

Participant No. 2
No deviations from the work schedule.

Participant No. 3
The establishment of laboratory cultures of the earthworm species *A. caliginosa* and *A. ora chlorotica* could not be satisfactorily completed in 1993 due to poor weather conditions at the time of sampling. In addition, earthworm activity was generally low during the reproduction tests.

Participant No. 4
No deviations from the work schedule.

Participant No. 5
Recently, the genus *Plectus* has been revised by nematode taxonomists. The species which was selected, *P. parietinus*, appears to be *P. acuminatus*. Also a second species of nematode *Heterocephalobus pauciannulatus* has been reared instead of *Cephalobus persegnis*. These modifications do not influence the development of the test or the ecological realism.

Participant No. 6
Studies concerning sexually reproducing enchytraeid species have been delayed until 1994. Investigations concerning the effects of copper on fragmentation, survival and growth are currently underway.

Participant No. 7
During the present year a second Collembolan species, *F. candida*, was introduced into the investigations. It was considered that this would provide a dual benefit to the project by firstly, enabling tests with dimethoate, copper and DHTDMAC at an early stage

thus enabling us to develop handling and soil incorporation procedures while establishing *I. viridis* cultures and carrying out life-history studies. Secondly, the inclusion of this species will eventually enable us to compare and contrast the responses of *I. viridis* (a new test species) and *F. candida* (a widely used test species) to the pollutants. This is of particular interest as they have markedly different life-histories, for example *I. viridis* is a sexually reproducing, surface-active species whereas *F. candida* is a parthenogentically reproducing, subterranean species.

3.2 Modifications to the Work Plan for 1994

A general modification to the work plan for all participants in 1994 is the change of test detergent from DHTDMAC to LAS.

Participant No. 1
Individual species tests with the detergent, LAS, will be carried out in 1994. The pilot studies with copper and *H. succinea* and the individual species tests with copper will be completed in 1994.

Participant No. 2
No modifications required.

Participant No. 3
Further sampling of earthworms will be carried out in spring and autumn 1994 to establish new cultures.

Participant No. 4
In 1993 good progress was made with the development of extraction techniques for adult mites in both OECD artificial soil and the LUFA 2.2 soil. Because of a lack of juveniles, such extraction experiments could not be performed with juveniles. These experiments will be carried out in 1994.

Participant No. 5
No modifications required.

Participant No. 6
Growth studies with *C. sphagnetorum* were initiated in 1993. In 1994 further studies concerning the reproductive behaviour of this species will be carried out to determine if fragmentation is seasonal. Reproductive studies will begin in the second half of 1994.

Participant No. 7
Because of the inclusion of the second Collembolan species and the time taken to establish *I. viridis* cultures and gather life-history data concerning the performance of *I. viridis* under laboratory conditions it was decided to delay development of population models until the second year of the project. This will have no effect on the planned project time schedule as progress has already been made in other areas planned for 1994, for example with chemical bioassays on standard soils and the impact of abiotic factors, such as soil type, on the toxicity of the test chemicals.

4 Standardization

Standardization is a central aspect of the SECOFASE project. The cooperative nature of work within this project will enable the development of a battery of standardized tests for a range of important soil organisms. These are based on standardizing test conditions and measurements which are essential if the test methods are to be implemented to set international standards or be used for regulatory purposes. Guidelines were set out in the Manual of SECOFASE (Løkke and van Gestel, 1993) to describe culturing techniques, choice of test substrates and test chemicals, chemical treatment, test conditions and procedures for Good Laboratory Practice. In this report, the first section (4.1) outlines progress with standardization in 1993. The following section (4.2) describes additional chemical residue analysis studies that has been or will be carried out to determine the behaviour of the test chemicals in the test systems and aid interpretation of test results. The final section (4.3) describes a novel method of sensitivity analysis of fitness parameters for the test species to provide ecological insights into the implications of the test results.

4.1 Progress with Standardization

Working methods for culturing and testing organisms

During the initial development of culturing and test procedures for the test organisms it was agreed that each participant should focus on determining the specific requirements of their own test species. Therefore at present different species are being reared at different temperatures, under different light-regimes and in/on different substrates with different moisture contents. These investigations are still proceeding for many participants and will lead to the development of standard operating procedures for culturing and testing each organism during 1994. Initial tests, therefore, may be carried out at different temperatures however all participants intend to carry out the final tests at 15°C to ensure the comparability of results of toxicity tests between species.

Test conditions and soils

At the onset, two soils were chosen as standard test soils for the SECOFASE project: an artificial OECD soil and a natural LUFA Speyer soil (LUFA 2.2). Standardized procedures concerning the determination of soil moisture contents, pH and soil preparation are being followed by participants. A water content of approximately 50% of the water holding capacity (WHC) will be used in the final standardized tests. During the current workshop it was noted that some species have different demands concerning the structure of the soil. For example, millipedes require compacted soils whereas earthworms may require a loose soil. At present, however, no standardized procedures have been outlined for determining soil texture characteristics.

Exposure characteristics

All of the test chemicals are being mixed homogeneously into the test soils. Each participant has developed their own standard

operating procedures for incorporating the test chemicals. An intercalibration exercise between participants (see section 4.2) will be carried out to ensure that all the mixing procedures are adequate. The main exposure route under investigation by all participants is contact with chemical residues. In some cases investigations using contaminated diets may also be carried out. All participants are allowing periodic ventilation of their test systems.

Data analysis and statistical methods

At present there are no additional recommendations to the statistical procedures outlined in the SECOFASE manual (Løkke and van Gestel, 1993).

