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Preface

The present investigation was funded by the Danish National Pesticide Research Programme and was conducted in cooperation between the following institutions and scientists:

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Summary

Aims

The project "Effects of Pesticides on Soil Meso- and Microfauna" was started in 1990 and finished by the end of 1992. The objectives were to develop and if possible intercalibrate laboratory methods for measuring sublethal effects or effects disturbing the reproduction of species representative of soil meso- and microfauna. Concurrently, it was the aim to develop a semi-field method for selected species (Collembola and mites). The uncertainty at extrapolation from laboratory to field conditions was to be studied, partly by comparing the laboratory test methods to the semi-field experiment, partly by applying statistical methods on results from the "battery" of laboratory test methods.

Test organisms and test compounds

The project has focussed on developing test methods for single species of protozoans, Collembola and mites, and on developing semi-field methods for validation of the laboratory tests. As far as possible, identical organisms and methods have been applied by the participating institutions. The project has been carried out, using common test substances, i.e. the insecticides dimethoate and pirimicarb, and the fungicide fenpropimorph, from the same batches of the trade products "Dimethoate 28" from DLG, "Pirimor" from ICI and "Corbel" from Schering Ltd., respectively.

Tests with Protozoa

A soil microcosm test deals with the non-specific functional impact of pesticides on soil protozoa at the community level. This test has the advantage of integrating the pesticide effects on survival and growth of protozoa in soil under realistic conditions. The disadvantage on the other hand is the difficulties in standardizing the test, mainly because identification of the soil protozoa is difficult. Of the three pesticides tested dimethoate and fenpropimorph delayed soil respiration as well as protozoan multiplication in presterilized soil. Pirimicarb showed no such effect.

The single species test is easier to interpret than the soil community test but conversely the results are difficult to translate into soil effects. Single culture tests performed with the flagellate *Cercomonas sp.* the amoebae *Acanthamoeba sp.* and the ciliate *Colpoda sp.* revealed that *Cercomonas sp.* was the most suitable test organism because of ease of identification and counting, relatively high sensitivity and ease of interpreting dose-response curves. *Cercomonas sp.* showed EC_{10} and EC_{50} similar to *Acanthamoeba* and *Colpoda* for dimethoate, but 10-30 times lower EC_{50} values than *Acanthamoeba* and *Colpoda* for pirimicarb and fenpropimorph. Current work is in progress on the diversity of soil protozoan community as dependent on soil conditions *in situ* which will improve the choice of

organisms for single species tests and thereby these tests can be further refined.

The ideal evaluation of pesticide effects on soil protozoa will be obtained by combination of an easily interpretable and reproducible single species test with a more realistic soil microcosm test. Thereby a more comprehensive understanding of pesticide effects on soil protozoa is obtained than by conducting twice as many single species tests or soil microcosm tests.

Test with Enchytreids

A seven days test for sublethal effects was developed for *Enchytraeus bigeminus*. The method is based on the formation of new segments in an active growth phase and it does not involve manipulation of the test organisms during this period which is the case in similar test methods. The test showed that the fungicide fenpropimorph was found to have the highest toxicity. Among the two tested insectides, dimethoate was the more toxic compared with pirimicarb. At its present stage the method needs further standardization, especially the number of worms and the amount of food supplied should be better balanced. The method may be improved by the use of digital image processing for measuring the length and size of the test animals.

Tests with Collembola

The reproduction of two closely related soil microarthropods, *Folsomia candida* Willem and *Folsomia fimetaria* L. (Insecta: Collembola), was tested under the influence of the insecticides dimethoate and pirimicarb and the fungicide fenpropimorph. Only dimethoate had an adverse effect on survival of adults and their reproduction in concentrations not exceeding five times the recommended field dose. The experimental conditions were altered to evaluate the realism in the basic single species/single chemical reproductive test system. Combinations of three pesticides and yeast either present or absent in three different soil types pointed to soil type and food availability as crucial for reproduction and for the expression of pesticide effects.

The importance of the spatial distribution of dimethoate was studied with food applied to the surface (original procedure), mixed homogeneously in the whole soil profile or just in the top-layer or mixed heterogeneously into the soil preserving the small granula of yeast originally in the commercial formulation. Toxicity decreased significantly when exposure could be avoided in an uncontaminated bottom layer and even more if food was available in this soil horizon.

The study suggests some important recommandations in ecotoxicological studies with Collembola: 1) To simulate environmental conditions in Denmark, a soil with a low content of organic matter should be preferred rather than the OECD soil; 2) Application of the test chemical should

result in realistic heterogeneous spatial distribution; 3) The species *F. fimetaria* is recommended as test organism; 4) Food should be mixed heterogeneously with the soil at a sub-optimal level.

Tests with predatory mite

A single species reproductive test system with the predaceous mite *Hypoaspis aculeifer* Canestrini (Gamasida: Laelapidae) is presented. This mite breeds successfully on the prey organisms *Folsomia fimetaria* L. or *Folsomia candida* Willem (Collembola). Trials testing fenpropimorph and pirimicarb showed no negative effects on the reproductive output of the mite up to the maximum dosage of approximately five times the recommended field dosage. Dimethoate caused a stimulation at 0.4 mg/kg (LOEC) and a reduction at 1.3 mg $\cdot$ kg^{-1} corresponding to 3 times the recommended field dosage (LOAEC), and EC_{50} was found to be in the range of 0.8-1.0 mg/kg.

The study shows that the following points may be of importance when performing toxicity testing with Gamasida: 1) Stimulations of the reproductive output of the mite at low doses of pesticide below and around field rates; 2) An uneven distribution of pesticide due to surface application compared to a homogeneous distribution in the test soil.

Microbial respiration

CO_2 was used as a measure of net respiration in samples collected at the field site as well as in a 2^5 factorial laboratory experiment with dimethoate, fenpropimorph, pirimicarb, two soil types and +/- addition of yeast. The 2^5 factorial experiment included Collembola of which the reproduction was estimated. Furthermore, CO_2 evolution was compared to a Cotton Strip Assay, an alternative method of estimating soil microbial activity.

Neither of the three pesticides were found to have any significant effect on respiration in either of the two types of experiments. In the factorial study, addition of yeast significantly raised respiration, and respiration was generally higher in the natural loamy soil than in OECD standard soil, probably due to the addition of inoculate in the former. Fenpropimorph was found to decrease degradation of cotton strips significantly at the highest dose, which indicates that this method may be more sensitive for detecting chemical effects on microorganisms than CO_2 production. The reproduction of the Collembola in the factorial experiment corresponded well with that found in the collembolan test described above.

Semi-field test

A semi-field method was developed for testing to which extent extrapolation of ecotoxicological laboratory tests to field conditions is valid. The test was specifically addressed at side-effects of agricultural pesticides on the microarthropod component of the soil fauna. The method was tested with populations of a single species of *Collembola*, mixed populations of two collembolan species or simplified microarthropod communities includ-

ing one or two collembolan species together with a predator belonging to the mite order *Gamasida*.

A methodology based on field microcosms/enclosures containing defaunated field soil was chosen in order to make registration of reproduction and population development possible and reduce variability between sample units in comparison with an unconfined field population.

Seven different types or variants of field microcosms were tested in order to find a method which combine an efficient confinement of the test population with a high degree of similarity between the environment inside the microcosm and that of the unmanipulated field soil.

A cylindrical microcosm type closed in the bottom with 45 micron mesh polyester web and open top end surrounded by a "collar-trap" proved to be highly efficient for isolating the test populations of eu- and hemiedaphic species. Moisture content and volume weight of the soil inside microcosms was a little higher than the surrounding soil while the soil temperature was similar inside and outside the microcosms. A high version of this type of microcosm was used as standard method in 1992. A low "closed" microcosm buried below 2 cm soil was used as standard in 1991. The soil in the high "open" microcosms was divided into two 10 cm strata at extraction.

A relatively gentle defaunation method using alternately freezing, heating, drying and rewetting proved to eliminate the microarthropods efficiently. A filtered soil suspension was added in order to facilitate the recovery of the soil microflora.

The collembolan *Folsomia fimetaria* and the gamasid mite *Hypoaspis aculeifer* were chosen as principal test species but in 1991 the collembolan *Isotoma notabilis* was also tested. The gamasids used in 1991 were not identified. The choices were based on experience with distribution and behaviour in the soil, generation time, ease of culturing and success of population development in microcosms.

Twenty to thirty collembolan specimens of equal size/age were added to each microcosm in the laboratory. 5 specimens of gamasid mites were further added to a selection of the microcosms.

Microcosms were placed in the experimental field according to a randomized block's design with 10 replications. Treatments included control, dimethoate (recommended dosage for agricultural use), dimethoate (5 times recommended dosage), pirimicarb (5 times recommended dosage), and fenpropimorph (5 times recommended dosage).

Spraying was done by means of an experimental boom sprayer. Measurement of deposition of pesticides showed significant differences between treatments and between squares within treatments. The deposition was lower inside than outside the projecting top end of the "open" type microcosms.

No effect of pesticide treatment was observed 3 or 14 days after spraying. This indicates that these pesticides do not have acute toxic effects on the tested soil microarthropods when sprayed on the soil surface. However, negative effects on the test species appeared later and were observed from 5 - 6 weeks (38 - 39 days) until 10 weeks (70 days) after treatment:

Microcosms sprayed with 5 times recommended dosage of dimethoate contained significantly lower numbers of *F. fimetaria* and *I. notabilis* than untreated control microcosms at all 3 sampling dates between 5 and 10 weeks after treatment. The same treatment caused significantly lower numbers of non-identified gamasids compared to the control microcosms 10 weeks after spraying in 1991. No effect was observed on *H. aculeifer* (measured 10 weeks after spraying, 1992).

The lower (recommended) dosage of dimethoate had a similar but weaker effect which was statistically significant at the 3 sampling dates between 5 and 10 weeks after spraying for *F. fimetaria* and at the first sampling date for *I. notabilis* (39 days after spraying).

Effects of pirimicarb and fenpropimorph on the test species are less convincingly documented by the present study than the effects of dimethoate. However, negative effect of both pesticides on *F. fimetaria* was suggested at a few sampling occasions and a positive, i.e. stimulating, effect of fenpropimorph on *I. notabilis* was suggested at a single sampling date.

The short persistence of dimethoate (half-life: 4.8 - 9.7 days at 20° C) makes it plausible that the effects recorded 5 - 10 weeks after treatment are delayed indirect effects due to reduced reproduction of the originally introduced test animals and the first generation hatched in the microcosms.

The high "open" type microcosms which were subdivided vertically into two 10 cm horizons during extraction made a comparison between the surface and the deeper soil layer possible. The pesticide effects observed were mostly restricted to the surface horizon but at one sampling date in each year the population size in the deep horizon was significantly smaller in microcosms treated with the high dosage dimethoate than in control microcosms.

The majority of the *F. fimetaria* test population had moved down to the 10 - 20 cm horizon 2 weeks after treatment in 1992. At later sampling dates the general vertical distribution had changed so that most of the population was in the upper 10 cm horizon. These vertical changes were the same irrespective of treatment except for dimethoate treated microcosms recovered from the field 5 - 6 weeks after spraying. In these microcosms the number of specimens was about equal in the two horizons thus indicating a deeper distribution than in the other treatments. This could be the result of a repellent effect or higher rates of mortality or immobilization in the upper than in the lower soil horizon.

No significant changes of the pesticide effect on *F. fimetaria* were observed when another collembolan species *I. notabilis* was added. However, the EC_{50} values for the dimethoate dosage effect on *F. fimetaria* were higher for microcosms with *I. notabilis* than without this additional species. Addition of gamasids appeared to decrease the population size of *F. fimetaria* in all treatments. Presence of gamasids did not alter the effect of pesticides on *F. fimetaria.*

EC_{50} values for dimethoate dosage effect on *F. fimetaria* calculated on the assumption of a decreasing exponential relationship were lower than the recommended dosage for agricultural use in Denmark 5 weeks after treatment in 1991 and 8 - 10 weeks after treatment in 1992.

A proposal for a test-procedure using the semi-field technique is outlined. It is recommended to use high or low "open" type microcosms which are sprayed in the field and extracted 2 weeks and 6 - 10 weeks after treatment in order to test for acute and long-term effects, respectively.

Extrapolation from laboratory to field

Results from laboratory tests and semi-field tests were compared for all three compounds. The single-species tests in the laboratory with fenpropimorph and pirimicarb showed no effects. However, in the semi-field situation significant effects were observed at single sampling dates. Laboratory results with dimethoate indicated that effects could be expected in the field. In this case the two different methods proved to be in agreement: a significant reduction was observed in the semi-field test five to ten weeks after treatment. In general, the semi-field test was more sensitive compared with the laboratory tests.

The Wagner & Løkke extrapolation method

The concentrations of dimethoate affecting less than five percent of the species in the soil system with a probability of 95% were calculated using the statistical extrapolation method of Wagner and Løkke. EC_{10} for decrease in reproduction was chosen as effect parameter and log-normal distribution of the data is assumed.

Statistical extrapolation

The dataset obtained in the present study was poorly fitted to the log-normal distribution. However, to demonstrate the procedure the calculations were accomplished.

The statistical extrapolation of single species laboratory data to the field was done by varying the leaching depth of dimethoate from 1 to 10 cm. Further, the fraction of dimethoate which was deposited on the soil surface was varied from 10 to 100%. If the deposition on the soil was 100%, and the leaching depth was 1 cm, the "safe dosage" for 95% of the soil dwelling species was estimated with 95% confidence to be 0.01% of the recommended field dosage at 280 g/ha. By changing the deposition to 10% of the field dosage, and the leaching depth to 10 cm, the "safe dosage" was 1.25% of the recommended field dosage. At 10% deposition, and 5 cm leaching, the "safe dosage" was estimated to be 0.61% of the recommended field dosage. The values should be regarded as premature due to the lack of proper log-normality of the data. By analysing a larger dataset, Løkke et al. (1994) estimated a "safe dosage" of dimethoate at 0.35 g/ha corresponding to 0.125% of the recommended dosage assuming for soil-dwelling species that 10% of the spray hitted the soil, and that the leaching depth was 5 cm. This value is lower than the corresponding estimate from the present study. However, the larger study included species living on the soil and in the vegetation where they are more exposed to pesticides than the soildwelling organisms.

Dansk sammendrag

Formål

Projektet "Pesticiders effekter på meso- og mikrofaunaen i jorden" blev igangsat i 1990 og forløb til udgangen af 1992. Formålet var at udarbejde og om muligt interkalibrere laboratoriemetoder til bestemmelse af subletale eller reproduktionsforstyrrende effekter hos repræsentative arter for meso- og mikrofaunaen i jorden. Sideløbende skulle udvikles en semifeltmetode for udvalgte arter (collemboler og mider). Usikkerheden ved ekstrapolation fra laboratoriet til felten skulle belyses dels ved sammenligning af laboratoriemetoderne og semifeltforsøget, dels ved anvendelse af statistiske metoder på resultater fra "batteriet" af laboratorietestmetoder.

Testorganismer og teststoffer

Projektet har været centreret om udviklingen af testmetoder for enkeltarter af protozoer, collemboler og mider og om udviklingen af semifeltmetoder til validering af laboratoriemetoderne. Der er så vidt muligt anvendt samme organismer og metoder af de deltagende institutioner i projektet. Projektet er udført med fælles teststoffer, nemlig insekticiderne dimethoat og pirimicarb samt svampemidlet fenpropimorph, idet samme batches af handelspræparaterne "Dimethoat 28" fra DLG, "Pirimor" fra ICI og "Corbel" fra Schering A/S er anvendt.

Protozotests

En jordtest i mikrokosmer viste pesticidernes non-specifikke indflydelse på jordbundsprotozoerne på samfundsniveau. Fordelen ved denne test er, at den viser den samlede pesticideffekt på overlevelse og formering under realistiske jordbetingelser. Testen er imidlertid vanskelig at standardisere på grund af vanskelighederne med at identificere protozoerne til art. Dimethoat og fenpropimorph forsinkede respirationsaktiviteten og protozoernes formering, mens pirimicarb ikke viste en sådan effekt.

Enkeltartstesten er lettere at aflæse, men på den anden side sværere at tolke i relation til jordmiljøet. Enkeltartstest med flagellaten *Cercomonas*, amøben *Acanthamoeba* og ciliaten *Colpoda* viste, at *Cercomonas* var den mest velegnede testorganisme; den var let at identificere og tælle, relativt følsom for pesticider og gav den dosis-respons- kurve, der var lettest at tolke for alle tre pesticider. *Cercomonas* havde de samme EC_{10}- og EC_{50}-værdier som *Acanthamoeba* og *Colpoda* for dimethoat, men derimod 10-30 gange lavere værdier for pirimicarb og fenpropimorph. Igangværende arbejde med jordbundsprotozoernes diversitet viser, hvilke protozoer der dominerer afhængigt af jordmiljøet *in situ*. Enkeltartstestens værdi vil yderligere kunne øges, hvis man inddrager resultaterne af disse undersøgelser i organismevalget til enkeltartstesten.

Den optimale vurdering af pesticideffekter på jordbundsprotozoer fås ved at kombinere en reproducerbar enkeltartstest med en mere realistisk mikro-

kosmostest med jord. Herved fås en mere uddybende viden om pesticideffekterne end ved at udføre det dobbelte antal enkeltarts- eller jordmikrokosmostests.

Enkytrætest

For enkytræen *Enchytraeus bigeminus* er udviklet en 7 dages test for subletalitet. Metoden er baseret på dannelsen af segmenter i den aktive vækstfase. Testen udføres i vandkultur og indebærer ingen omflytninger af testdyrene under testen i modsætning til lignende metoder. Under afprøvningen udviste svampemidlet fenpropimorph den største toksicitet. Af insektmidlerne var dimethoat mere toksisk end pirimicarb. På dens nuværende stade kræves en bedre standardisering, især bør antallet af testdyr afbalanceres bedre med tilførslen af næring. Metoden kan forbedres ved anvendelse af elektronisk billedbehandling til måling af længden og størrelsen af enkeltindivider.

Collemboltest

Med udgangspunkt i en eksisterende protokol for collembolarten *Folsomia candida*, der formerer sig ved parthenogenese, blev der udarbejdet en protokol for collembolarten *Folsomia fimetaria*. Arten, som formerer sig kønnet, er almindeligt forekommende i Danmark og blev udvalgt ved screening af en række arter, idet der blev taget hensyn til muligheden for at etablere en laboratoriekultur med høj produktion af individer og til reproducerbarheden af kontrolforsøg. Som en del af projektet blev udviklet en metode til optælling og måling af dyrenes størrelsesfordeling ved anvendelse af elektronisk billedbehandling (DIP).

Undersøgelserne viste, at dimethoat påvirker de undersøgte collembolarters reproduktion signifikant ved et eksponeringsniveau, som svarer til almindelig landbrugspraksis, medens dette ikke var tilfældet for pirimicarb og fenpropimorph.

Interaktionen mellem de tre afprøvede pesticider, fødeudbuddet og jordtypen (OECD-standardjord, en sand- og en lerholdig jord) blev undersøgt i 2^5-faktorforsøg. Undersøgelsen viste, at fødeudbuddet og jordtypen er af afgørende betydning for testens resultat, medens interaktionen med pesticiderne og deres indbyrdes interaktion var af mindre betydning. En særlig undersøgelse af den rumlige fordeling af pesticidkemikaliet dimethoat i jordprofilen og den samtidige placering af fødeudbuddet viste, at toksiciteten aftog signifikant, når dyrene kunne undgå eksponeringen, og i særlig grad hvis føden befandt sig i jordlag uden indhold af pesticidet.

På grundlag af undersøgelsen kunne det konkluderes, at der i internationale testprotokoller for det første bør anfales jordtyper med lavere indhold af organisk stof end OECD-jorden for bedre at simulere typiske naturlige forhold i Danmark. For det andet bør teststoffet applikeres til jorden ved en metode, som simulerer naturlige forhold. For det tredje bør *Folsomia*

fimetaria vælges som testorganisme, da den er en naturligt forekommende art. For det fjerde bør fødeudbuddet være sub-optimalt og heterogent opblandet med testjorden.

Rovmidetest

Der blev i projektet udarbejdet en testprotokol for den jordboende rovmide *Hypoaspis aculeifer*, som formerer sig kønnet. Denne art kan holdes i kultur ved fodring med en af collembolarterne *Folsomia candida* eller *Folsomia fimetaria*, der anvendes som testdyr i den her beskrevne collembolteste. Rovmiden viste sig at være mindre følsom end collembolerne over for dimethoat. Pirimicarb og fenpropimorph forårsagede ikke påviselig toksisk effekt i doseringer op til 5 gange anbefalet behandlingsniveau. Der blev i undersøgelsen observeret stimulation af reproduktionen ved lave behandlingsniveauer. Også den rumlige fordeling af teststoffet i jordprofilen spillede en vigtig rolle, hvilket der bør tages hensyn til i den videre standardisering af testprotokollen.

Mikrobiel respiration

Som mål for den mikrobielle aktivitet blev CO_2-produktionen bestemt både i prøver fra et pesticidbehandlet feltområde og i et 2^5 faktorielt laboratorieforsøg omfattende de tre pesticider, to jordtyper og +/- tilsætning af gær. I det faktorielle forsøg indgik reproduktionsmålinger på collemboler. Desuden blev respirationsmålingerne for pesticidet fenpropimorphs vedkommende sammenlignet med Cotton Strip Assay udført i laboratoriet, en alternativ metode til bestemmelse af mikrobiel aktivitet.

Der blev ikke fundet nogen effekt af pesticiderne på respirationen, hvorimod jordtypen og tilsætning af gær havde signifikant betydning for respirationens størrelse. Fenpropimorph hæmmede nedbrydningen af cottonstrips signifikant ved den højeste koncentration, hvilket indikerer, at denne metode måske er mere følsom end respirationsmålinger og dermed bedre til at forudsige mulige toksiske effekter på mikroorganismer. Collembolernes reproduktion i det faktorielle forsøg stemte godt overens med resultaterne fra collembolteste beskrevet ovenfor.

Semi-feltmetode

En semi-feltmetode til vurdering af, hvorvidt økotoksikologiske laboratorietests kan ekstrapoleres til feltforhold, er udviklet og afprøvet. Metoden er specielt beregnet på vurdering af sideeffekter på jordbundens mikroleddyr som følge af behandling med bekæmpelsesmidler. Den er testet på populationer af en enkelt collembolart (= springhaler), blandede populationer af 2 collembolarter eller forenklede mikroleddyrsamfund bestående af en eller to collembolarter samt et rovdyr tilhørende mideordenen *Gamasida* (= rovmider).

Metoden bygger på anvendelse af feltmikrokosmer, d.v.s. indelukker fyldt med defauneret markjord (markjord hvorfra mikroleddyrene er elimineret) anbragt i marken. Dette muliggør måling af formering og populations-

udvikling og forventedes at reducere variationen mellem prøveenheder i forhold til den store horisontale variation i artssammensætning og populationsstørrelse, man generelt finder ved prøvetagning af jordbunden.

7 forskellige typer eller varianter af feltmikrokosmer blev afprøvet med henblik på at finde en metode, som effektivt kan isolere en testpopulation fra faunaen i den omgivende markjord og samtidigt vil sikre, at miljøbetingelserne for testpopulationen inden for mikrokosmerne i så høj grad som muligt svarer til miljøbetingelserne i markjorden.

En cylindrisk mikrokosmos type (høj eller lav "åben" type), der er lukket i bunden med fintmasket polyestervæv (45 mikron maskevidde), og hvor den øverste ende over jorden er omgivet med en "krave-fælde" (fig. 7.1.), viste sig at kunne isolere testpopulationer af eu- og hemi-edaphiske arter effektivt. Jordens fugtighed og rumvægt var lidt højere inden i mikrokosmerne end udenfor (fig. 7.7., 7.8.). Der blev ikke konstateret forskelle mellem temperaturen inden for og uden for mikrokosmerne. Den høje "åbne" type blev anvendt som standardmetodik i 1992, medens en lav "lukket" type dækket af 2 cm jord blev anvendt som standard i 1991.

Det blev påvist, at en relativ "blid" metode til defaunering baseret på afvekslende frysning, opvarmning, tørring og genfugtning kunne eliminere mikroleddyrfaunaen effektivt. En filtreret jordsuspension blev tilført for at støtte genetableringen af en naturlig mikroflora.

Collembolarten *Folsomia fimetaria* og rovmiden *Hypoaspis aculeifer* blev valgt som primære testarter, men i 1991 blev der også gennemført forsøg med collembolen *Isotoma notabilis*. De rovmider, som blev anvendt i 1991, var ubestemte. Valget af testarter var baseret på erfaring vedrørende fordeling og adfærd i jorden, generationstid, mulighed for opformering i kultur og trivsel i mikrokosmer.

Der blev tilsat 20 - 30 collemboler af ens størrelse/alder til hvert mikrokosmos i laboratoriet. Derudover blev der til et udvalg af mikrokosmerne tilsat 5 rovmider.

Mikrokosmerne blev placeret i forsøgsmarken ifølge et randomiseret blokdesign med 10 gentagelser (fig. 7.2). Behandlingerne omfattede kontrol (ingen sprøjtning), insektmidlet dimethoat (anbefalet dosering til landbrugsafgrøder), dimethoat (5 gange anbefalet dosering), insektmidlet pirimicarb (5 gange anbefalet dosering) og svampemidlet fenpropimorph (5 gange anbefalet dosering).

Sprøjtningen blev udført med en forsøgsbomsprøjte. Måling af sprøjtemiddeldepositionen viste statistisk signifikante forskelle mellem de forskellige sprøjtemiddelbehandlinger og mellem forsøgsfelter behandlet med samme

middel (tabel 7.5, 7.6). Depositionen var mindre inden for end uden for den øvre ende af den "åbne" type mikrokosmos.

Mikrokosmer optaget fra marken 3 eller 14 dage efter sprøjtningen viste ingen effekt af bekæmpelsesmidlerne (fig. 7.12.A., 7.13.A., table 7.7 - 7.10). Der synes derfor ikke at være akut toksisk virkning af disse stoffer på de valgte jordlevende testarter, når bekæmpelsesmidlet sprøjtes på jordoverfladen.

Mikrokosmer behandlet med 5 gange anbefalet dosering af dimethoat havde signifikant lavere antal *F. fimetaria* og *I. notabilis* end ubehandlede mikrokosmer på alle 3 tidspunkter mellem 5 og 10 uger efter sprøjtning, hvor mikrokosmer blev optaget fra marken og uddrevet (fig. 7.12.B-C., 7.13.B-C., 7.17., 7.33., table 7.7., 7.8., 7.10., 7.12.). Samme behandling havde signifikant negativ effekt på rovmider 10 uger efter sprøjtning i 1991 (fig. 7.37). I 1992 blev der ikke konstateret effekt af dimethoat på rovmiden *H. aculeifer* (målt 10 uger efter sprøjtning).

Dimethoat i anbefalet dosering havde en tilsvarende men svagere virkning, der var statistisk signifikant for *F. fimetaria* på de 3 tidspunkter for inddragning af mikrokosmer mellem 5 og 10 uger efter sprøjtning og for *I. notabilis* på det første tidspunkt (39 dage efter sprøjtning).

Effekter af pirimicarb og fenpropimorph var noget mindre overbevisende end virkningen af dimethoat. Resultaterne af de statistiske beregninger peger dog på, at *F. fimetaria* populationerne på nogle få tidspunkter var negativt påvirket af pirimicarb, og ved en enkelt lejlighed tyder en statistisk test på en positiv, d.v.s. stimulerende, virkning af fenpropimorph på *I. notabilis*.

Den korte persistenstid for dimethoat (halveringstid: 4.8 - 9.7 dage ved 20° C) gør det overvejende sandsynligt, at den konstaterede virkning 5 - 10 uger efter sprøjtning er en forsinket indirekte effekt foranlediget af nedsat reproduktion hos de oprindeligt udsatte dyr og den første generation klækket i mikrokosmerne.

De observerede pesticideffekter var overvejende begrænset til de øverste 10 cm af de høje "åbne" mikrokosmostyper, men 5 uger efter sprøjtning i 1992 og 8 uger efter sprøjtning i 1991 var populationsstørrelsen i 10 - 20 cm horisonten signifikant mindre i mikrokosmer behandlet med dimethoat i høj dosis end i ubehandlede mikrokosmer (table 7.9., 7.10.).

Hovedparten af *F. fimetaria* test-populationen havde bevæget sig ned i det nederste 10 cm jordlag 2 uger efter sprøjtningen i 1992. På senere optagningstidspunkter var dybdeudbredelsen ændret, så det meste af popula-

tionen var i det øverste 10 cm jordlag. Disse vertikale ændringer var ikke påvirket af sprøjtemidlerne, bortset fra at der i de dimethoatbehandlede mikrokosmer optaget 5 - 6 uger efter sprøjtning var omtrent samme antal individer i det øverste og det nederste jordlag, d.v.s. en dybere udbredelse end ved de øvrige behandlinger. Dette kunne tolkes som resultat af flugt fra de højeste koncentrationer af bekæmpelsesmidlet, men det kan også være forårsaget af større dødelighed eller immobilisering i det øverste jordlag (Fábián og Petersen, 1994).

Der blev ikke konstateret signifikante ændringer af pesticideffekten på *F. fimetaria* som følge af tilstedeværelsen af en anden collembolart, *I. notabilis*. Dog var EC_{50}-værdien for dimethoat højere for mikrokosmer med end uden *I. notabilis*. Tilsætning af rovmider synes at nedsætte populationsstørrelsen af *F. fimetaria* i alle behandlinger. Tilsætning betød af rovmider ingen ændring af pesticideffekten på *F. fimetaria*.

EC_{50}-værdier for effekten af dimethoat dosering på *F. fimetaria*'s populationsstørrelse blev beregnet på grundlag af en aftagende eksponentiel relation mellem dosering og populationsstørrelse (fig. 7.28., 7.29.). Værdierne var noget lavere end den anbefalede dosering til behandling af landbrugsafgrøder (DLG 1991) 5 uger efter behandling i 1991 og 8 - 10 uger efter behandling i 1992 (table 7.11.).

På grundlag af undersøgelsesresultaterne foreslås en testprocedure baseret på den høje eller lave "åbne" type mikrokosmer tilsat 30 unge voksne individer. Mikrokosmerne sprøjtes i marken og inddrages og uddrives 2 uger efter sprøjtning med henblik på konstatering af akutte effekter, henholdsvis 6 - 10 uger efter sprøjtning med henblik på konstatering af langtidsvirkninger.

Ekstrapolation fra laboratoriet til felten

Resultaterne fra laboratorietestene og semifeltforsøgene blev sammenlignet for alle tre teststoffer. Ud fra laboratorieforsøgene kunne det vurderes, at stofferne pirimicarb og fenpropimorph tilsyneladende ikke ville resultere i signifikante effekter i semifeltforsøgene. Dette viste sig ikke at være tilfældet, idet der blev påvist signifikante effekter af pirimicarb og fenpropimorph i semifeltforsøget. Derimod kunne der ud fra laboratorieforsøgene forventes effekter af dimethoat i felten ved det anbefalede doseringsniveau. Der var her god overensstemmelse, idet semifeltforsøgene viste en signifikant effekt af dimethoat 5-10 uger efter behandlingen.

Wagner & Løkkes ekstrapolationsmetode

Ved anvendelse af Wagner & Løkkes statistiske ekstrapolationsmetode blev der for dimethoat beregnet den koncentration, hvor mindre end 5% af arterne i jordsystemet påvirkes af stoffet med 95% sandsynlighed. Som parameter for effekt anvendtes EC_{10} for reproduktionshæmning. Det er en forudsætning for anvendelsen af metoden, at data er log-normalfordelte.

Dette var kun tilnærmelsesvis tilfældet, men beregningerne blev gennemført for at vise fremgangsmåden.

Statistisk ekstrapolation

Den statistiske ekstrapolation af data fra enkeltartstestene til felten blev udført ved at variere dimethoats nedsivningsdybde i jorden fra 1 til 10 cm. Desuden blev den brøkdel af den udbragte dimethoat, som rammer jordoverfladen, varieret fra 10 til 100%. Beregningerne viste, at hvis 100% af sprøjtemidlet rammer jorden og nedsiver 1 cm med en homogen koncentration, vil 95% af arterne være beskyttet med 95% sandsynlighed ved en dosering svarende til 0,01% af den anbefalede dosering på 280 g/ha. Ved at ændre depositionen til 10% af doseringen og nedtrængningsdybden i jorden til 10 cm, beregnede den 95%-beskyttelsesniveauet tilsvarende til 1,25% af den anbefalede dosis. Ved 10% deposition og 5 cm nedtrængning beregnedes 95%-beskyttelsesniveauet til 0,61% af den anbefalede dosering. Disse værdier skal dog betragtes som foreløbige, da datamaterialet ikke var tilfredsstillende log-normalfordelt. Løkke et al. (1994) beregnede på grundlag af et større datasæt en 95%-beskyttelsesdosering af dimethoat til at være 0,35 g/ha svarende til 0,125% af den anbefalede dosering. Det blev her antaget for de jordboende organismer, at 10% af sprøjtemidlet ramte jorden og at nedsivningsdybden var 5 cm. Denne værdi er lavere end den tilsvarende i denne undersøgelse. Imidlertid omfatter den primært arter, som lever på jorden eller i vegetationen, hvor de er mere udsat for sprøjtemidler end de jordboende organismer.

1 Introduction

Scope of the project

There is a need for methods to assess the hazard and risk of pesticides in the arable land. Only few tests for hazard and risk assessment of chronic effects have been developed and standardized for non-target terrestrial species. Most of the few existing tests estimate acute effects only, e.g. the EC/OECD earthworm and honey bee tests. For beneficial organisms a programme is running within IOBC, the International Organization for Biological Control, West Palaearctic Regional Section (Hassan, 1992). However, in this work no concentration-effects relationships are established.