4.2 Chemical Analysis

During 1993 residue analysis of dimethoate was carried out in both of the proposed test soils (OECD and LUFA 2.2). These studies were conducted jointly by participants No.s 2 and 3 in collaboration with a chemical analyst (Dr. J. Siebers, BBA Braunschweig). Residue analysis (GC) was carried out at different times after incorporating doses of dimethoate into samples of the soils. The experiments included the test design for earthworms (26.9 mg a.i./kg dried soil) of participant 3 and that for staphylinid larvae (1 mg a.i./kg dried soil) of participant 2. Residues were extracted with acetone/water (total residues) and with water only.

The total residues of dimethoate declined after 22 days to about 25% (LUFA 2.2) and 40% (OECD) using the earthworm test design and to about 50% (LUFA 2.2) and 60% (OECD) in the staphylinid larvae test design. Both experiments were conducted at 20°C. Water extracted residues were in most cases lower than the total residue values. The total reduction in residues over 22 days was up to 30% less at 15°C compared with 20°C.

The degradation of dimethoate will be regarded as a general decay pattern in the standard soils of all sub-projects of SECOFASE. The decay curves and the biological effects observed will be published elsewhere.

It is proposed that residue analysis of copper will be conducted for the standard test soils (e.g. OECD and LUFA 2.2 soils) in order to determine the behaviour of copper in the standardized test systems. Assessments to determine the homogeneity of copper in the tests soils may also be carried out. An intercalibration exercise will be co-ordinated between participants to ensure standardization of soil incorporatation procedures for copper.

4.3 Sensitivity Analysis of Fitness Parameters for Test Species

During 1993 progress was made with the development of a mathematical model for life-history analysis (Kammenga *et al.* in prep)

at the Department of Nematology, Wageningen Agricultural University (participant No. 5). The model incorporates juvenile and adult life cycle variables into one parameter of fitness which is unique for each species at certain environmental conditions. The model provides a tool for investigating the relative sensitivity of fitness to pollutant induced changes in life-history traits.

At present the model has been applied to the life-history strategy of *P. acuminatus*. A sensitivity analysis of the model illustrated that the length of the juvenile period and the daily reproduction greatly influence fitness of this nematode species. Therefore selection of suitable test parameters will include these life cycle components to enhance ecological realism.

During the workshop it was agreed that all of the SECOFASE participants will attempt to gather the necessary life-history data so that the model may be applied to their test species. This may result in a unique comparison of different life-history strategies, indicating which variables are important for each species in terms of maximizing their fitness. The findings may be published at a later date as a joint paper.

5 Meetings Attended by Project Participants

All participants attended the first SECOFASE Workshop held at NERI, Silkeborg, Denmark, from 18th to 19th January 1993. Several participants also attended the SERAS Group meeting in Lisbon, Portugal in March 1993.

Participant No. 1
MSc. Paul Henning Krogh presented a paper entitled "Test for effects of pesticides on micro-arthropods with single species, a predator-prey system and a semi-field system" at the First SETAC World Congress at Lisbon, Portugal in March 1993. He also attended the 4th Annual meeting of SETAC-UK, at The University of Keele, UK, in September 1993 and visited Dr. J. Weeks at the Institute of Terrestrial Ecology, Monkswood, UK and Dr. J.A. Wiles and Dr. P.C. Jepson at The University of Southampton, UK.

MSc. M. Holmstrup visited Dr. C.A.M. van Gestel at Vrije Universiteit, Amsterdam and Dr. L. Posthuma at the R.I.V.M, Bilthoven, The Netherlands.

Dr. Hans Løkke chaired the session "Soil Ecotoxicological Hazard Assessment (II), at the First SETAC World Congress in Lisbon, Portugal in March 1993. He also presented a paper entitled: "Extrapolation of the Effects of Dimethoate from the Laboratory to the Field" at a Central and Eastern European Regional Meeting of SECOTOX held in Poland, 23rd to 26th August 1993.

Participant No. 2
MSc. K. Metge presented a paper entitled "First Experiences with Culturing of Staphylinid Species and Testing the Effects of Chemicals" at a meeting of the IOBC/WPRS working group "Pesticides and Beneficial Organisms" held in Vienna, Austria, 12th to 14th October 1993.

Dr. U. Heimbach visited Dr. J.A. Wiles and Dr. P.C. Jepson at The University of Southampton in September 1993.

Dr. U. Heimbach and MSc. K. Metge have been in frequent contact with Prof. Dr. O. Larink and MSc. H. Kula from the Technical University of Braunschweig during the current year.

Participant No. 3
Prof. Dr. O. Larink and MSc. H. Kula attended the First SETAC World Congress at Lisbon, Portugal in March 1993. MSc. H. Kula presented a joint paper entitled "Sensitivity differences of soil organism groups of agroecosystems towards pesticides."

Participant No. 4
MSc. A. Augustsson, from the University of Lund, and MSc. M. Holmstrup from NERI, Denmark visited Vrije Universiteit of Amsterdam to learn techniques.

Participant No. 5
Ir. J.E. Kammenga attended the First SETAC World Congress at Lisbon, Portugal in March 1993 and presented a poster: "Availability of food influences the effect of cadmium on biomass production in nematode populations". In addition a presentation was given on: "The principle of the dose-response-fitness relationship" at the 5th congress of the Netherlands Integrated Soil Research Programme.

Participant No. 6
MSc. A. Augustsson visited Dr. C.A.M. van Gestel at The Vrije Universiteit of Amsterdam, The Netherlands to learn techniques.

Dr. S. Rundgren and MSc. A. Augustsson attended a workshop on soil organisms held in the Department of Ecology, University of Lund in August 1993. They also also made a presentation entitled "Reproduction, growth and survival in the enchytraeid *Cognettia sphagnetorum*" at the 6th Nordic Symposium on Soil Ecology in Finse, Norway in September 1993.

Participant No. 7
Dr. P.C. Jepson and Dr. J.A. Wiles attended the First SETAC World Congress of Ecotoxicology, in Lisbon, Portugal in March 1993. Dr. P.C. Jepson presented a platform paper.