Aim: method development

The aim was to develop and if possible to intercalibrate laboratory methods for the determination of sublethal effects on species of meso- and microfauna which could be regarded as representative of soil ecosystems. The project embodied the following animals: Protozoans, Enchytraeids, Collembola and mites. Earthworms were not included in the present project because these species were investigated in other projects within the Danish National Pesticide Research Programme. Laboratory test methods were developed for all species included in the project, and protocols were drafted when possible. Reproduction or growth were employed as endpoints, and the EC_{10} values were estimated. In general, a 95% confidence level was applied.

Semifield method/validation of laboratory methods

As an important part of the project a semi-field method was developed including springtails and mites. The uncertainty of extrapolation from the laboratory to the field was elucidated by comparison of laboratory and semi-field experiments, and by the use of statistical extrapolation methods on the results from the battery of laboratory single species tests.

Changes of the project

The sub-project with laboratory tests for microarthropods was extended with techniques using high gradient extraction in combination with digital image processing for measurement and counting of microarthropods, and with studies on spatial distribution of food and pesticide and bioavailability of the pesticides. Furthermore, several species of Collembola were screened for use as laboratory test organisms. In the original project design the fresh water protozoan *Tetrahymena* sp. was chosen as test organism. However, this species was substituted by soil living species representing three major groups of protozoa, i.e. the ciliates, the flagellates and the amoebae. Additionally, protozoa were studied in a microcosm test at community level. In the sub-project on semi-field methods the field site had to be moved during the period due to road building, and the original design

of the microcosms used was further developed. However, the time schedule of the project did not allow for intercalibration of the methods.

The project dealt with two insecticides, dimethoate and pirimicarb, and with one fungicide, fenpropimorph. All pesticides were used as commercial formulations. Exposure conditions were assessed when possible. In most cases the tests were designed to simulate natural conditions as closely as possible. Especially the spatial conditions and the importance of food ressources were included in the studies.

Chapters in the report

The investigations were initiated in 1990 and carried on for three years until the end of 1992. In the present report the project is introduced, and the results from the different sub-projects are presented, partly as scientific pre-publications. Chapter 1 "Introduction" is written by Hans Løkke. Chapter 2 entitled "Effects on Protozoa" is written by Flemming Ekelund, Regin Rønn, Søren Christensen, and Hans Løkke. Chapter 3 entitled "Toxicity of Pesticides to Enchytraeus bigeminus" is written by Bent Christensen and Lisbeth Overgaard Jensen. Chapter 4 entitled "Toxicity Testing with Collembola" is written by Paul Henning Krogh and Marianne Bruus Pedersen. Chapter 5 entitled "Toxicity testing with a predaceous mite" is written by Paul Henning Krogh. Chapter 6 entitled "Microbial respiration" is written by Marianne Bruus Pedersen. Chapter 7 entitled "Development of a semi-field method for evaluation of laboratory tests as compared to field conditions" is written by Henning Petersen and Peter Gjelstrup. Chapter 8 entitled " Extrapolation from the Laboratory to the Field" is written by Hans Løkke. Finally, Chapter 9 "Conclusions" on the entire project contains contributions from all participants in the project.

2 Effects on Protozoa

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2.1 Introduction

Soil protozoa are in a key position in the soil ecosystem as important consumers of bacteria and to a smaller extent of fungi, and as food for larger organisms e.g. nematodes (Hunt et al. 1987). A limited number of protozoa can be grown axenically, and many toxicity experiments have been carried out under these conditions. Test organisms commonly used for toxicity research in axenic culture are the ciliate *Tetrahymena pyriformis* and the amoeba *Acanthamoeba castellanii* (Dive et al. 1984, Lord & Wright 1984, Otsuka et al. 1988, Prescott et al. 1977).

In the present project, experiments were conducted with soil microcosms of indigenous communities of protozoa. Further, liquid culture experiments were carried out on three common indigenous soil organisms isolated a few weeks before the tests were conducted. The species present represented the three major groups of protozoa inhabiting agricultural soils: a flagellate of the genus *Cercomonas*, an amoebae of the genus *Acanthamoeba*, and a ciliate of the genus *Colpoda*. *Acanthamoeba* sp. and *Colpoda* sp. were isolated from the same soil that was used in the microcosm experiments while *Cercomonas* sp. was isolated from soil from the Danish National Agricultural Research Centre at Askov. By choosing few weeks old isolates, laboratory degeneration should be avoided. The experiments are described in detail in a paper by Ekelund et al., see Annex I.

In the present chapter, draft test guideline protocols are outlined for a microcosm procedure at protozoa community level, and single species liquid culture procedures for three different species groups, and the main ecotoxicological results are presented. The procedures were developed by use of three commercial pesticides containing the active ingredients dimethoate, pirimicarb, and fenpropimorph, respectively.

2.2 Outline of test guidelines

2.2.1 Soil microcosm procedure

Microcosms and materials

Microcosms were prepared by adding 5.0 g of soil to 116 ml serum bottles followed by dry sterilisation (18 h 180°C). For the development of the method, a coarse sandy soil from the Danish National Agricultural Research station at Jyndevad was used as inoculum (Table 2.1). The soil was air dried, and sieved twice through a 1 mm mesh. The number of protozoa in the air dried soil was assessed by the most probable number (MPN) method (Darbyshire et al. 1974), using 1/100 Tryptic Soy Broth (Difco) as nutrient medium. Micro titer plates were used for enumeration of protozoa in soil (Costar, No 3598, 8 x 12 wells).

Table 2.1

Soil characteristics.

clay[a]	(%)	3.9
silt[a]	(%)	4.1
fine sand[a]	(%)	12.2
coarse sand[a]	(%)	76.8
humus[a]	(%)	3.0
soil water capacity#	(%)	34.0
pH	(%)	6.5

[a]: Data from Hansen (1976).

#:Container Capacity determined according to Cassel & Nielsen (1986).

Incubation, sampling, and analyses

Protozoan inocula and pesticides dissolved in water were added to the sterilised microcosms as slurries, prepared from air dried but unsterilised soil. Each microcosm was inoculated with an amount of unsterilised soil corresponding to 10% of the sterilised soil. Finally the microcosms contained 5.5 g soil (d.w.) and 1.375 ml water (25% on d.w. basis). (In the initial experiments 1.87 ml water (34% on d.w. basis) were used). The bottles were sealed with airtight rubberstoppers and incubated at 10 or 15°C in darkness. The number of protozoa was determined by the MPN method (Darbyshire et al. 1974) three times during a three week incubation period by destructively sampling three replicate microcosms.

Determination of carbon dioxide

During the incubation carbon dioxide was measured in the microcosm systems that were subsequently used for enumeration of protozoa. The carbon dioxide content in three replicate microcosms of each treatment was determined by five repeated analyses, each of 1 ml headspace air

from the microcosms by use of a gas chromatograph with thermal conductivity detector.

Seven weeks test

The test was prolonged to seven weeks with enumeration of protozoa and measurement of carbon dioxide on six occasions during the incubation.

2.2.2 **Liquid culture procedures**

Liquid culture experiments were developed for three common indigenous soil organisms. The organisms were isolated a few weeks before the test to avoid laboratory degeneration.

Normalization of data

The results were normalized to soil concentrations of a standard soil of 1.7% organic carbon, density 1.44 kg dm^{-3}, and with pore water content w = 15% (d.w. basis). For the calculations the following soil adsorption partitioning coefficients (K_d values) were used: for dimethoate 0.46, for pirimicarb 8.5, and for fenpropimorph 53 (National Agency of Environmental Protection).

By establishing a mass balance equation for the soil in the microcosms the total soil concentration on dry matter basis (c_t) is calculated from the water concentration (c_w) (Løkke, 1993), (Chapter 8.3):

$$c_t = c_w \cdot (K_d + w)$$

Set of the test

Liquid culture experiments were carried out in micro titer plates (Costar, No 3598, 8 x 12 wells). 200 µl (0.3 $g \cdot l^{-1}$ pesticide in test solution) were added to the eight wells in row 1 on the titer plates. Tryptic Soy Broth was used as growth substrate and the pesticide was added in a concentration twice the maximum concentration required. The 8 wells in each of the next 11 rows were then added 0.3 $g \cdot l^{-1}$ tryptic soy broth without pesticide. Successive dilutions were carried out from row 1 to row 11 by transferring 150 µl in each step. Finally 50 µl protozoan culture was added to each well. The result of this procedure was that each well contained 100 µl liquid with protozoa. The wells in row n+1 contained pesticide in a concentration 3/4 times that in row n, except row 12 where no pesticide was added.

Quantification of protozoa

The protozoa were counted four days after inoculation. For each dilution quantified, at least four wells were counted at 200 times magnification in 12 light fields randomly selected in each well. The results were expressed as number of protozoa for each concentration divided by the number of protozoa in the control treatments.

2.3 **Results and discussion**

Microcosm procedure

The insecticides dimethoate and pirimicarb were tested at 15°C and 34% water (d.w.) and at dosage levels on soil dry matter basis at 316, and 170 mg kg^{-1}, respectively. The fungicide fenpropimorph was tested at 15° C at 85 mg kg^{-1} and further, a concentration series was tested at 10°C and 25% water (d.w.) at 6.25, 18.5, 62.5, and 185 mg kg^{-1} soil (d.w.).

In the microcosm procedure the number of protozoa was significantly reduced on day six in the dimethoate treated microcosms compared to the control. Pirimicarb had no significant effects on the protozoa community. During all the period until 20 days after application, fenpropimorph reduced the number of protozoans significantly at all sampling dates. Table 2.2 shows the number of protozoa after 20 days.

Table 2.2

Number of protozoa in microcosm tests 20 days after treatment. " = significantly different from control. For further details, see Annex I.

Treatment (mg kg^{-1})	Number of protozoa
Control	853,683
Dimethoate (316)	317,580
Pirimicarb (170)	731,683
Fenpropimorph (85)	153,106[a]

By treatment with fenpropimorph, a large decrease in the number of protozoa was observed during the first 2 weeks at concentrations ranging from 6.25 to 185 mg kg^{-1}. However, after 7 weeks no significant differences were observed between treated microcosms and the control microcosms.

The microcosm procedure needs further development

The procedure needs further development and standardization. Especially the inoculation should be more replicable. A culturing technique for a standard protozoan community might be considered. The choice of test temperature and humidity needs further investigation. The determination of the respiration by measuring carbon dioxide is a valuable control of the functioning of the microcosms. This technique should be further developed aiming at the detection of effects on the microcosm ecosystems.

The liquid culture procedures

The results from the liquid culture procedures are shown in detail in Annex I. In Table 2.3 values are given of the EC_{10} and EC_{50} for population decrease, and the $EC_{10.hormesis}$ for the increase in number of the test species (hormesis, Stebbing 1982) caused by the test compounds. The values for the EC_{10} and EC_{50} for population decrease, and the $EC_{10.hormesis}$ for stimulation are calculated from c_w-values given in mg $\cdot l^{-1}$ as found by the liquid culture procedures.

Table 2.3

EC_{10} and EC_{50} for population decrease, and $EC_{10,hormesis}$ for the increase in number of the test species, the flagellate of the genus Cercomonas, the amoebae of the genus Acanthamoeba, and the ciliate of the genus Colpoda. The results are shown for the three test compounds dimethoate, pirimicarb, and fenpropimorph and given in mg kg^{-1} soil.

mg a.i. kg^{-1}	Dimethoate	Pirimicarb	Fenpropimorph
Cercomonas			
EC_{10}	50	260	27
EC_{50}	420	960	110
$EC_{10,\ hormesis}$	a	a	a
Acanthamoeba			
EC_{10}	130	3,900-9,600	50-270
EC_{50}	320	13,000	740
$EC_{10,\ hormesis}$	a	1,400	a
Colpoda			
EC_{10}	70	7,000	430
EC_{50}	190	7,300	480
$EC_{10,\ hormesis}$	12	180	27

a: No stimulation (hormesis) was observed

Comparison of the microcosm and the liquid solution

By comparing the results obtained from the microcosm tests with those from the liquid procedure it can be concluded from the results of the liquid procedure that evidently no effects should be expected in the microcosm test by exposure with pirimicarb at the applied dosage of 170 mg kg^{-1} which corresponds to the level obtained by agricultural practise. The results obtained with dimethoate and fenpropimorph in the water culture procedures are also in general agreement with the results from the microcosms. However, a detailed interpretation is difficult due to the unknown species, and the population dynamics involved in the microscosm.

The degradation of the test compounds during the test period may have favoured the observed increase of the number of protozoa in the microcosms. However, the exposure may also have favoured species within the community of protozoa which were more tolerant of the compounds. The effects shown in the single species tests reflect the impact on specific species and thereby structural aspects in comparison with the rather unspecific outcome of the microcosm tests which might show the impact of the chemicals on the functioning of the protozoa community. The effects on the structure of the protozoa community may be studied by identification and quantification of the species present. However, such procedure will be

impossible as long as knowledge on identification of soil protozoa is on its present stage.

In general, the observed effects may be lethal or sublethal effects on reproduction, or the behaviour of the organisms. The fungicide fenpropimorph may have caused direct effects on the protozoa. However, indirect effects on bacteria and other food items cannot be excluded.

2.4 **Conclusions**

A preliminary guideline for a microcosm test procedure has been outlined. This method simulates natural conditions. The method as it stands is a test for non-specific functional impact on protozoa at the community level. The procedure may be extended to include structural aspects of the impact of chemical compounds. A standardization of the method is very difficult, mostly due to difficulties in identification of the species of the soil protozoan community.

Procedures for single species tests on three major groups of soil dwelling protozoa were developed. The flagellate *Cercomonas* was the most suitable organism for pesticide tests of the three animals tested. The shape of *Cercomonas* cells is very characteristic compared with e.g. *Acanthamoeba* which can sometimes be difficult to distinguish from particles of organic matter. *Cercomonas* is easy to culture and will grow as soon as a suitable food source is offered. It is easy to count, also when alive, because it moves rather slowly as opposed to *Colpoda* which swims rather fast. It showed to be the most sensitive, and it produces the dose response curves that is most easy to interpret.

At the present stage the liquid culture procedure testing single species may be preferred for community experiments carried out in soil microcosms. Single species based tests are much easier to interpret than community tests, but much more remote from the natural system they are supposed to reflect. However, the ideal approach might be to combine the two types of test in order to get a broader understanding of the effect of pesticides on the soil protozoa and thereby on the soil ecosystem as a whole.

3 Toxicity of pesticides to Enchytraeus bigeminus

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3.1 Introduction

Enchytraeids are present in high numbers in most soil types and play an important role in the decomposing process. Since they mostly occur in the upper 5 cm of the horizon they are potential non-target organisms which may be affected by some pesticides applied to the field.

A recently developed sublethal laboratory test measures cocoon production and hatchability in an enchytraeid species (Westheide and Bethge-Beilfuss, 1991). The duration of this test is approximately 30 days, and it requires the transference of all test organisms to a new culture medium every 5th day.

The test procedure proposed here measures the formation of new segments in an active growth phase. Its duration is seven days and it does not involve manipulation of the test organisms during this period. Both sublethal and lethal effects are assessed.

3.2 Material and methods

Various aspects of the reproductive biology of *E. bigeminus* are dealt with in Christensen (1964 and 1973). In dense cultures the reproduction is exclusively asexual through transverse fission and subsequent regeneration of new anterior and/or posterior ends. Fully grown worms (45-60 segments) divide into 3-4 fragments. The original anterior and posterior ends are somewhat longer than the intermediary fragments which are of fairly even length. The latter regenerate both a new anterior and a new posterior end. At 20 - 22°C regeneration of a new anterior end is completed in six days and is immediately followed by a rapid growth phase lasting appr. 8 days. We measure the effect of the chosen chemicals on the formation of new segments during this growth phase.

Mass cultures of *E. bigeminus* are kept in glass jars with appr. 3 cm of wet sand. The worms are fed with oatmeal under a small piece of glass placed on the surface of the sand. Since the proportion of test animals

(appr. 2 mm long and with newly regenerated anterior ends) is fairly low in the ordinary mass cultures, attempts were made to increase the proportion of this stage. New cultures were established in ordinary petridishes with 1 cm of wet sand, a teaspoonful of sand containing worms from a mass culture was added and supplied with a teaspoonful of oatmeal. The idea is that when the oatmeal has been consumed a large number of fragments is in the process of regenerating a new anterior end and when this is completed they are arrested in this stage due to the lack of food. After six weeks the proportion of suitable test animals may reach 40-60% compared to only a few percent in ordinary mass cultures.

Test organisms are kept in small petridishes with a diameter of 5 cm. The culture medium is 0.01 g ground oat flakes in 1 ml water (control) or the chosen concentrations of the test substances. The cultures are stored in the dark at high humidity and 21 °C. An experiment contains a control and different concentrations of the substance to be tested each comprising five replicates with twenty worms. After seven days the number of surviving worms are registered (acute tests) and in the case of sublethal tests they are fixed in 70% alcohol, imbedded in 44581 DPX and mounted on a slide for later counting of segments under the microscope.