Dr. J.A. Wiles attended a BSI/ISO "Biological Methods" Committee meeting in London in April 1993. He also attended the 4th Annual meeting of SETAC-UK, held at Keele University in September 1993 and presented a platform paper concerning the extrapolation of effects from the laboratory to the field.

Dr. P.C. Jepson attended the 14th Annual Meeting of SETAC in Houston, USA, during November 1993 and presented two papers concerning indirect effects of pesticides and validation of predictions.

Annex 1 - Test System Standardization Forms

Participant No. 1

Test protocol no. 1.1:

Single species reproductive test with *Folsomia fimetaria* (Collembola: Isotomidae)

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

Test organism

Taxonomic group:	Collembola: Isotomidae
Species:	*Folsomia fimetaria*
Life stage:	Adult
Sex:	♂♀
Age/weight/size:	16-19 days
Genetic composition:	Sexual populations
Source:	Field collected population

Type of surroundings and ambient climate

Description of surroundings

Type:	Microcosm

Ambient climate

Temperature (°C):	20±1
Rel. humidity:	95-100%
Light wave length:	not indicated
Light intensity:	100-200 lux
Light regime:	(L:D) 12:12

Type of test system and test conditions

Description of test system

Type:	Cylinder
Material:	Perspex, PVC lid
Openess:	Discontinously opened for gaseous exchange (Opened after 2 weeks)
Species/Test system:	Single species
Indv./test system:	25

Annex 1 - Test System Standardization Forms 1.1

Type of habitat

Soil:	LUFA 2.2 soil

Habitat characteristics

Soil:	Defaunated and reinoculated agricultural soil
Hydrology:	40-60% of Water Holding Capacity
Food supply:	15 mg yeast at day 0 and day 14
Feeding rate:	Every fortnight
Type of food:	Granulated baker's yeast

Exposure characteristics

Exposure route:	Soil
Composition of test soil:	LUFA 2.2 soil + deionized water + chemical
Composition of control soil:	LUFA 2.2 soil + deionized water + chemical

Duration of the experiment

Acclimat. time:	None
Exposure time:	3 weeks
Recovery period:	None

Exposure concentrations

Replicates:	4 per treatment
Concentrations:	5 in geometric series

Exposure responses

Effect parameters:	Survival of original test animals per replicate; Number of extractable offspring
Measurement of variables:	Adult survival and reproduction during incubation; water content after 2 weeks
Toxic endpoint:	NOEC, LOEC, EC_{10}, EC_{50}
Unit:	mg/kg dry soil
Working methods:	Enumeration of extracted adults and juveniles
Analytical methods:	-

Miscellaneous information needed for application of criteria

Practicability:	The species are easily cultured from populations obtained from the field. All steps in the test procedure can be performed by technicians. A MacFadyen high gradient extraction or similar apparatus must be available.
Cost-effectiveness:	The work that must be done before during and after the performance of the experiments takes 1 week and the amount of material, energy and test organisms that is required is normal.
Sensitivity:	EC_{50} (reproduction) for dimethoate is 0.3 mg/kg
Reproducibility:	Work in progress
Validity:	A) Determination NOEC and LOEC by ANOVA, and estimation of EC_{10} and EC_{50} by curve fitting B) Work in progress
Standardization:	Work in progress
Ecological realism:	The species is widely distributed in European soil ecosystems, e.g. agricultural land and grassland.
Broad chemical responsiveness:	The test system is in principle able to test any chemical that can be incorporated into soil.

Participant No. 1

Test protocol no. 1.2:

Single species reproductive test with *Hypogastrura succinea* (Collembola: Poduridae)

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

Test Organism

Taxonomic group:	Collembola:Poduridae
Species:	*Hypogastrura succinea*
Life stage:	Adult
Sex:	♂ and ♀ together
Age/weight/size:	6-9 days
Genetic composition:	Sexual populations
Source:	Laboratory reared population

Type of surroundings and ambient climate

Description of sourroundings

Type:	Microcosm

Ambient climate

Temperature (°C):	20±1
Rel. humidity:	95-100%
Light wave length:	not indicated
Light intensity:	100-200 lux
Light regime:	(L:D) 12:12

Type of test system and test conditions

Description of test system

Type:	Cylinder
Material:	Perspex, PVC lid
Openess:	Discontinously opened for gaseous exchange (opened after 2 weeks)
Species/Test system:	Single species
Indv./test system:	25

Type of habitat

Soil:	LUFA 2.2 soil

Habitat characteristics

Soil:	Soil defaunated and reinoculated agricultural soil
Hydrology:	40-60% of Water Holding Capacity
Food supply:	15 mg yeast at day 0
Feeding rate:	Only at day 0
Type of food:	Granulated baker's yeast

Exposure characteristics

Exposure route:	Soil
Composition of test soil:	LUFA 2.2 soil + deionized water + chemical
Composition of control soil:	LUFA 2.2 soil + deionized water + chemical

Duration of the experiment

Acclimat. time:	None
Exposure time:	2 weeks
Recovery period:	None

Exposure concentrations

Replicates:	4 per treatment
Concentrations:	5 in geometric series

Exposure responses

Effect parameter:	Survival of original test animals per replicate; Number of extractable off-spring
Measurement of variables:	Adult survival and reproduction du-ring incubation; water content after 2 weeks
Toxic endpoint:	NOEC, LOEC, EC_{10}, EC_{50}
Unit:	mg/kg dry soil
Working methods:	Enumeration of extracted adults and juveniles
Analytical methods:	-

Miscellaneous information needed for application of criteria

Practicability:	The species are easily cultured from populations obtained from the field. All steps in the test procedure can be performed by technicians. A MacFadyen high gradient extraction or similar apparatus must be available.
Cost-effectiveness:	The work that must be done before during and after the performance of the experiments takes 1 week and the amount of material, energy and test organisms that is required is normal.
Sensitivity:	Sensivity comparble to *F. candida* and *F. fimetaria*
Reproducibility:	Work in progress
Validity:	A) Determination NOEC and LOEC by ANOVA, and estimation of EC_{10} and EC_{50} by curve fitting B) Work in progress
Standardization:	Work in progress
Ecological realism:	The species is widely distributed in european soil ecosystems.
Broad chemical responsiveness:	The test system is in principle able to test any chemical or mixtures of chemicals that can be incorporated into soil.

Participant No. 1

Test protocol no. 1.3:

Single species reproductive test with *Hypoaspis aculeifer* (Acari: Gamasida)

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

<u>Test Organism</u>

Taxonomic group:	Acari: Gamasida
Species:	*Hypoaspis aculeifer*
Life stage:	Adults
Sex:	♂♀
Age/weight/size:	16-19 days
Genetic composition:	Clone?
Source:	Laboratory population

Type of surroundings and ambient climate

<u>Description of surroundings</u>

Type:	Microcosm

<u>Ambient climate</u>

Air temperature (°C):	20±1
Rel. humidity:	95-100%
Light wave length:	not indicated
Light intensity:	100-200 lux
Light regime:	(L:D) 12:12

Type of test system and test conditions

<u>Description of system</u>

Type:	Cylinder
Material:	Perspex, PVC lid
Openess:	Closed
Species/Test system:	Single species
Indv./test system:	15

<u>Type of habitat</u>

Soil:	LUFA 2.2 soil

Habitat characteristics

Soil:	Defaunated and reinoculated agricultural soil
Hydrology:	40-60% of Water Holding Capacity
Food supply:	700-900 prey indv. (per. week)
Feeding rate:	Weekly
Type of food:	Synchroneous 16-19 days old *F. fimetaria*

Exposure characteristics

Exposure route:	Soil
Composition of test soil:	LUFA 2.2 soil + deionized water + chemical
Composition of control soil:	LUFA 2.2 soil + deionized water + chemical

Duration of experiment

Acclimat. time:	None
Exposure time:	3 weeks
Recovery period:	None

Exposure concentrations:

Replicates:	4 per treatment
Concentrations:	5 in geometric series

Exposure responses

Effect parameter:	Survival of original test animals per replicate; Number of extractable offspring
Measurement of variables:	Adult survival and reproduction during incubation; water content after 2 weeks
Toxic endpoint:	NOEC, LOEC, EC_{10}, EC_{50}
Unit:	mg/kg dry soil
Working methods:	Enumeration of extracted adults and juveniles
Analytical methods:	-

Miscellaneous information needed for application of criteria

Practicability:	The species are easily cultured from populations obtained from the field. All steps in the test procedure can be performed by technicians. A MacFadyen high gradient extraction or similar apparatus must be available.
Cost-effectiveness:	The work that must be done before during and after the performance of the experiments takes 1 week and the amount of material, energy and test organisms that is required is normal.
Sensitivity:	EC_{50} (reproduction) for dimethoate is 0.9 mg/kg
Reproducibility:	Work in progress
Validity:	A) Determination NOEC and LOEC by ANOVA, and estimation of EC_{10} and EC_{50} by curve fitting B) Work in progress
Standardization:	Work in progress
Ecological realism:	The species is widely distributed in european soil ecosystems.
Broad chemical responsiveness:	The test system is in principle able to test any chemical that can be incor porated into soil.

Participant No. 1

Test protocol no. 1.4:

Predator-prey test with *Hypoaspis aculeifer* (Acari: Gamarida) and *Folsomia fimetaria* (Acari: Collembola)

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

Test organism

Taxonomic group:	Acari/Collembola
Species:	*Hypoaspis aculeifer* + *Folsomia fimetaria*
Life stage:	Adults
Sex:	♂♀
Age/weight/size:	16-19 days/16-19 days
Genetic composition:	Sexual populations
Source:	Laboratory reared populations

Type of surroundings and ambient climate

Description of surroundings

Type:	Microcosm

Ambient climate

Temperature (°C):	20±1
Rel. humidity:	high
Light wave length:	not indicated
Light intensity:	low
Light regime:	(L:D) 12:12

Type of test system and test conditions

Description of test system

Type:	Cylinder
Material:	Perspex, PVC lid
Openess:	Discontinously opened for gaseous exchange (Opened after 2 weeks)
Species/Test system:	Two species
Indv./test system:	15 *H. aculeifer*/100 *F. fimetaria*

Type of habitat

Soil:	LUFA 2.2 soil

Habitat characteristics

Soil:	Defaunated and reinoculated agricultural soil
Hydrology:	40-60% of Water Holding Capacity
Food supply:	100 at day 0/Surplus yeast
Feeding rate:	Once at day 0/every fortnight
Type of food:	Granulated baker's yeast

Exposure characteristics

Exposure route:	Soil + food chain
Composition of test soil:	LUFA 2.2 soil + deionized water + chemical
Composition of control soil:	LUFA 2.2 soil + deionized water + chemical

Duration of the experiment

Acclimat. time:	None
Exposure time:	3 weeks
Recovery period:	None

Exposure concentrations

Replicates:	4 per treatment
Concentrations:	5 in geometric series

Exposure responses

Effect parameter:	Survival of original test animals per replicate; Number of extractable offspring
Measurement of variables:	Adult survival and reproduction during incubation; water content after 2 weeks
Toxic endpoint:	NOEC, LOEC, EC_{10}, EC_{50}
Unit:	mg/kg dry soil
Working methods:	Enumeration of extracted adults and juveniles
Analytical methods:	-

Miscellaneous information needed for application of criteria

Practicability:	The species are easily cultured from populations obtained from the field.