Test organisms used in a given experiment were isolated from the same culture on the same day. The total mean length of 5 x 20 such worms was found to be 24.74 segments. There was no significant difference between the means of the five subsamples indicating that the test organisms used in a given experiment are of uniform length. However, the length of the control animals after seven days differs significantly between experiments and this indicates that the test organisms might differ in length and/or physiological state between the above cultures.

LC_{50} is estimated by a computer programme (M.K.Lauridsen, Danish Pest Infestation Laboratory) based upon a maximum likelihood approach. Estimates of the substance concentrations causing a 10% reduction in the number of new segments are based upon the linear regression of number of segments on substance concentrations causing no or moderate mortality. Otherwise conventional statistics is used.

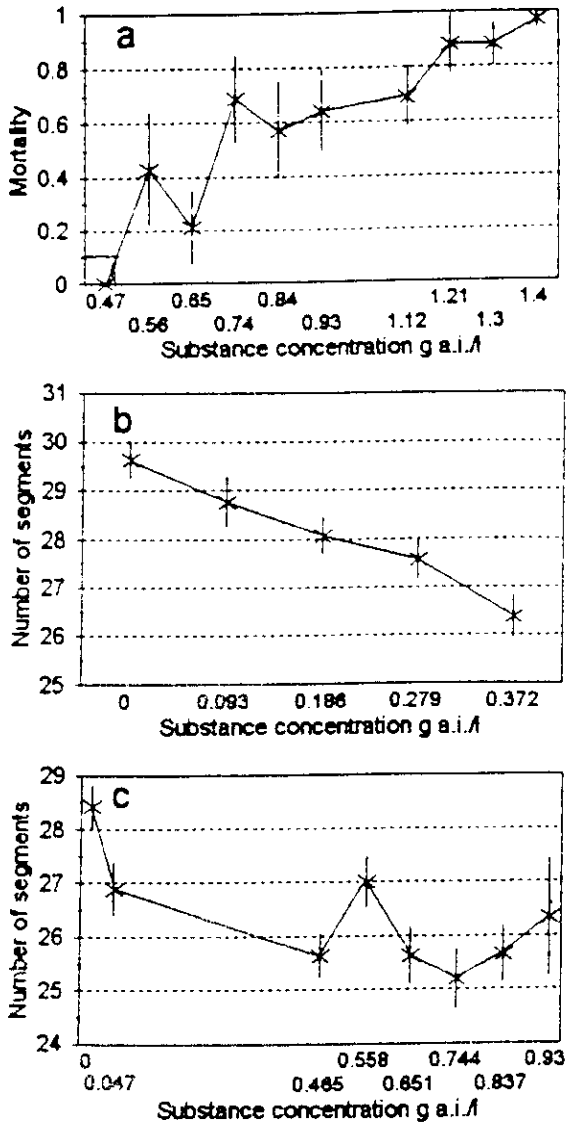
The following pesticides are tested: Dimethoate, given as the formulation "DLG Dimethoat 28"; pirimicarb, given as the formulation "Pirimor", ICI and fenpropimorph given as the formulation "Corbel", Schering A/S. The two former are insecticides, the latter is a fungicide.

3.3 **Results**

Dimethoate

The LC_{50} value is estimated to 0.78 g a.i. l^{-1}. The observed distribution is shown in Figure 3.1a.

Figure 3.1
The effect of dimethoate on mortality and growth of E.bigeminus. a) Mortality. b and c) The effect on growth measured in two different experiments (A and B resp.). Five replicates each with 20 worms. Vertical bars: std. error.



Experiment Dim.A (Figure 3.1b) shows a gradual decline in the number of segments with increasing substance concentrations. Apparently a more or less linear correlation is found at concentrations causing no or moderate mortalities, whereas a more irregular pattern is seen at higher concentrations (Exp. Dim.B, Figure 3.1c) with no general trend in the number of segments and with great variations in the mean numbers. The latter may be caused by small sample sizes due to the high mortality at these concentrations. Another feature at these high concentrations is that the mean number of segments in some cases was only slightly above that of the test organisms at the start of the experiment (appr. 25 segments), indicating that many individuals do not add new segments.

A significant reduction in growth was observed at a concentration of 0.047 g a.i. l^{-1}. Based upon the regression observed in Experiment Dim.A the concentration causing a 10% reduction in the number of new segments was estimated to 0.06 g a.i. l^{-1}.

Pirimicarb

The LC_{50} value is estimated to 0.6 g a.i. l^{-1}. The observed distribution is shown in Figure 3.2a.

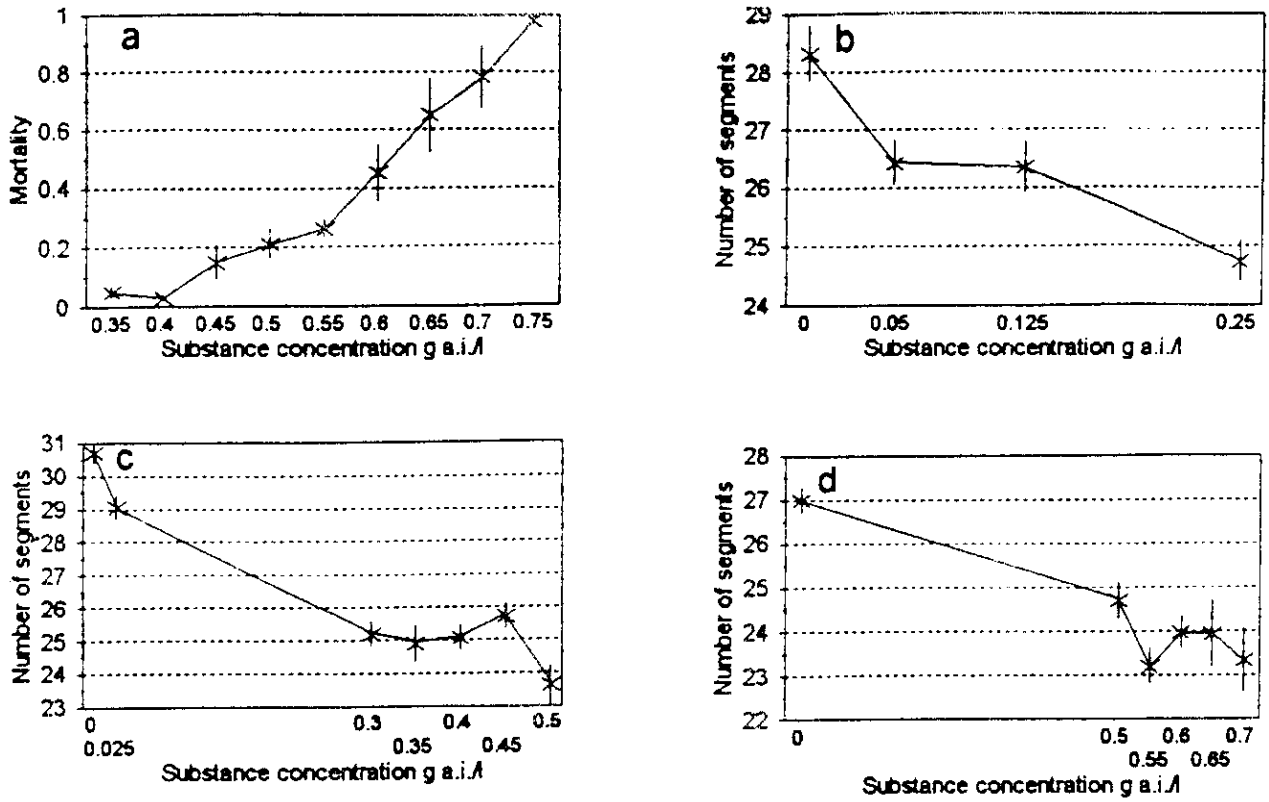


Figure 3.2

The effect of pirimicarb on mortality and growth of E. bigeminus. a) Mortality. b, c and d) The effect on growth measured in three different experiments (A, B and C resp.). Five replicates each with 20 worms. Vertical bars: std. error.

As in the case of dimethoate is seen a more or less gradual decline in the number of segments at concentrations causing no or low mortalities, and no regular trend and practically no overall growth at higher concentrations.

A significant reduction in growth was observed at a concentration of 0.025 g a.i. l^{-1}. An estimate of the concentration causing a 10% reduction in growth was not possible because none of the individual experiments included a sufficient number of concentrations at the sublethal level.

Fenpropimorph

The LC_{50} value is estimated to 0.225 g a.i. l^{-1}. The observed distribution is shown in Figure 3.3a.

Experiment Cor.A (Figure 3.3b) shows the same gradual decline in number of segments at substance concentrations with no or moderate mortalities as observed in the experiments with dimethoate (Figure 3.1b). Due to the high toxicity of fenpropimorph only a few observations are available at concentrations causing medium to high mortalities, but apparently they also follow the pattern described above.

A significant reduction in growth was observed at a concentration of 0.0025 g a.i. l^{-1} (Figure 3.3d). However, the mean number of segments at this concentration did not differ significantly from that obtained at a ten

times higher concentration (0.025 g a.i. l^{-1}), which may indicate either the existence of a thresshold value or perhaps that one or both results obtained in this experiment may not be fully reliable. The concentration causing a 10% reduction in growth was estimated to 0.02 g a.i. l^{-1} from the regression based on substance concentrations of ≤ 0.2 g a.i. l^{-1} in Exp. Cor.A (Figure 3.3b).

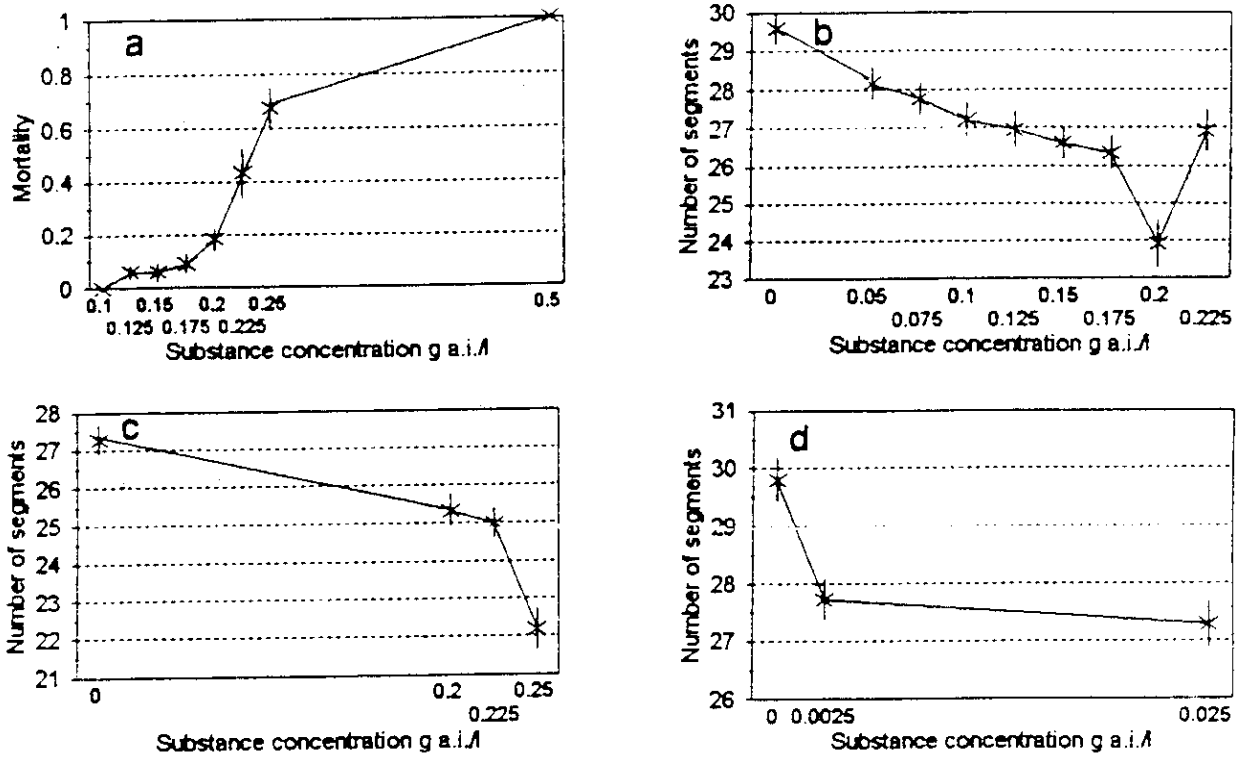


Figure 3.3

The effect of fenpropimorph on mortality and growth of E. bigeminus. a) Mortality. b, c and d) The effect on growth measured in three different experiments (A, B and C resp.). Five replicates each with 20 worms. Vertical bars: std. error.

3.4 **Discussion**

In a series of recent papers Westheide and his group has thouroughly described a test method using species belonging to the socalled *Enchytraeus buchholzi* complex as test organisms (Westheide et al., 1989 and 1991 and Westheide and Bethke-Beilfuss, 1991). This procedure measures the effect of chemicals on reproduction i.e. the number of eggs produced and their hatching frequency, whereas the present method measures the effect upon the somatic growth i.e. the formation of new segments during individual growth. Both processes are vital elements in the life cycle of any organism.

The *E. buchholzi* test requires the transference of test individuals to a new culture medium (agar) every 5th day, and during each inspection the positions of the cocoons produced are marked on the bottom of the culture dishes, from which the worms are removed. The duration of the test is 30

days. The duration of the present E. bigeminus test is only 7 days and it does not involve manipulation of the test worms in this period. Thus, the latter method is much less time and labour consuming than the former.

Direct comparison of the sensitivity of the two methods is not possible because there is no overlap in the chemicals tested (the funding agency specified those used in the present study). However, both methods indicate that apparently enchytraeid worms are less sensitive to insecticides than to the fungicides, benomyl (Westheide et al, 1991) and fenpropimorph (see above). In the case of benomyl effects were observed at a concentration of approximately 0.006 g a.i. l^{-1}, a figure very similar to those recorded for fenpropimorph in the present study, of Figure 3.3d, and this may indicate that there is no major difference in the sensitivity of the two methods.

The assessment of segment numbers is technically the simplest way to quantify the size of *E. bigeminus*. However, an increase from appr. 25 segments in the test worms to 29-30 segments in the controls only amounts to a relative increase of one fifth in a week. Within the same period a fivefold increase in biomass takes place under control conditions (Christensen, 1973), or approximately a three-fold increase in absolute length. Thus, assessing either weight or absolute length might provide a much more powerful measure than the number of segments. As a matter of fact the immediate visual comparison of controls and experimental cultures usually indicated a much stronger effect of a given chemical than the small difference in means of segment numbers showed. A computer based scanning procedure to measure the size of the worms is an obvious way to increase the sensitivity, and if properly automated this may at the same time reduce the labour costs.

Another suggestion is to reduce the differences in length and/or physiological state between test worms from different cultures by standardizing the balance between number of worms and amount of food supplied when these cultures are established.

3.5 **Conclusions**

A week long laboratory test is described which measures both sublethal and lethal effects of chemicals on *Enchytraeus bigeminus*. Sublethal effects are measured as the reduction in number of new segments formed during a rapid growth phase.

The following results were obtained: Dimethoate: LC50 = 0.78 g a.i. l^{-1} and EC10 = 0.06 g a.i. l^{-1}; Pirimicarb: LC50 = 0.6 g a.i. l^{-1}; Fenpropimorph: LC50 = 0.225 g a.i. l^{-1} and EC10 = 0.02 g a.i. l^{-1}.

4 Laboratory toxicity testing with Collembola

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4.1 Introduction

New test-systems with soil fauna

Laboratory test systems with species representative of the soil invertebrate community were demanded by an OECD workshop (OECD, 1989) and advocated by a European workshop (Eijsackers & Løkke, 1992). Present experience with single species reproductive test systems counts for *F. candida* (Collembola: Isotomidae) (Biologische Bundesanstalt (BBA) 1989), *Eisenia fetida andrei* (van Gestel 1989), *Platynothrus peltifer* (Acarina: Oribatida) (Denneman & van Straalen 1991), *Orchesella cincta* (Collembola: Entomobryidae) (Badejo & Van Straalen 1992) and *Hypoaspis aculeifer* (Acarina: Gamasida) (Krogh in press). The present study uses the BBA procedure with *Folsomia candida* as a starting-point for tests and evaluates the protocol with respect to selected factors of primary importance to the ecotoxicity.

Important factors for the outcome of tests

This chapter presents a feasible way to perform laboratory studies on Collembola and pursues questions about the importance of factors that are believed to influence the outcome of toxicity tests. The motivation for this lies in the fact that the recognized structural complexity of the biotic and abiotic compartments in the natural soil environment is extremely in contrast with the simplism in the design of laboratory test systems. This has stimulated the investigation of simple simulations of these aspects in the test systems. Hence, the basic questions to be clarified is whether soil types, mixtures of pesticides, starvation, and manipulations of the distribution of food and pesticide result in significant changes of the toxicity in comparison with a simple test system.