	All steps in the test procedure can be performed by technicians. A MacFadyen high gradient extraction or similar apparatus must be available.
Cost-effectiveness:	The work that must be done before during and after the performance of the experiments takes 1 week and the amount of material, energy and test organisms that is required is normal.
Sensitivity:	Both species are sensitive to dimethoate at slightly different concentration levels.
Reproducibility:	Work in progress
Validity:	A) Determination NOEC and LOEC by ANOVA, and estimation of EC_{10} and EC_{50} by curve fitting B) Work in progress
Standardization:	Work in progress
Ecological realism:	The species are widely distributed in european soil ecosystems. The test system will include an additional exposure route namely from prey to predator. This is a highly realistic route of exposure for predators. By having two interacting species the interaction in forms of predation can be developed as a more realistic endpoint, compared to artificially isolated single-species systems.
Broad chemical responsiveness:	The test systems are able to test any chemical that can be incorporated into soil.

Participant No. 2

Test protocol no. 2:

Toxicity of dimethoate to staphylinid larvae

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

Test organism

Taxonomic group:	Staphylinidae
Species:	*Philonthus fuscipennis* Mannh. (*P. cognatus* Steph.)
Life stage:	larvae LI
Sex:	not determinable for larvae
Age/weight/size:	1-2 days old
Gen. composition:	no information
Source:	parental generation from natural population region Braunschweig (Germany), first generation in laboratory

Type of surroundings and ambient climate

Description of surroundings

Type:	climatic chamber

Ambient climate

Air temperature:	15°C
Relative humidity:	85-100%
Light wave length:	to be determined
Light intensity:	to be measured
Light regime:	complete darkness

Type of test system and test conditions

Description of test system

Type:	19 ml glasstubes (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 (Charge no.: F 21593)

Habitat characteristics

Soil/litter charact.:	see analysis data (section 6.2); dried soil was stored at 5°C.
Hydrology:	50% WHC
Food supply:	during the complete test
Feeding rate:	continously 3 times a week during the first week, 2 times a week during the following weeks
Type of food:	1/2 *Calliphora*-pupae at each feeding data

Exposure characteristics

Exposure route:	by contaminated soil
Comp. of test hab.:	1 week before the test begins the soil was moistened with dist. water up to 20% water holding capacity. 1 day before the larvae were put into the soil the chemical with an amount of water, corresponding to 10-20% of the WHC, was mixed for 30 min. into the soil. Afterwards the soil was wetted up to 50% WHC and stored at 5°C. At the day the test started soil amounts corresponding to 20 g dried soil were filled into 19 ml glasstubes that were also used for culturing the beetles.
Composition of control habitat:	same procedure without chemical (see above)

Duration of the experiment

Acclimat. time:	no acclimatisation time
Exposure time:	the complete time of larval develompement (mean at 15°C: 63 days)
Recovery period:	adults were kept in uncontaminated habitat

Exposure concentration

Replicates:	20 per concentration and control
Concentrations: (control excluded)	5, minimum 100 µg/kg, maximum 5 mg/kg dried soil
Series:	1
Ref. chemical:	none available

Exposure response:

Effect parameters:	survival, affected animals (legs or mandibles trembling, laying on the back, uncoordinated movement, dead) sublethal effects, (growth, weight of hatching beetles, development time, sex, later reproduction)
Frequency of measurements:	observations after 1, 2, 3, 4, 5, 7, 10, 14, 21, 28 days and weekly thereafter
Toxic endpoint:	LD_{50}, EC_{50}
Unit:	mg a.i./kg dry soil
Working methods:	counting survivals, measurement of weight, determination of sex
Analytic methods:	no

Miscellaneous information needed for application of criteria

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 fuscipennis* Mannh.
Cost-effectiveness:	time for culturing the beetles, no expensive material
Sensitivity:	not known
Reproducibility:	not known
Validity of the test:	mortality of the control should not be higher than 30%
Standardization:	at the moment the test itself is in progress. It is easy to standardize expect of test animals and soil.
Ecological realism:	the test method mimics quite well the natural habitat
Broad chemical responsiveness:	not known

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Participant No. 3

Test protocol no. 3.1:

Reproduction toxicity test with the earthworm *Eisenia fetida* (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:	(work in progress)
Sex:	hermaphrodite
Age/weight/size:	adult age: min. 2 months, max. 12 months
Weight:	250-600 mg
Juvenile:	work in progress
Gen. composition:	natural population
Source:	laboratory-bred

Type of surroundings and ambient climate

Description of surroundings

Type:	climate chamber

Ambient climate

Air temperature:	20°C
Relative humidity:	≥ 80%
Light wave length:	light from fluorescent lamps
Light intensity:	400-800 lux
Light regime:	light dark cycle 8:16 - 16:8 hours

Type of test system and test conditions

Description of test system

Type:	rectangular container (1000 cm^3)
Material:	plastic
Openess:	continously open for gas exchange
Spec./test system:	single species test system
Ind./test system:	10

Type of habitat

Soil/litter:	artificial soil, homogeneously mixed
Medium:	artificial culture medium

Habitat characteristics

Litter/soil charact.:	artificial soil (OECD-guideline No. 207): ca. 8% O.M. content, pH (KCl) 6.0-6.5, CEC 10.8 mval/100g LUFA Speyer 2.2: ca. 4% O.M. content, pH (KCl) 6.0-6.5, CEC 9.7 mval/100 g
Hydrology:	art. soil: 50 % of water holding capacity, water content: 30-35% of dry weight LUFA Speyer 2.2: work in progress
Food or substrate supply:	during breeding, acclimatization and test
Feeding rate:	0.5 g/worm/week (work in progress)
Type of food:	finely ground dried cattle manure

Exposure characteristics

Exposure route:	by soil habitat
Composition of test habitat:	aqueous solution (in deionized water) of test chemical homogeneously mixed in soil substrate
Composition of control habitat:	same amount of deionized water

Duration of the experiment

Acclimat. time:	1 d
Exposure time:	28 d
Recovery period:	-

Exposure concentrations

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

Exposure responses

Effect parameters:	survival, feeding rate, weight development, cocoon production, hatching rate

Other parameters:	general behaviour (burrowing, repellent effects) measurement of number of individuals, semi-quantitative estimation of feeding rate
Variables:	fresh weight, number of cocoons, number of juveniles, number of infertile cocoons
Toxic endpoint:	A) EC_{50} B) based on nominal concentration
Unit:	mg test substance per kg dry weight test substrate
Working methods:	-
Analytical methods:	-

Miscellaneous information needed for application of criteria

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 the technical and academic personnel without the necessity to follow specific courses.
Cost-effectiveness:	the amount of material, energy, test organisms and time to prepare and conduct the test are normal.
Sensitivity:	work in progress
Reproducibility:	work in progress
Validity of the test:	work in progress
Standardization:	work in progress
Ecological realism:	occurence 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.

Participant No. 3

Test protocol no. 3.2:

Reproduction toxicity test with the earthworm *Aporrectodea caliginosa* and *Allolobophora chlorotica*

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

Test organism

Taxonomic group:	Earthworms (Annelida: Oligochaeta)
Species:	*Aporrectodea caliginosa* or *Allolobophora chlorotica*
Life stage:	juvenile or adult (work in progress)
Sex:	hermaphrodite
Age/weight/size:	work in progress
Gen. composition:	natural population
Source:	laboratory-bred (animals for starting culture sampled on not contaminated sites)

Type of surroundings and ambient climate

Description of surroundings

Type:	climate chamber

Ambient climate

Air temperature:	10°C (work in progress)
Relative humidity:	≥ 80%
Light wave length:	darkness
Light intensity:	-
Light regime:	-

Type of test system and test conditions

Description of test system

Type:	rectangular container (1000 cm^3)
Material:	plastic
Openess:	continously open for gas exchange
Spec./test system:	single species test system
Ind./test system:	work in progress

Type of habitat

Soil/litter:	artificial soil, homogeneously mixed
Medium:	artificial culture medium

Habitat characteristics

Litter/soil charact.:	Artificial soil (OECD-guideline No. 207): ca. 8 O.M. content, pH (KCl) 6.0-6.5, CEC 10.8 mval/100 g LUFA Speyer 2.2: ca. 4% O.M. content, pH (KCl) 6.0-6.5, CEC 9.7 mval/100 g
Hydrology:	art. soil: 50% of water holding capacity, water content: 30-35% of dry weight LUFA Speyer 2.2: work in progress
Food or substrate supply:	during breeding, 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:	by soil habitat
Composition of test habitat:	aqueous solution (in deionized water) of test chemical homogeneously mixed in soil substrate
Composition of control habitat:	same amount of deionized water

Duration of experiment

Acclimat. time:	1 d
Exposure time:	28 d
Recovery period:	-

Exposure concentrations

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

Exposure responses

Effect parameters:	survival, feeding rate, weight development, cocoon production, hatching rate

Other parameters:	general behaviour (burrowing, repellent reaction), measurement of number of individuals, semiquantitative estimation of feeding rate, fresh weight, number of cocoons, number of juveniles, number of infertile cocoons
Toxic endpoint:	A) EC_{50} B) based on nominal concentration
Unit:	mg test substance per kg dry weight test substrate
Working methods:	-
Analytical methods:	-

Miscellaneous information needed for application of criteria

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 the technical and academic personnel without the necessity to follow specific courses.
Cost-effectiveness:	the time to prepare and conduct the test takes about 10 weeks. The amount of material, energy, test organisms and time to prepare and conduct the test are large.
Sensitivity:	work in progress
Reproducibility:	work in progress
Validity of the test:	work in progress
Standardization:	work in progress
Ecological realism:	*A. caliginosa* is a common earthworm species of great importance especially for agro-ecosystems.
Broad chemical responsiveness:	no restrictions are given with respect to the chemical substances which may be evaluated by this test.

Participant No. 4

Test protocol no. 4:

Development of Sublethal Test Methods for *Platynothrus peltifer* (Acari: Oribatida)

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

Test organism

Taxonomic group:	Oribatidae
Species:	*Platynothrus peltifer*
Life stage:	adult
Sex:	parthenogenetic
Age/weight/size:	about 1 mm
Genetical comp.:	?
Source:	cultured in the laboratory

Type of surroundings and ambient climate

Description of surroundings

Type:	15 cm plastic culture boxes with a bottom of plaster of Paris

Ambient climate

Air temperature:	20°C (the suitability of a temperature of 15°C needs further investigation)
Relative humidity:	75%
Light wave length:	no specification needed; normal light is suitable
Light intensity:	400-800 lux
Light regime:	light dark cycle 12:12 hours

Type of test system and test conditions

Description of test system

Type:	test container (diameter 5 cm, height 3 cm)
Material:	perspex ring, with on the bottom a gauze (1 mm mesh)
Openness:	lid with an opening covered with cheese cloth
Spec./test system:	single species test system

Ind./test system:	10 (starting point; work in progress on the desirability of using higher numbers)

Type of habitat

Soil/litter:	OECD artificial soil, made up with sieved moist peat

Habitat characteristics

Soil characteristics:	OECD artificial soil consists of sphagnum peat (9%), kaolin clay (20%), sand (70%), green algae (1%; *Pleurococcus*) and $CaCO_3$ to adjust the soil pH. Work in progress on the preferred pH of the soil, also work in progress on the influence of soil characteristics.
Hydrology:	moisture content: 30-40% of water holding capacity
Food supply:	yes, algae mixed in with the soil (see above)
Feeding rate:	not applicable
Type of food:	green algae, *Pleurococcus*, sampled in the field from tree bark

Exposure characteristics

Exposure route:	soil
Composition of test soil:	artificial soil with deionized water, test substance and auxiliary agent (if used), all constituents well homogenized
Composition of control soil:	artificial soil and deionized water (including auxiliary agent, if applicable)

Duration of the experiment

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

Exposure concentrations

Replicates:	work in progress
Concentrations:	5 and a control
Series:	?
Ref. chemical:	?