Intercalibration

A first attempt to intercalibrate between our laboratory and the Department of Ecology and Ecotoxicology at the Vrije Universiteit Amsterdam questioned the identity of breeding conditions. In that period our cultures were unstable in terms of variation in adult survival and reproduction. To find possible explanations for this we examined the effects of various test conditions on survival and reproduction of control animals (i.e. animals not treated with chemicals). Both acute and long term mortality were studied, and the parameters included were:

- substrate type
- food type
- aeration (+/-)
- age of adults at start of experiment

4.2 Materials and methods

4.2.1 Pesticides

Dimethoate

Dimethoate, O,O-dimethyl-S-(N-methylcarbomoyl-methyl)-phosphorodithionate, is an inhibitor of cholinesterase. It has a half life in soil of 5-9 days depending on soil texture (Kolbe *et al.* 1991). It is a broad spectrum insecticide with pronounced effects on the arthropod fauna at the recommended dose level (e.g. Frampton 1988, Goodwin 1984, Vickerman & Sunderland 1977, Powell *et al.* 1985). Dimethoate has been selected in our laboratory as a reference chemical in toxicity studies.

Pirimicarb

Pirimicarb, 2-dimethylamino-5,6-dimethylpyrimidin-4-yl dimethylcarbamate, is a selective aphicide, but direct mortality has been observed for *Phytoseiulus persimilis* (Acarina: Gamasida) at the recommended field concentration for both adults and nymphs (Goodwin 1984).

Fenpropimorph

Fenpropimorph, (±)-*cis*-4-[3-[4-(1,1-dimethylethyl)phenyl]-2-methylpropyl]-2,6-dimethylmorpholine, a systemic fungicide is considered harmless (mortality < 50%) to predaceous mites (Hassan *et al.* 1988).

4.2.2 Laboratory cultures and basic test protocol

Production of synchronized cultures

F. candida and *F. fimetaria* are established in permanent cultures and test animals are produced according to the schedule in Table 4.1. Stimulation of oviposition is obtained by moving adults to newly produced plaster/-charcoal substrate. When the Collembola have lived on the substrate for 5 weeks they are again stimulated to oviposit. If the culture is old or shows signs of decreasing health it will be disposed of. The cultures are renewed by surplus synchronized offspring and from the eggs not collected for production of synchronized cultures.

Table 4.1
The pesticides tested in the project.

Active ingredient	Commercial name	Recommmended dosage pr. ha (formulated product)	Active ingredient pr. ha	Calculated a.i. in soil (upper 5 cm)
Dimethoate	Dimethoate 28	1 *l*	280 g	0.389 mg/kg
Pirimicarb	Pirimor	0.3 kg	150 g	0.209 mg/kg
Fenpropimorph	Corbel	1 *l*	750 g	1.042 mg/kg

Table 4.2
Weekly schedule for the breeding of collembolan test animals.

Monday	Removing unhatched eggs from synchrone culture. Feeding and watering synchrone culture
Tuesday	Production of substrates (Gypsum/charchoal)
Wednesday	Changing the substrate with *F. candida* and *F. fimetaria* for stimulation of oviposition
Thursday	Feeding and watering the cultures
Friday	Collecting eggs from *F. fimetaria* and *F. candida* stimulated to oviposit last week Feeding and watering synchrone culture

A synchronized culture is produced by collecting about one week old eggs which are allowed to hatch over the week-end from Friday to Monday. Tests with *F. candida* are carried out on the Wednesday when the animals are 9-12 days old and with *F. fimetaria* on the Wednesday when they are 16-19 days old.

Soil treatment

In order to eliminate undesired fauna in the soil used in the tests, the soil was alternately dried (60 °C), frozen (-35 °C) and incubated (20 °C) in wetted condition. The soil was then sieved through a 2 mm mesh.

Inoculation

The soil was moistened and inoculated with a suspension of soil extract. The extract was produced from ½ kg fresh soil which had been stored at 5° C for no longer than 2 months, added ½ l demineralized water, incubated for 24 hours for extraction of microbes and then sieved through a 40 µm mesh.

Dosage range

Concentrations of pesticides were prepared in an increasing geometric series with a factor of 1.5-1.8 depending on the interval covered. Concentrations were calculated on the basis of the recommended dosage for the pesticides used, assuming even distribution in the uppermost 5 cm of the soil. In the case of dimethoate this leads to a concentration of 0.389 mg a.i./kg soil based on a soil density of 1.44 kg/dm^3 of the actual soil (Table 4.1). The results may be recalculated to other soil layer depths, see section 8.3.

Test procedure

The test scheme for the testing of chemicals is shown in Table 4.3. 10 parthenogenitically reproducing *F. candida* females or 25 individuals of a random mixture of the two sexes of *F. fimetaria* were added to each of the 4 replicates per concentration. A replicate thus consisted of contaminated soil in a closed container, i.e. a microcosm, with food and test Collembola. When the test was terminated after 4 weeks the animals were extracted from the microcosms in a high gradient extractor (HGE) of the Mac-

Fadyen type and collected into a cooled vessel (2° C) with plaster of Paris/charcoal. (A detailed description of the extraction procedure will appear in Krogh & Holmstrup (*in prep.*)). The extraction was started at 25° C and the temperature was increased automatically every 12 hours. After 40° C the extraction was over.

Table 4.3

Test scheme for the testing of chemicals with F. candida and F. fimetaria.

Day no.	Day of week	Operation
1	M	1. Making inoculate from fresh undisturbed soil 2. Weighing of defaunated dry soil in portions of 26 g for each test container
2	T	3. Pesticides and inoculate mixture (4 ml) is added to 100 ml beakers with automate pipette, mixed thouroughly into soil to obtain even humidity and distribution of chemical 4. Soil is incubated in fume cupboard until next day
3	W	5. Weighing of 5 beakers to adjust water content 6. Transfering soil to microcosm and weighing microcosms 7. Transferring test-animals with exhaustor from synchroneous culture to black lid for counting and addition to microcosm 8. Addition of 15 mg granulated dried baker's yeast to each microcosm, weighing of 5 microcosms 9. Incubation in climate chamber at 20° C
17	W	10. Addition of 15 mg granulated dried yeast to each microcosm, weighing of 5 microcosms to adjust for lost water
31	W	11. Termination of test. Extraction from soil in high gradient extractor
33	F	12. Removal of collection vessels from extractor. Storage at 5 °C until counting

The pesticides were dissolved or suspended in water and the amount of water was adjusted to about 50 % of water holding capacity (see Table 4.4 concerning soil characteristics). Thirty g of moist soil was used per replicate consisting of 26 g dry soil plus 4 ml pesticide/inoculate solution.

4.2.3 2^5-factorial design

To study the interaction between the three pesticides and food and soil type a 2^5-factorial design was set up with the factors either present or absent, i.e. at two levels: ± dimethoate (D), ± fenpropimorph (F), ± pirimicarb (P), ± baker's yeast (Y), OECD soil/agricultural soil (soil type). Two series were performed, one with the OECD soil vs. a sandy soil and one

with the OECD soil vs. a sandy loam. The level of the pesticide when present was the calculated concentration based on the recommended dose (see Table 4.1), assuming even distribution in the upper 5 cm of the soil.

As it was only possible to do a limited number of treatments on one occasion or test session, due to the actually available test animals and manpower, the 32 possible treatments were split into 2 blocks carried out at 2 different occasions.

Table 4.4
Soil characteristics of the soil types used in the tests.

Origin	Water Holding Capacity %	pH	% Content					
			Total C	Humus	Clay <2 µm	Silt 2-20 µm	Sand 20-200 µm	Coarse sand 0.2-2 mm
OECD	60	5.61	5.3	9.0	10.0	10.5	67.9	2.5
Ødum	30	6.28	1.0	1.8	12.6	15.0	48.5	22.1
Kalø	30	5.75	1.0	1.7	11.1	12.5	48.1	26.6
Jyndevad	N.D.	6.34	1.2	2.1	4.0	4.6	13.4	75.9

F. candida was used in this experiment in the same way as in the reproductive test with 4 replicates per treatment.

The tests were planned in accordance with a factorial design with the above mentioned 5 factors and the time at two 'levels', i.e. performed at two different occasions.

4.2.4 **Spatial distribution of yeast and dimethoate**

Reproductive tests with *F. candida* were performed with dimethoate applied in 0, 0.4 (calculated field dosage) and 1.3 mg · kg^{-1}. Food and dimethoate were distributed in the following ways with all treatments replicated 5 times:

a. Control:

Addition of 15 mg yeast to the surface of the soil (Biologische Bundesanstalt 1989).

b. Homogeneous distribution of yeast and dimethoate in the soil:

- 15 mg yeast
- 60 mg yeast

The yeast was suspended in the dimethoate solution and mixed into the soil to obtain a homogeneous distribution. The same amount as in the base

test system, 15 mg, was suspended. Furthermore, a set of replicates received a dose of 60 mg as 15 mg was expected to be sub-optimal because the availability would be considerably lower in the mixed form.

c. Homogeneous distribution of dimethoate in the top 1 cm of soil:

- Homogeneous distribution of yeast in the top layer
- Homogeneous distribution of yeast in both top and bottom layer

An uneven vertical distribution of dimethoate was obtained by placing 30 g treated soil on top of 30 g untreated soil. The treatment consisted of addition of a solution of dimethoate with 60 mg yeast in suspension to the soil. The bottom soil was either added demineralized water or a water suspension containing 60 mg yeast.

d. Heterogeneous distribution of yeast:

- Heterogeneous distribution of yeast in top layer.
- Heterogeneous distribution of yeast in both top and bottom layer

Fifteen mg yeast was distributed as intact granules by carefully mixing it into the moist soil. This should resemble an intermediate distribution between completely homogeneous distribution and the confinement of yeast to the surface.

4.2.5 **Survival and reproduction of "control" *F. candida***

The basic setup was identical with the one used in the basic test system. The organism studied was *F. candida*, 10 per petri dish or microcosm. Two age-groups of adults were used, 9-12 and 23-26 days old at the start of the experiments, respectively, in order to study the influence of start age.

Acute mortality was studied by counting surviving adults after one day on two types of substrate: natural soil and compressed natural soil, the latter hindering the animals from hiding in the soil and thus excluding the effect of extraction. Subacute mortality was estimated after 7 and 14 days, respectively.

Long-term mortality and reproduction were estimated after 28 days in separate experiments with varying substrate type (plaster of Paris/charcoal, natural soil (Kalø soil) and OECD soil), food type (Danish baker's yeast and Dutch baker's yeast) and aeration (+/-) by opening the lid once every week.

The whole setup is summarized in Table 4.5

4.2.6 **Statistical analysis**

Table 4.5
Experimental setup in study of mortality and reproduction in controls
Substrate: P = plaster of Paris/charcoal, K = Kalø soil, CK = compressed Kalø soil, O = OECD soil.
Food type: DK = Danish baker's yeast, NL = Dutch baker's yeast.

Duration	Substrate				Food type		Aeration		Measure		No. of experiments	No. of replicates
days	P	K	CK	O	DK	NL	+	-	adults	juveniles		
1		x	x		x		x		x		4	10
7			x		x		x		x		2	10
14		x			x		x		x		2	10
28	x	x		x #	x	x	x	x	x	x	16	10/5 *)

#) Only with adults aged 23-26 days at start of experiment
*) 10 for adults aged 23-26 days, 5 for adults aged 9-12 days at the start of the experiment.

Calculations of EC_{10} and EC_{50} were done by fitting a model to the data, (Lacey & Mallett 1991):

$$y = c \cdot (1 - e^{\alpha} \cdot x^{\beta})^{-1} \qquad (1)$$

y: reproductive output
c: control reproduction
x: dose of chemical
α: 'slope' parameter
β: curvature parameter

This formula was reparameterised by substitution of α with an expression including EC_X (Ann Gould, Shell Research Ltd, pers. comm.):

$$e^{\alpha} = \frac{1-(X/100)^{-1}}{EC_x^{\beta}} \qquad (2)$$

This allows the calculation of 95% confidence intervals when estimation of the parameters including EC_X is done with the SAS procedure NLIN (SAS Institute Inc. 1988A).

Statistical tests for NOEC and LOEC were made with a Williams Test (Gelber *et al.*) by the use of the program Toxstat (Gulley *et al.* 1988).

Analysis of the 2^5 factorial design was done with SAS using the GLM procedure (SAS Institute, 1988A). All main, second order and confounded interactions were specified in the model, but significant confounded interactions were considered evidence for dependence on time, i.e. block ef-

fects. The ANOVA is computed on data using the log(x+1) transformation to make the variance independent of the mean.

Statistical analyses of data from control survival and reproduction were performed using SAS STAT's GLM procedure, and differences were compared with Bonferroni and LSD test at the 5 % significance level.

4.3 Results

4.3.1 Dosis-response toxicity data from basic test protocol

Dimethoate had an adverse effect on survival and reproduction of the Collembola (Figure 4.1) but neither pirimicarb nor fenpropimorph had a toxic effect at the range of concentrations tested. The derived toxicity measures are shown in Table 4.6. EC_{50} was about the calculated field dosage. *F. fimetaria* was slightly more sensitive to dimethoate than *F. candida*. For both species a sublethal effect was observed above 0.4 mg · kg^{-1}, while concentrations higher than 1.0 mg · kg^{-1} led to high mortality of the adults. Quantitatively, this was expressed in the reproductive rate per adult, which was decreasing with the concentration starting with a control rate of about 100 per female.

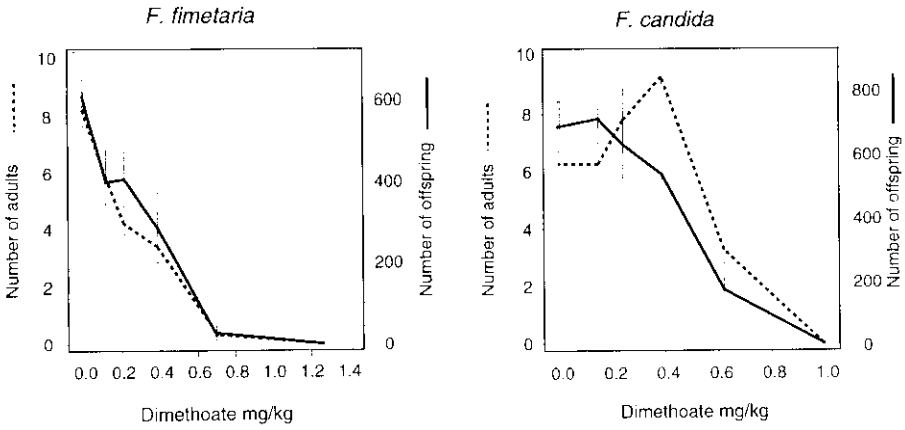

Figure 4.1
The effects of a concentration series of dimethoate on the survival and reproduction of adult F. fimetaria and F. candida. 0.4 mg · kg^{-1} is the calculated recommended field application dose. Vertical lines are ±1 standard error of the mean (n = 4).

4.3.2 Interactions between pesticides, soil type and optimal feeding/starvation

The ANOVA revealed that the effects of the main factors and the factor combinations are significant (Table 4.7). Only main effects and second order interactions are included in the analysis apart from the time/block effect, which only proved significant for the sandy vs. OECD soil trial.

Inspection of Figure 4.2 showing reproductive output in the factorial experiment demonstrates the overall importance of food and soil type. The artificial soil supports a generally higher production of *F. candida* offspring than the sandy soil and dimethoate had a significant negative im-

pact on the number of offspring in the latter soil irrespective of food availability. In addition, there was a stimulation by pirimicarb, and the offspring numbers were also dependent on the date of performing the test. Furthermore, there was an interaction between pirimicarb and fenpropimorph indicating a decrease in reproduction in comparison with the single pesticides, but still a stimulation in comparison with the control.

The difference between the data concerning OECD soil in the two experimental series may be due to less experience in performing the test as this was run in the beginning of the project.

The results with the loamy soil compared to the OECD soil generally shows less marked pesticide effects.

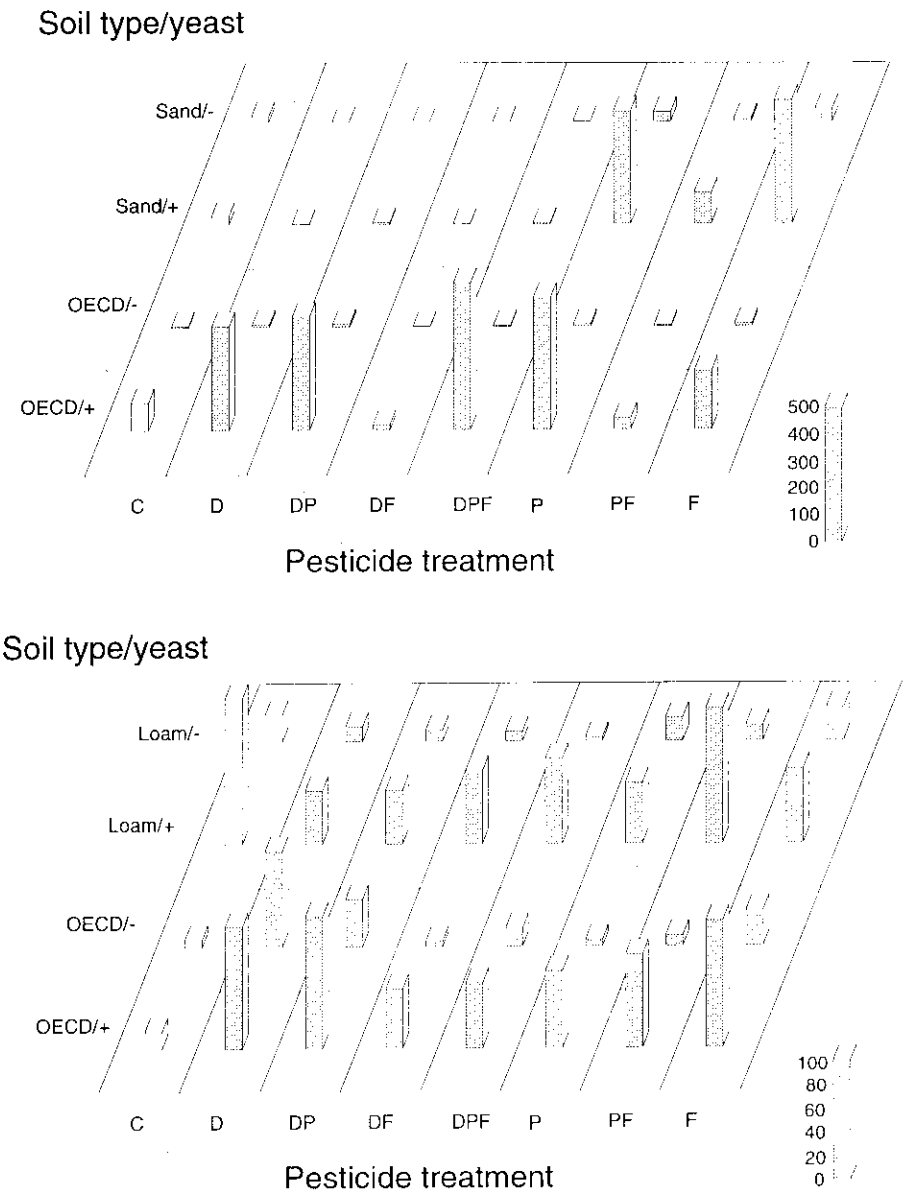

Figure 4.2
2^5 factorial experiment with 4 weeks reproduction of F. candida subject to combinations of three pesticides, presence (+) or absence (-) of food (yeast) and two different soil types. Upper graph: comparison of a sandy soil with the OECD soil; lower graph: comparison of a loamy soil with the OECD soil. Blocks represent the mean number of offspring (n=4). C: control, D: Dimethoate, P: Pirimicarb, F: Fenpropimorph.

Table 4.6

EC_{50}, EC_{10} , NOAEC and LOAEC of dimethoate (mg/kg soil) for reproduction of the two Collembolan species. For comparison the gamasid mite Hypoaspis aculeifer Canestrini (Acarina: Gamasida) has been included. C.I.: Confidence Interval. NOAEC: No Observed Adverse (≡ reduction) Effect Concentration; LOAEC: Lowest Observed Adverse Effect Concentration.

	EC_{10}	95% C.I.		EC_{50}	95% C.I.		NOAEC	LOAEC
	$mg \cdot kg^{-1}$			$mg \cdot kg^{-1}$			$mg \cdot kg^{-1}$	
		lower	upper		lower	upper		
F. fimetaria	0.1	0.01	0.2	0.3	0.1	0.5	<.1	.1
F. candida	0.3	0.2	0.4	0.5	0.4	0.6	0.4	.6
H. aculeifer	0.7	0.6	0.8	0.9	0.8	1.0	0.7	1.3

4.3.3 **Spatial distribution of yeast**

The most obvious effect of mixing Baker's yeast homogeneously into the soil was a considerable decrease in the reproduction compared with the surface application in the base test (Figure 4.3).

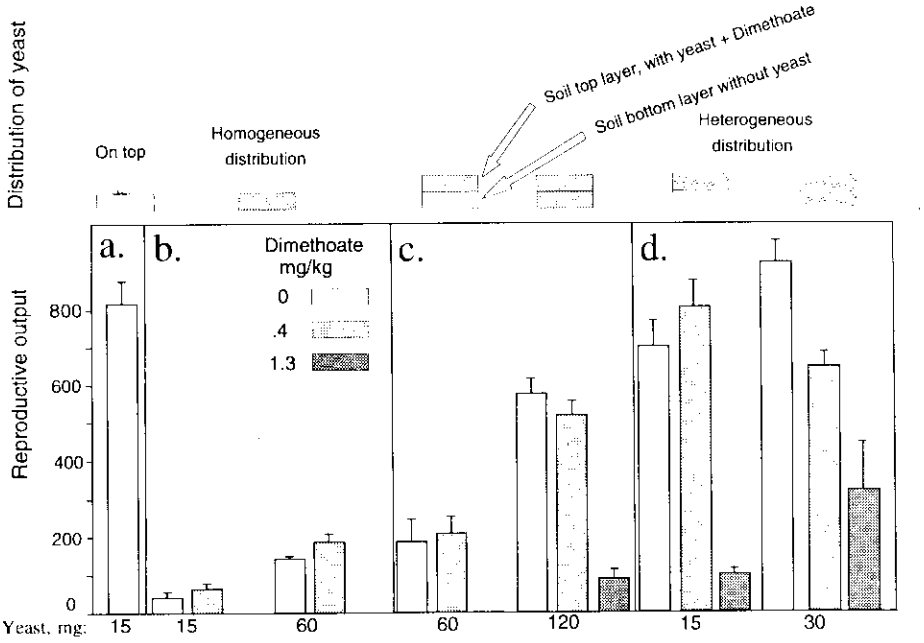

Figure 4.3

The effect of spatial distribution of yeast and dimethoate on the reproduction of F. candida. a: Control reproduction with yeast on top. b. Homogeneous distribution of yeast and dimethoate. c. Homogeneous distribution of dimethoate in the top 1 cm of soil. Left: Bottom layer without yeast. Right: Layer with yeast. d. Heterogeneous distribution of yeast. With two layers present (c. and d.) dimethoate was confined to the top layer. In experiment c. and d. right bars represent yeast in bottom layer. Vertical lines ± 1 SE (n=4).

There was a tendency to stimulation at 0.4 mg dimethoate · kg^{-1}, but above this concentration reproduction was negatively affected. Raising the amount of yeast to 60 mg increased the reproduction. When dimethoate was confined to the top layer (Figure 4.3c) the effect was identical to the single layer situation. But when the food was made available in the bottom layer toxicity decreased. Granula of yeast in the top layer increased the reproduction to normal levels (Figure 4.3d), i.e. as in the case of yeast on top. When granulae were added to the bottom layer as well, there was a reduction at 0.4 mg dimethoate · kg^{-1}, but the reproduction at 1.3 mg · kg^{-1} increased. The adults survived at the highest concentration, but their reproduction was sublethally affected as the control level was usually in the order of 100 juveniles per female but had now been reduced to 50%.

The observed effects were sublethal (reduction in average reproduction per 10 adult females) at the heterogeneous yeast distribution and at 60 mg yeast in both top and bottom layer.

The adults were significantly affected at the highest dose, but the addition of yeast to the bottom layer increased the survival.

4.3.4 **Survival and reproduction of "control" *F. candida***

Generally, three types of comparison were performed:

1) Effects of adult start age for each combination of substrate type, food type and aeration

2) Effects of combination of substrate type, food type and aeration for each age group

3) Test for interactions between the two types of effects

Acute mortality

The results are summarized in Figure 4.4.

Significantly more older than younger adults survive after one day on compressed Kalø soil, whereas there are no differences between age-groups on un-compressed soil. When the two types of substrate were compared for each age group, no statistically significant differences were found.

Adult mortality after 7 and 14 days

No effects of age were found after neither one nor two weeks. Only one type of substrate was used in these experiments (plaster of Paris/charcoal).

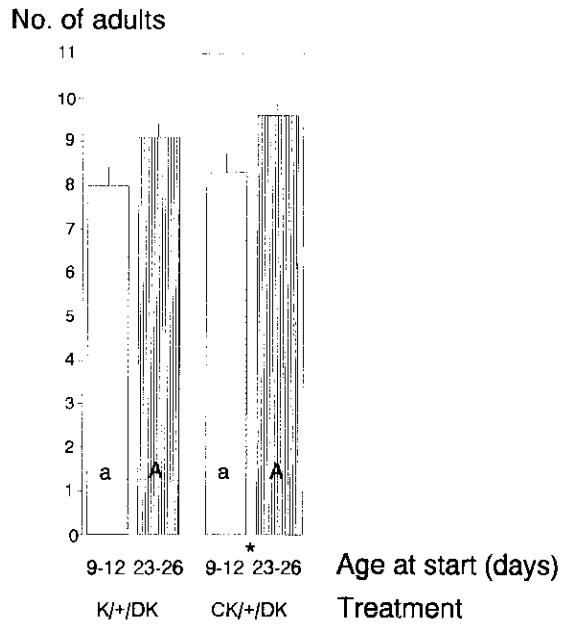

Figure 4.4

Acute mortality of adults from the two age-groups after one day (9-12 days old at start shown in left columns, 23-26 days old in right columns). Substrate types: K (Kalø soil), CK (compressed Kalø soil). Aeration: +. Food type: DK (Danish baker's yeast). Ten adults added per sample at start of experiment, 4 replicates per treatment. Standard error of the mean indicated by vertical lines. Means with same letters are not significantly different (small letters for comparison of survival of younger adults, capital letters for comparison of survival of older adults). Stars indicate statistically significant differences (p<5%) between the two age-groups.

Adult mortality after 28 days

Results are presented in Figure 4.5.

Generally, there were no statistically significant differences between the different combinations of substrate type, food type and aeration.

Only for one combination (plaster of Paris with Danish yeast and aeration) is there a statistically significant difference between age groups, more older than younger adults surviving. No interactions between the two types of effects were found.

Reproduction after 28 days

Results are shown in Figures 4.6-4.8.

For reproduction data, there were interactions between age group effects and effects of combination of substrate type, food type and aeration, younger adults generally producing significantly fewer offspring than older adults.

For older adults, OECD soil as well as plaster of Paris with Dutch yeast resulted in fewer offspring compared to Kalø soil with Danish yeast (+/- aeration). Additionally, plaster of Paris with Danish yeast gave more offspring than plaster of Paris with Dutch yeast.

The tendencies were identical for the two age-groups, but no OECD soil experiments were performed with younger adults.

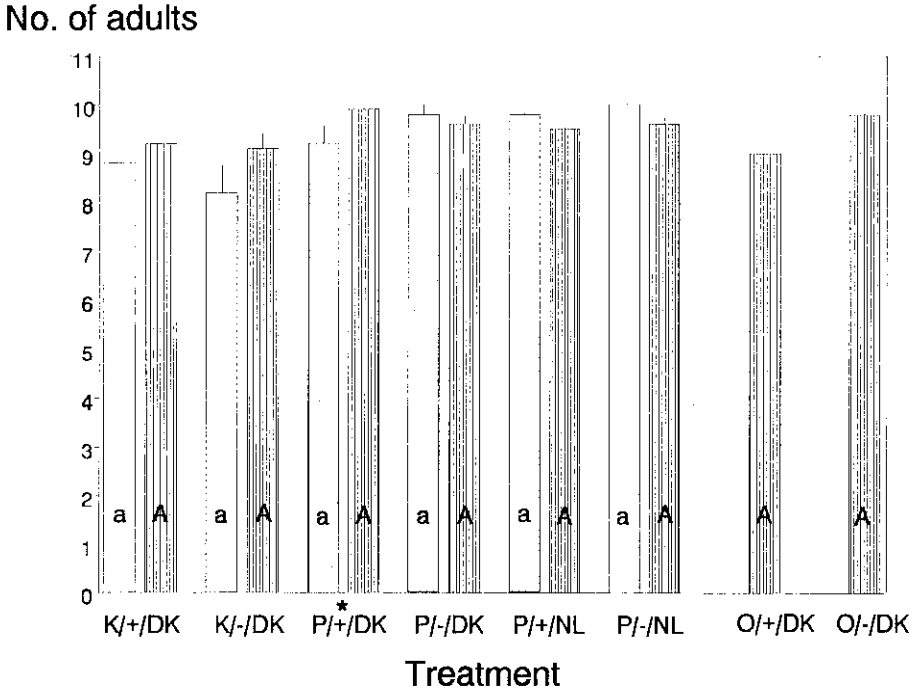

Figure 4.5

Adult survival of the two age-groups after 28 days (9-12 days old at start shown in left columns, 23-26 days old in right columns). Substrate types: K (Kalø soil), P (plaster of Paris/charcoal), O (OECD soil). Aeration: +, -. Food type: DK, NL (Danish and Dutch baker's yeast, respectively). Ten adults added per sample at start of experiment, 4 replicates per treatment. Standard error of the mean indicated by vertical lines. Means with same letters are not significantly different (small letters for comparison of survival of younger adults, capital letters for comparison of survival of older adults). Stars indicate statistically significant differences ($p<5\%$) between the two age-groups. Experiments with OECD soil were not performed with younger adults.

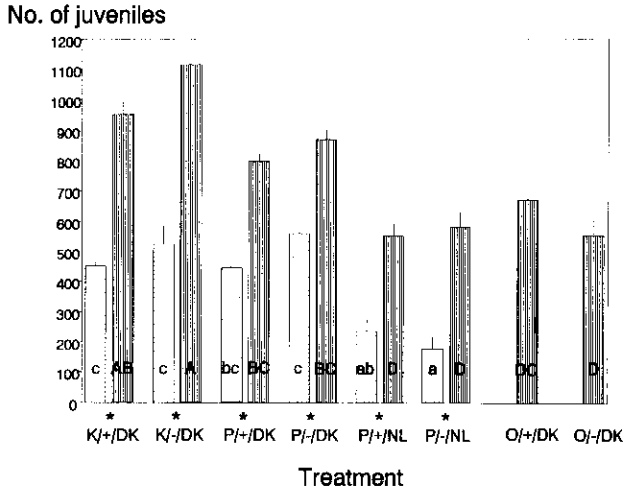

Figure 4.6

Reproduction of the two age-groups after 28 days (9-12 days old at start shown in left columns, 23-26 days old in right columns). Substrate types: K (Kalø soil), P (plaster of Paris/charcoal), O (OECD soil). Aeration: +, -. Food type: DK, NL (Danish and Dutch baker's yeast, respectively). Ten adults added per sample at start of experiment, 4 replicates per treatment. Standard error of the mean indicated by vertical lines. Means with same letters are not significantly different (small letters for comparison of number of offspring from younger adults, capital letters for comparison of offspring from older adults). Stars indicate statistically significant differences (p<5%) between the two age-groups. Experiments with OECD soil were not performed with young adults.

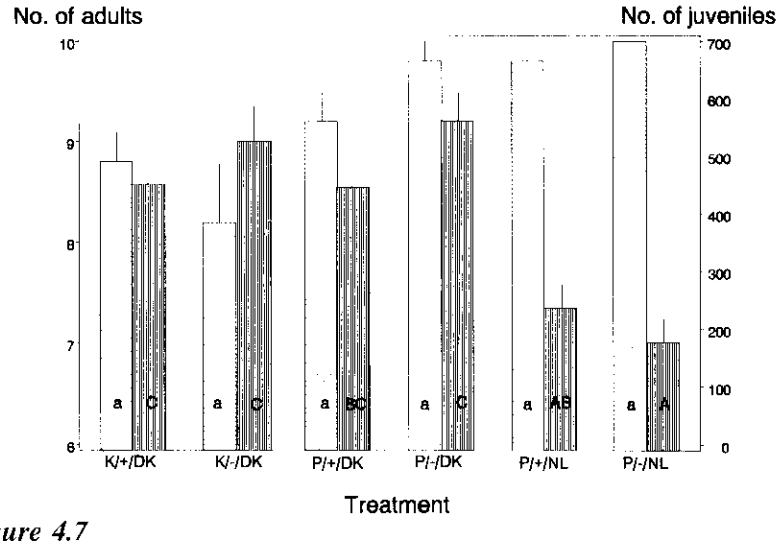

Figure 4.7

Survival (left columns) and reproduction (right columns) of younger adults after 28 days. Substrate types: K (Kalø soil), P (plaster of Paris/charcoal). Aeration: +, -. Food type: DK, NL (Danish and Dutch baker's yeast, respectively). Ten adults added per sample at start of experiment, 4 replicates per treatment. Standard error of the mean indicated by vertical lines. Means with same letters are not significantly different (p>5%) (small letters for comparison of number of adults, capital letters for comparison of juveniles).