Exposure responses

Effect parameter:	reproduction (number of offsprings produced)
Other parameters:	survival of adults; growth
Measurement of variables:	moisture content and pH of the soil at start and end of the test
Toxic endpoint:	NOEC and EC50 for reproduction; LC50
Unit:	mg/kg dry soil
Working methods:	use Tullgren apparatus for extraction of mites from soil at the end of test
Analytical methods:	not relevant

Miscellaneous information needed for application of criteria

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, the best way to obtain animals is by extraction from leaf or needle litter sampled in the field (deciduous forest, mainly oak and coniferous forest, mainly *Pinus* spp.). Work on a culture method is in progress. Identification of the mites, and especially of the different juvenile stages requires some training.
Cost-effectiveness:	as it looks now, both the culture and the toxicity test will be rather laborious.
Sensitivity:	from feeding studies, *P. peltifer* appeared to be sensitive to heavy metals compared to other soil arthropods. Due to its slow reproduction rate and long life cycle, even minor effects on reproduction may have great impact at the population level. This point needs, however, further research, and hopefully results of SECOFASE will allow for conclusions about the relati-

	ve sensitivity of *P. peltifer* compared to other soil invertebrates upon exposure in (artificial) soil.
Reproducibility:	needs to be investigated
Validity of the test:	work in progress
Standardization:	work in progress
Ecological realism:	*P. peltifer* is mainly living in forest soils, and can be considered representative of the group of oribatid mites. It is the first oribatid mite species with which ecotoxicological research has been done. It has a long life cycle, and is from that point of view a realistic choice. In the test, soil moisture characteristics (and maybe also pH) will be adapted to optima for *P. peltifer*.
Broad chemical responsiveness:	the test should be applicable to all chemical substances, maybe with exclusion of the very volatile ones.

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Participant No. 5

Test protocol no. 5:

Effects of soil pollutants 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)
Species:	*Plectus acuminatus* (Bastian, 1865) *Heterocephalobus pauciannulatus*
Life stage:	adult
Sex:	only female, parthenogenetic
Age/weight/size:	*P. acuminatus* 20 days, *H. pauciannulatus* 8 days
Gen. composition:	not known
Source:	test specimens obtained from laboratory culture

Type of surroundings and ambient climate

Description of surroundings

Type:	climate chamber

Ambient climate

Air temperature:	20°C
Relative humidity:	?
Light wave length:	not relevant
Light intensity:	0 (darkness)
Light regime:	24 h/day darkness

Type of test system and test conditions

Description of test system

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 (OECD) soil

Habitat characteristics

Soil:	10% peat, 20% kaolin clay, 69% sand, 1% $CaCO_3$ (dry weight)
Hydrology:	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:	possibly by pore water through cuticle
Composition of test soil:	artificial soil with water, test substance
Composition of control soil:	artificial soil 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:	work in progress
Other parameters:	work in progress
Measurement of variables:	work in progress
Toxic endpoint:	EC-value
Unit:	mg test substance per kg dry weight test soil
Working methods:	work in progress
Analytical methods:	not used

Miscellaneous information needed for application of criteria

Practicability:	in progress
Cost-effectiveness:	in progress
Sensitivity:	in progress
Reproducibility:	in progress
Validity of the test:	in progress
Standardisation:	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

References

Kammenga, J.E., van Gestel, C.A.M. & Bakker, J. (in press): Patterns of sensitivity to cadmium and pentachlorophenol among nematode species from different taxonomic and ecological groups. Arch. Environ. Contam. Toxicol.

Participant No. 6.

Test protocol no. 6:

Effects of soil pollutants on Enchytraeidae: determination of the impact on fragmentation, growth and survival in artificial soil

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

Test organism

Taxonomic group:	Enchytraeidae
Species:	*Cognettia sphagnetorum* (Vejdovsky)
Life stage:	adult
Sex:	-
Size:	> 35 segments
Gen. composition:	not known
Source:	sampled in the field

Type of surroundings and ambient climate

Description of surroundings

Type:	climate chamber

Ambient climate

Air temperature:	15°C
Soil moisture:	27% (d.w.)
Light regime:	darkness

Type of test system and test conditions

Description of test system

Type:	vial, 5 ml
Material:	glass
Openess:	closed

Type of habitat

Soil:	soil, homogeneously mixed

Habitat characteristics:

Soil charact:	soil (LUFA 2.2)
Soil per vial:	2.5 ml (the vial should be half-filled)

Hydrol. charact:	wetted with deionized water until 27%
Indiv. per vial:	1
Fragment per vial:	1
Food supply:	The fungus *Mortierella isabellina* (Oudem) added as spore suspension (750,000 cells per ml) 1 wk before the start of the experiment to the soil (100 g) to be used during the experiment.
Feeding rate:	Soil with growing *M. isabellina* is to be changed once a week in connection with control of enchytraeids.
Type of food:	*Mortierella isabellina* (Dash and Cragg, 1972, Augustsson and Hedlund, MS).