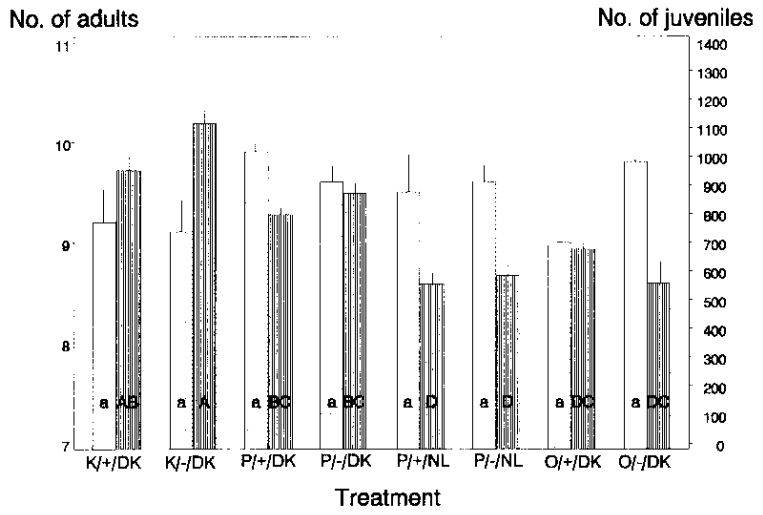

Figure 4.8

Survival (left columns) and reproduction (right columns) of older adults after 28 days. Substrate types: K (Kalø soil), P (plaster of Paris/charcoal), O (OECD soil). Aeration: +, -. Food type: DK, NL (Danish and Dutch baker's yeast, respectively). Ten adults added per sample at start of experiment, 4 replicates per treatment. Standard error of the mean indicated by vertical lines. Means with same letters are not significantly different (p>5%) (small letters for comparison of number of adults, capital letters for comparison of juveniles). Experiments with OECD soil were not performed with younger adults.

4.4 Discussion

4.4.1 Methodology

High gradient extraction

The use of high gradient extraction is preferable to water-flotation with loamy and organic soils, but may cause troubles with sandy soils because the mesh cannot retain dry sand particles. HGE is useful for mites which tend to be submerged in water when water-flotation is used as extraction technique. Another advantage of HGE is that collection of microarthropods on the plaster of Paris substrate enables determination of population size structure from undisturbed individuals in contrast with the collection from the surface of a liquid. Furthermore it is possible to make chemical analysis of contaminants in the single individuals. The extraction is of the dynamic type, thus the estimation of offspring is based on mobile individuals.

4.4.2 Dosis-response toxicity data from basic test protocol

Comparison with other studies

Numerous studies have dealt with dimethoate and mostly the application led to high mortality. Only few, however, are comparable to the present study covering well defined and computable dosage levels. The present

experiences, however, show that only dimethoate had a consistent negative effect on the Collembola compared with the two other pesticides in the study.

Sublethal effects

Sublethal effect of dimethoate was indicated through a decrease in reproductive rate per adult. If pure mortality had been the only observed effect then the reproductive rate per adult would have been constant, i.e. equal to the control.

Limitations of laboratory test systems

Test systems of this simple kind can only reveal direct effects as the elements which could mediate indirect effects are eliminated. Effects mediated through the microorganisms is possible but the importance of the microorganisms to the Collembola have not been estimated in this study. Effects of repulsion could be registered with an uneven vertical distribution of pesticide.

Comparison with field studies

Krogh (1991) observed effects on Collembola in a field study which indicated the importance of the spatial distribution of pesticide. There was a differential sensitivity of the basic life forms of Collembola corresponding with their vertical distribution. The laboratory and semi-field results (see chapter 7) await final validation from true field samples taken in the semi-field experimental area.

Modelling of dose-response enables prediction for any dose

The number of replicates (4) may seem inadequate, especially if pairwise comparisons should be done to exactly detect small (e.g. 10%) differences from the control with a high probability. The emphasize in this study has been on determining the dose-response relationship for dimethoate. This is of more general utility because knowledge of the relationship enables estimation of effects in all exposure situations that may differ concerning concentrations experienced by the organism. It was assumed that dimethoate was distributed in the upper 5 cm of the soil. If the distribution was confined to a shallow layer of, say, 1 cm, still the dose response relationship determined in this study would enable calculation of the exact effect, see section 8.3. For collembola living in this horizon a field dosage would be very harmful (i.e. LC_{90} would be exceeded).

4.4.3 **Factorial experiment**

The OECD soil was included in the factorial experiment because it is becoming a standard. However, the ingredients have not been explicitly defined and cannot be purchased as a standard from one company. E.g. a study by Van Gestel & Ma (1990) used a formulation, which resulted in a texture significantly different from ours concerning the silt and sand fractions. This points to the need for a more careful specification and perhaps production of the soil. This need could be met by a commercial or governmental supplier, if the artificial soil is supposed to be used in future test systems.

Variability in reproduction

In the factorial experiments the importance of time indicates that the conditions from one test session to another had changed. This has been improved later so the reproduction has become more reproduceable. Now the control reproduction always turns out to be between 500-1000 after 4 weeks. Thus, generally this experiment must be considered of preliminary nature so only conclusions concerning main trends can be drawn.

Bioavailability

The difference between the sandy soil and the OECD soil is illustrated in the many significant factors that includes the soil-factor (Table 4.7). Concerning the interaction between dimethoate and soil this is readily interpreted as the difference in bioavailability of dimethoate in the two soil types as dimethoate is sorbed onto sphagnum and clay in the OECD soil.

In the sandy soil there is an unknown factor which inhibits the reproduction. This inhibition is counteracted in pirimicarb and fenpropimorph treatments.

Table 4.7
Significant factors and second order interactions in the factorial design. ★: P<5 %; ★★: P<1 %; ★★★: P<1 ‰; ↔ indicates interaction. The dependent variable is the number of offspring produced during the test-period of 4 weeks. Dim: Dimethoate, Pir: Pirimicarb, Fen: Fenpropimorph.

	Artf. soil vs. sand	Artf. soil vs. loam
DIM	★★★	
PIR	★	
YEAST	★★★	★★★
SOIL	★★★	
DIM↔FEN		★
DIM↔SOIL	★★★	★★
PIR↔FEN	★	
FEN↔SOIL	★	
YEAST↔SOIL	★★★	
DIM↔PIR↔FEN	★★★	

Especially in an optimal growth situation (i.e. with surplus of food) the results with pirimicarb and fenpropimorph clearly demonstrates their potential for stimulating populations (Figure 4.2). This has also been hypothesized from a field experiment with pesticides and microarthropods (Krogh, 1991).

4.4.4 **Spatial distribution of yeast and dimethoate**

Mixing a dimethoate+yeast suspension into the soil did not enhance the toxicity of dimethoate. If this was the case, an effect at 0.4 mg $\cdot$ kg^{-1} ($\approx EC_{50}$) would be expected. On the contrary, a tendency to stimulation was observed. As the yeast is homogeneously distributed in the soil, *F. candida* is most probably not optimally fed and this may have changed the toxicity in terms of the dosis-response. This would have to be demonstrated in a test with different levels of food along with a concentration series.

When an uncontaminated bottom layer was offered as a refuge this seemed useless as the toxic pattern was identical to the one layer situation. It can be concluded that *F. candida* is not completely able to avoid dimethoate and it prefers eating in a contaminated soil instead of starving.

At the low levels of available food, the highest concentration of dimethoate was more harmful to the Collembola than when food was plenty. The results also indicate that contamination of the food can stimulate the reproduction. This was not seen when uncontaminated food was available in the bottom layer.

Conclusion

Thus, different heterogeneous distributions of dimethoate and food affected on the control reproductive level as well as the outcome of the toxicity test.

4.4.5 **Survival and reproduction of "control" *F. candida***

Acute mortality

Since differences in survival between age-groups are only found in compressed soil, where no extraction is needed to count the Collembola, younger adults are probably either less affected by extraction, or require a more natural environment than older adults (compressed soil offer only very few holes in which the animals can hide). The last explanation seems the most likely, since there are no differences in survival between substrate types for either age-group.

Long-term mortality

As there are no differences in adult survival after 28 days, the differences between age-groups in acute mortality are probably compensated for later on.

Reproduction

Reproduction is highly affected by the age of the adults at the start of the experiment, but there is no clear correlation between survival of adults and number of offspring produced (Figures 4.5-4.8), since reproduction in some instances is affected by experimental conditions, whereas adult long-term survival is not.

4.4.6. **Conclusion**

The reproduction of two closely related soil microarthropods, *Folsomia candida* Willem and *Folsomia fimetaria* L. (Insecta: Collembola), was tested under the influence of the insecticide dimethoate. Dimethoate had an adverse effect on survival of adults and their reproduction in concentrations at the recommended field dose corresponding to 0.4 mg $\cdot$ kg^{-1}, with *F. fimetaria* being more sensitive than *F. candida*.

The experimental conditions were altered to evaluate the realism in the basic single species/single chemical reproductive test system. The importance of the spatial distribution of dimethoate was studied with food applied to the surface (original procedure), mixed homogeneously in the whole soil profile or just in the top-layer or mixed heterogeneously into the soil preserving the small granula of yeast originally in the commercial formulation. Toxicity decreased significantly when exposure could be avoided in an uncontaminated bottom layer and even more if food was available in this soil horizon. But the results indicates that Collembola are not able to completely avoid dimethoate when they have the choice. For extrapolation purposes a simple test system may be sufficient as EC_{50} is changed less than one order of magnitude with the different test designs used in the study.

In terms of EC_{50} the outcome of toxicity tests with a heterogenous distribution of food and dimethoate is changed only slightly but the effects to suboptimally fed populations should be considered as they may be more vulnerable.

Reproducion and survival of *F. candida* were investigated under control conditions: differing substrate forms, two commercial forms of yeast, and weekly aeration. The results show that reproduction is probably more sensitive than mortality to sub-optimal test conditions, especially food source. This speeks in favour of using reproduction as test parameter rather than mortality, if other parameters can be kept sufficiently stable, since the importance of well-standardized test conditions in order to obtain comparable results is also strongly indicated. Consequently, when setting GLP standards should be well-defined and standardized for reproduction tests with Collembola, such as food source, age-groups, and substrate type, and the test organisms should be handled and extracted with care.

- *F. candida* and *F. fimetaria* respond to dimethoate exposed through soil at a dosage around the recommended field rate corresponding to 0.4 mg $\cdot$ kg^{-1}, assuming homogeneous distribution in upper 5 cm of soil.
- Dimethoate has a predominantly direct effect resulting in decreased reproduction due to adult mortality.

- *F. candida* is less sensitive to dimethoate than *F. fimetaria.*
- *F. candida* is not able to avoid dimethoate when offered an uncontaminated soil layer.
- In terms of EC_{50} the outcomes of toxicity tests with a heterogenous distribution of food and dimethoate are changed only slightly but the effects to suboptimally fed populations should be considered as they may be more vulnerable.
- Yeast type gave rise to the largest observed difference in reproductive output. Food source, age-groups and substrate type should be well-defined and standardized, and the test organisms should be handled and extracted with care.

5 Laboratory toxicity testing with a predaceous mite

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National Environmental Research Institute

See Annex II

The main experience with the predaceous mite is described in Annex II.

A new testsystems with a soil dwelling mite

A single species reproductive test system with the predaceous mite *Hypoaspis aculeifer* Canestrini (Gamasida: Laelapidae) has been developed. This mite breeds successfully on the prey organisms *Folsomia fimetaria* L. or *Folsomia candida* Willem (Collembola). Synchroneous cultures for the tests were produced as shown in Table 2, Annex II.

Characterization

To determine appropiate test conditions the chosen test species *Hypoaspis aculeifer* (Gamasida: Laelapidae) was characterized with respect to growth, egg production, consumption and satiation. The growth was determined by digital image proccessing, whereby the size could be measured without disturbing the mite. This is not possible with a determination of fresh weight. However, correlations between size parameters and weight have been determined elsewhere (Krogh and Holmstrup in prep).

Procedure

The procedure for the testing of chemicals on reproduction and survival of *H. aculeifer* was basically identical to the test with Collembola presented in chapter 4. Ten female and five male *H. aculeifer* of age 16-19 days old were added to the test soil and incubated for three weeks at 20° C. Every week 700-900 16-19 days old *F. candida* were added. This is close to the point of "satiation". Old adults of collembolans were added to secure surplus of food. Reproduction during this period is 200-400 juveniles. The procedure can be summarized in the following steps:

- Mixing of pesticides and inoculate with defaunated soil.
- Transfer of synchroneous 16-19 days old mites to test soil.
- Addition of 700-900 prey of 16-19 days old *F. candida* each week.
- Incubation in climate chamber at 20° C.
- Termination of test after 3 weeks. Extraction of microarthropods from soil in high gradient extractor at 25° C increasing to 40° C during two days.

Dose-response data

Trials testing fenpropimorph and pirimicarb showed no negative effects on the reproductive output of the mite up to the maximum dosage of approx. five times the recommended field dosage (cf. Table 4.1). Dimethoate sho-

wed a stimulatory effect at 0.4 mg · kg^{-1} (LOEC) and a reduction at three times the recommended field dosage (LOAEC), and an EC_{50} in the range of $0.8 < EC_{50} < 1.0$. The stimulation is believed to belong to a physiological response type: hormesis (Stebbing 1982).

Toxicity of dimethoate in the top-layer of the soil

When dimethoate was confined to the top-layer of the soil it still showed an effect, but now the hormesis was observed at 0.7 mg · kg^{-1}. Thus, *H. aculeifer* apparently received a smaller dose in this situation, but was not able to avoid the pesticide by adjusting its behaviour.

Factorial experiment

A factorial design with the three pesticides and presence/absence of prey, two soil types, the artifical OECD soil and a loamy soil, was tested in all possible combinations (Figure 5.1). The design was identical to the one described in paragraph 4.2.3. The fungicide had a stimulating influence in the loamy soil compared with the OECD soil. *H. aculeifer* depended completely on the added collembolan food source and therefore did not reproduce when no prey was available. The recommended dose levels showed no other trends.

Conclusions

The study shows that the following points may be of importance when performing toxicity testing with Gamasida 1) Stimulations of the reproductive output of the mite at low doses of pesticide below and around field rates 2) An uneven distribution of pesticide due to surface application compared to a homogeneous distribution in the test soil.

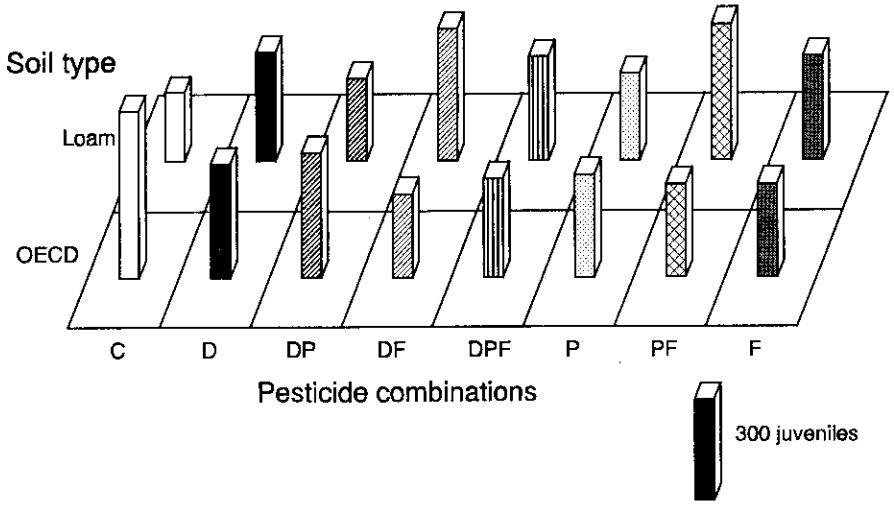

Figure 5.1
Reproductive output of H. aculeifer in a factorial experiment with the three pesticides in combination with the OECD soil and a loamy soil.

The study showed that dimethoate has two ways of affecting the predaceous mite *H. aculeifer*: an initial stimulation at low concentrations around field dosage and an adverse effect at doses from about two times the field dosage and upwards. It remains to be investigated if these results have any relevance to the field situation and are valid for other gamasid mites.

6 Microbial respiration in soil

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National Environmental Research Institute

6.1 General background

The aim of this part of the project is to study the influence of the three chemicals on soil respiration. CO_2 evolution is used as a measure of net respiration, both on soil cores from the sprayed field and in a laboratory test setup with natural soil and OECD standard soil. Additionally, CO_2 evolution is compared to Cotton Strip Assay, an alternative measure of microbial activity.

6.2 Methods

6.2.1 Field-treated soil

On four occasions (2 July, 28 July, 18 August and 1 September 1992), soil cores from the upper five cm were collected at the field site (Figure 7.2), one core from each of the 50 plots. The plots were sprayed on June 19, 1992. Cores from neighbour rows were mixed (treatment-wise) in order to half the number. The soil was sieved (2 mm sieve) to exclude stones and larger animals. Water content was determined and adjusted to 50 % of field capacity. 30 g wetted soil was placed in a 72 ml glass with a rubber stopper in which a silicone septum was inserted. Once a week the glasses were aerated by removing the stoppers.

For CO_2 analysis, 1 ml of air was removed from each glass with a syringe and analyzed in a gas chromatograph.

On 2 July and 1 September, half the soil from each core was amended with lucerne (Elmholt, 1992) before incubation, giving two series of assays for each date. CO_2 evolution was determined on 4 and 5 occasions, respectively (2 and 27 July, 17 and 31 August; 1, 7, 16, 21, and 28 September).