Exposure characteristics

Exposure route:	via soil and food
Composition of test soil:	artificial soil with deionized water, test substance, all constituents well homogenized (5 hrs)
Composition of control soil:	artificial soil and deionized water ho mogenized as above

Duration of the experiment

Acclimat. time:	work in progress
Exposure time:	10 weeks
Recovery period:	-

Exposure concentrations

Replicates:	30; each concentration and control
Concentrations:	0, 25, 50, 75, 100, 200, 400 Cu^{2+} mg per kg soil
Series:	1

Exposure responses

Effect parameters:	reproduction: average fragmentation rate and number of fragments per day
Other parameters:	growth of adults and fragments: average of increase of segments over 10 weeks. Survival: number of adults and fragments surviving over 10 weeks
Toxic endpoints:	a) NOEC b) LOEC

Unit: mg test substance per kg dry weight test soil

Working methods: extraction of individuals/fragments by flushing soil into Petri dish; determining the number of fragments and the number of segments per fragment

Miscellaneous information needed for application of criteria

Practicability: required material can easily be commercially obtained; the test organism is commonly found (Abrahamsen, 1972, Lundkvist, 1981) and easily extracted (O'Connor, 1967) from litter/-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 will be available in 1994.

Sensitivity: Data not available

Reproducibility: Data not available

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). The soil is artificial and food has to be supplied. It is not yet known how *C. spagnetorum* differs from other enchytraeid species with respect to ecological requirements. In its reproduction mode (being asexual) it differs pronouncedly from other enchytraeids. C. sphagnetorum inhabits humus rich soil (Standen, 1973).

References

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

Augustsson, A. & Hedlund, K., MS. Low levels of enchytraeid grazing can increase fungal growth and respiration.

Dash, M.C. & Cragg, J.B. (1972): Selection of microfungi by Enchytraeidae (Oligochaeta) and other members of the soil fauna. Pedobiologia **12**: 282-286.

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. Pol. **31**: 9-56.

O'Connor, F.B. (1967): The Enchytraeidae. In: Soil Biology, A Burges, 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. Animal Ecol. **42**: 219-245.

Participant No. 7

Test protocol no. 7.1:

Effects of soil pollutants on Collembola reproduction

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

Test organism

Taxonomic group:	Springtails (Collembola)
Species:	*Folsomia candida* (Willem) (Isotomidae)
Life stage:	Juvenile
Sex:	Probably only female (parthenogenetic)
Age/weight/size:	10-12 days old; mean weight/indiv. approx. 77 µg (SE ± 16 µg)
Source:	Laboratory culture

Type of surroundings and ambient climate

Description of surroundings

Surroundings:	Completely enclosed

Ambient climate

Air temperature:	15 ± 0.2°C
Relative humidity:	70-90%
Light intensity:	11-70 lux (depending upon the position in the incubator). Vessels placed on shelves in random order and rotated at approx. 7 day intervals.
Light regime:	Ambient

Type of test system and test conditions

Test system

Type:	120 ml jar with screw top
Material:	Glass
Aeration:	Approx. every 7th day
Species/test system:	Single
Indv./test system:	10

Type of habitat

Soil/Litter:	Artificial or natural soil (e.g. LUFA 2.2), homogeneously mixed

Habitat characteristics

Soil charact.:	Artificial soil - consisting of 10% sphagnum peat, 20% kaolin clay, 1% $CaCO_3$ and 69% industrial quartz sand (when expressed as dry weight)
	Natural soil - LUFA Speyer soil 2.2
Hydrology:	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 after 14 days

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. 100 g/kg dry weight of test soil)

Duration of experiment

Acclim. time:	2-5 days (if collembola transferred from different culturing temp.)
Exposure time:	28 days
Recovery period:	none

Exposure concentrations:

Replicates:	4 for each concentration and a control
Concentrations:	Usually 5 in a geometric series (e.g. $\sqrt{10}$); May be (excluding controls)necessary to repeat with a definitive range of doses (maximum recommended concentration 1000 mg/kg dry weight)
Ref. chemical:	None currently proposed

Exposure responses

Effect parameters:	Reproduction: a) average number of juveniles (alive) per test vessel after 28 days; b) average number of live juveniles/live adults per test vessel after 28 days
Other parameters:	Survival: average number of adults (alive) after 28 days
Measurement of variables:	a) Numbers of live juveniles and adults b) Water content of test soil before and after the test c) Measurement of pH (H_2O or KCL) before and after the test
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 per kg dry weight (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:	Not currently used

Miscellaneous information needed for application of criteria

Practicability:	The required apparatus (e.g. incubators, insect culturing facilities and laboratory mixers) are easily obtainable. The test protocol can be perfor-

	med 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). Little information is available for other chemicals and test invertebrates.
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 the test: (From ISO guidelines test at 20°C)	a) statistical methods (analysis of ho mogeneity, U-test of Mann & Whitney b) parental mortality within the control group should be less than 10 per cent c) parental mortality within the treatment groups should not be significantly higher than parental mortality within the control group d) reproduction rate per control vessel should reach a minimum of 100 juveniles NB. All of these criteria may not be appropriate for tests at 15°C.
Standardization:	At the moment the test protocol is described as a working draft by the ISO, and recently an international ring test has been performed (Riepert, 1992 & 1993).

Ecological realism:	*F. candida* is a panthenogenetic, subterranean species of Collembola that is widely distributed in European 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.

Participant No. 7

Test protocol no. 7.2:

Effects of soil pollutants on Collembola growth

The test framework may be similar to that mentioned above for reproduction.

Some of the main differences include:

a) Increased duration of exposure, for example 9-10 weeks for *F. candida* (Crommentuijn *et al.*, 1993) or possibly longer for species with a longer generation time, e.g. *I. viridis*, to determine effects on adult and juvenile growth

b) Increased replication to allow for periodic measurements of growth parameters, such as body length or body weights. At the same time measurements of soil characteristics, e.g. pH and soil moisture, should be made

c) Analytical techniques may be required to measure chemical body burdens and chemical concentrations in the soil at each sampling period

References

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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.

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