6.2.2 2^5 factorial experiment

The design was the same as used in the reproduction experiments with *Folsomia candida* (see Section 4.2.3), except that the treated soil was placed in air-tight glasses of the same type as for the field-treated soil.

Kalø soil and OECD standard soil were used. 2 × 2 replicates were started with 2 days interval, and CO_2 evolution was measured four times with intervals of one week.

Air extraction and CO_2 analyses proceeded as for field treated soil.
In addition to respiration, the reproduction of *F. candida* was determined after four weeks (see Section 4.2.2) in order to compare the sensitivity of these two parameters.

A pilot project proved that animal respiration was negligible compared to microbial respiration.

6.2.3 **Laboratory cotton strip assay**

As an alternative way of quantifying pesticide effects on soil microorganisms, the loss of tensile strength in cotton fabric placed in soil treated with four different concentrations of fenpropimorph (1.04, 2.08, 5.21, 10.42 mg $\cdot kg^{-1}$) plus one control was measured (Harrison et al., 1988). For each concentration, ten strips 2cm×8cm (equal numbers of longitudinal threads) were placed in plastic boxes on one cm of pesticide treated Kalø soil and then covered with one cm of treated soil. After three weeks at 20° C, the strips were washed in demineralized water, and tensile strength was determined.

6.3 **Statistics**

Differences between treatments were analyzed using SAS/STAT GLM procedure (SAS Institute Inc. 1988A). Additionally, the influence of replicate number was tested in the factorial experiment, since the replicates were started with two days interval.

6.4 **Results**

6.4.1 **Field-treated soil**

When respiration in soil from each treatment was compared, no statistically significant differences were found for any of the dates, neither with nor without lucerne amendment (Figures 6.1-6.3).

6.4.2 **2^5 factorial experiment**

The results of the four replicates differed, but since grouping according to start date did not give a more uniform result, all four replicates were used in the tests.

Figure 6.1
Mean soil respiration in field samples from all treatments collected on July 2 (upper part) and September 1 (lower part), respectively. Half the samples were amended with lucerne meal. N = 25, vertical bars indicate SEM.

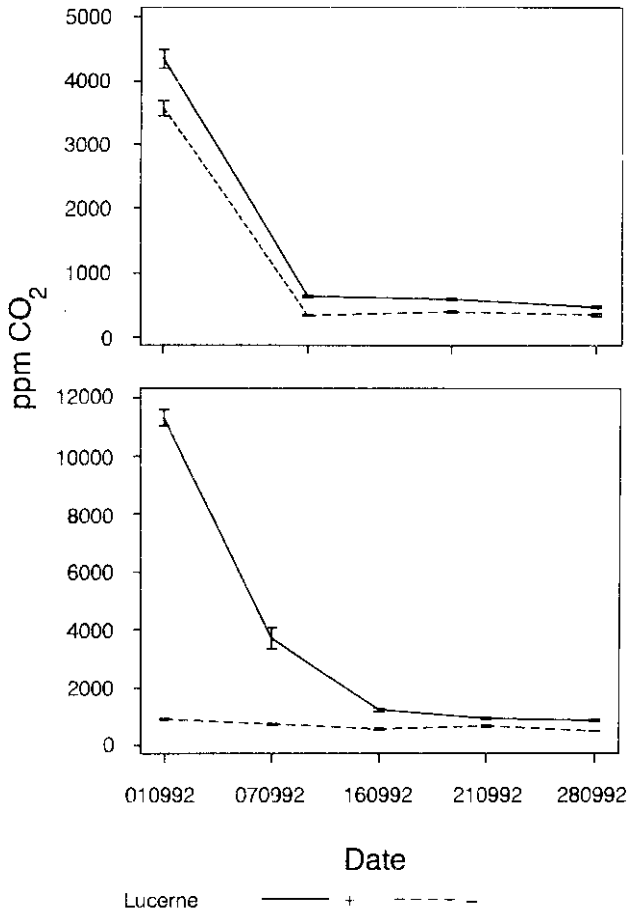
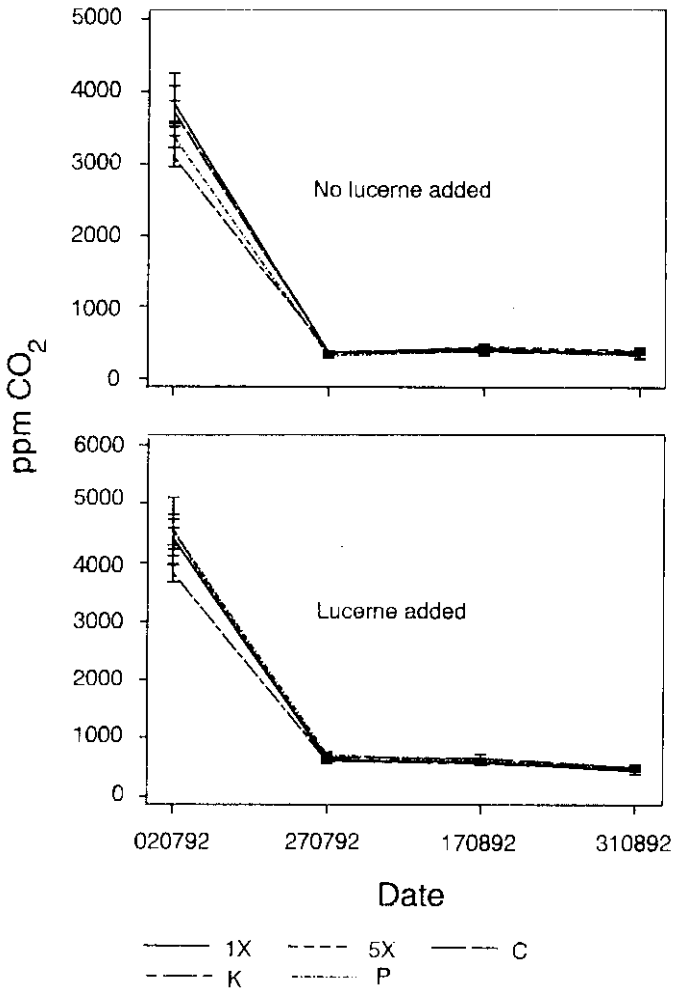


Figure 6.2
Soil respiration in field samples collected on July 2. Neither samples amended with lucerne (lower part) nor unamended samples (upper part) show any significant differences between treatments (p > 0.05). N = 10, vertical bars indicate SEM.
Signatures: K = control, C = Corbel (fenpropimorph) at recommended field dose, P = pirimicarb at recommended field dose, 1× = dimethoate at re-commended field dose, 5× = dimethoate at five times recommended field dose.



As shown in Figure 6.4, the only significant differences in respiration were caused by soil type and addition of food (+/- yeast), loamy soil and especially addition of yeast giving higher respiration throughout the four weeks.

For reproduction, only addition of yeast caused significant differences, giving a mean reproduction of 21 juveniles per ten adults without yeast and 696 with yeast.

6.4.3 **Laboratory cotton strip assay**

The tensile strength of cotton strips was affected by fenpropimorph, as shown in Figure 6.5. Strips placed in soil treated with 5.21 mg · kg^{-1} soil were significantly more degraded than strips from untreated soil.

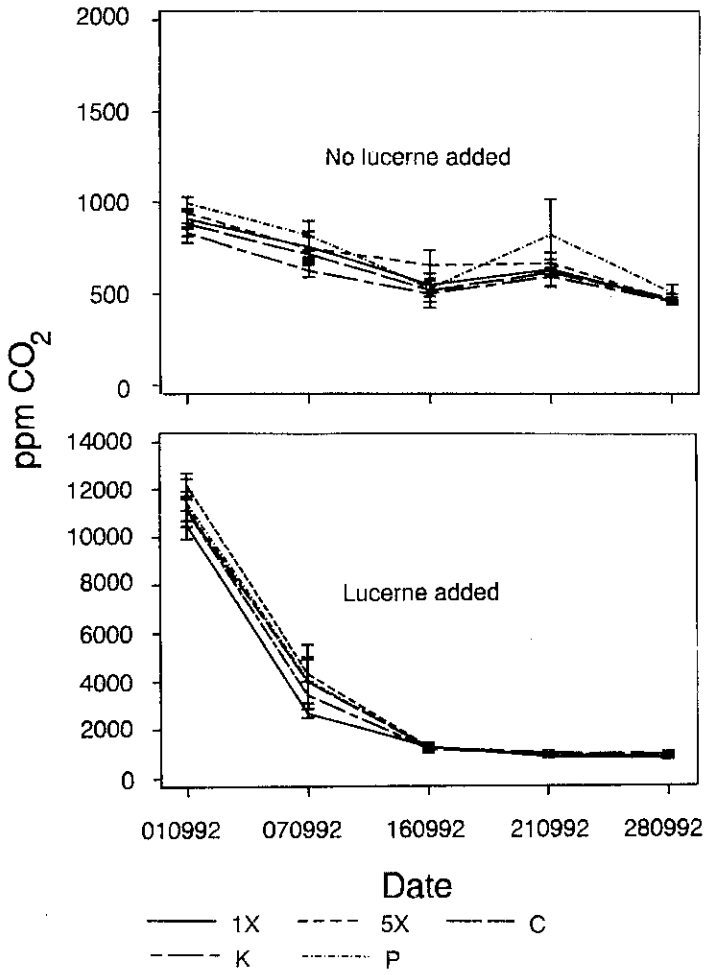


Figure 6.3

Soil respiration in field samples collected on September 1. Neither samples amended with lucerne (lower part) nor unamended samples (upper part) show any significant differences between treatments ($p > 0.05$). N = 10, vertical bars indicate SEM. Signatures: K = control, C = Corbel (fenpropimorph) at recommended field dose, P = pirimicarb at recommended field dose, 1× = dimethoate at recommended field dose, 5× = dimethoate at five times recommended field dose.

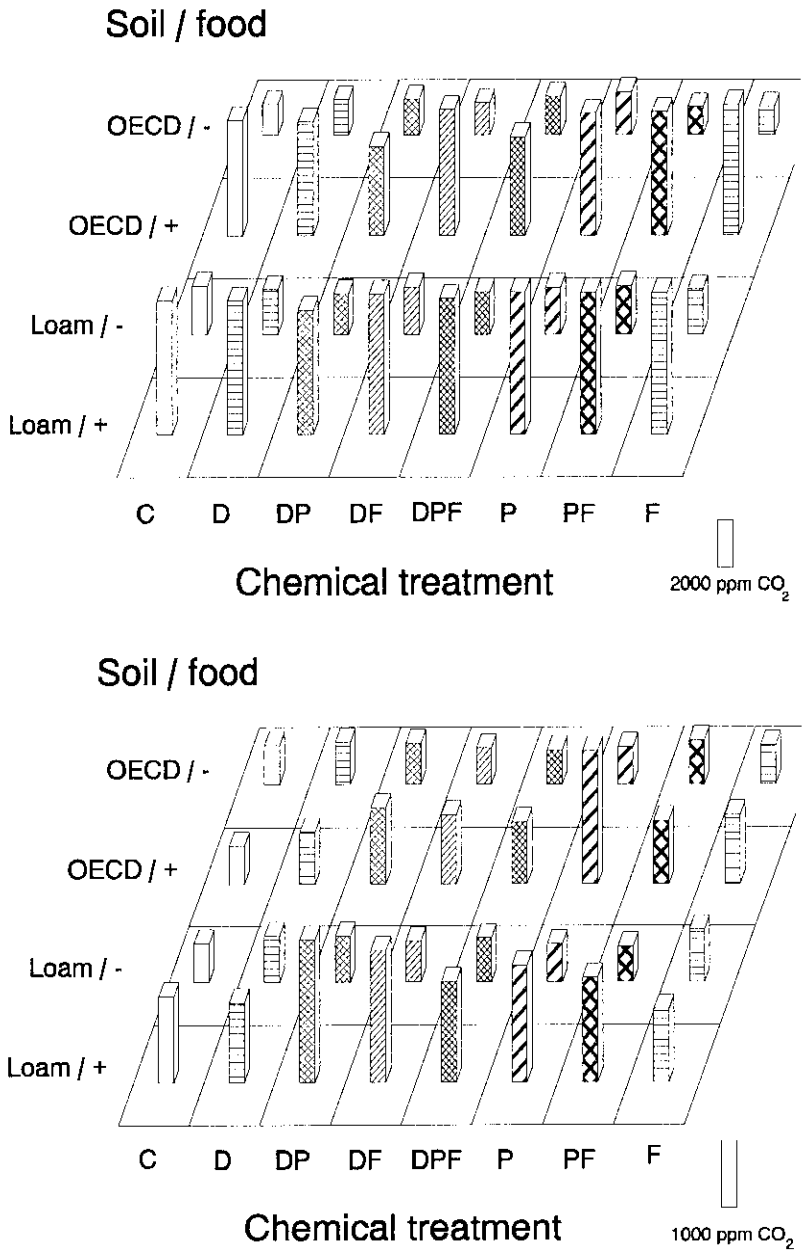


Figure 6.4

Soil respiration at the start of the 2^5 factorial test (upper part) and after four weeks (lower part). Height of columns indicate ppm CO_2. N = 4. Signatures: OECD = OECD standard soil, Loam = natural loamy soil, + = yeast added as food, - = no yeast added; C = control, D = dimethoate at recommended field dose, P = pirimicarb at recommended field dose, F = fenpropimorph at recommended field dose.

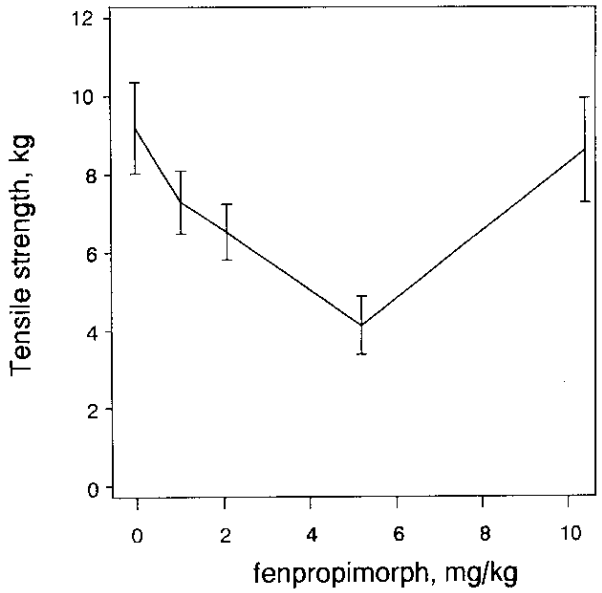


Figure 6.5
Effects of fenpropimorph on the decomposition of cotton strips, measured as tensile strength. N = 10, vertical bars indicate SEM.

6.5 Discussion and conclusions

Either soil microorganisms are rather insensitive to treatment with the three pesticides, or soil respiration measured as CO_2 evolution is a parameter too general for detecting pesticide effects, new groups of organisms taking over when one group is affected. The latter is in agreement with Elmholt (1992) and Helweg (1988), who found no or only negligible effects on soil respiration at realistic pesticide dosages.

The results from the laboratory cotton strip assay indicate that this or other methods may be more adequate than soil respiration procedures for estimating effects of pesticides or other chemicals on microorganisms. Fenpropimorph might be expected to hamper the breakdown of cotton strips through its fungicidal effects. However, this is not the case, probably either because fungi are not the main decomposers of cellulose, or because unaffected species take over.

In accordance with Chapter 4.5, yeast is highly decisive for the number of offspring produced by Collembola.

7 Development of a semi-field method for evaluation of laboratory tests as compared to field conditions

Henning Petersen and Peter Gjelstrup
Natural History Museum, Mols Laboratory

7.1 Introduction

The main purpose of the subproject has been to develop a test which can be used to evaluate whether extrapolations of ecotoxicological laboratory results to complex field situations are justified.

An evaluation of this question should ideally be done by studying effects of pesticide treatment on field populations. One main obstacle to such true field experiments is the high degree of variability in composition and population size which naturally occurs within short distances in a field soil. This "noise" reduces the possibility for detecting treatment effects with a given sampling effort. Therefore, experiments based on the census of field populations tend to be very time demanding because a high number of replicate samples has to be treated in order to attain a reasonable degree of statistical confidence. Field samples provide information on the result of population dynamic events following a treatment but it is rarely possible to deduce population dynamic parameters such as survival, reproduction and body growth from that information.

It was believed that a semi-field method could be developed which overcomes the above mentioned problems associated with traditional field experiments while at the same time preserving close to natural living conditions for the soil fauna. This method should ensure that a known start population be kept isolated from the field populations through the experiment thus making a direct measurement of population growth possible. That goal was attempted by means of field microcosms (mesocosms following the terminology of Teuben and Verhoef, 1992), i.e. small enclosures containing field soil and the original microflora but without microarthropods except for the test species. The microcosms were exposed to natural temperatures, precipitation and other environmental conditions of the experimental field.

Teuben and Verhoef (1992) compared results of microbial respiration, enzyme activities and availability of macronutrients obtained from laboratory microcosms, field mesocosms and unmanipulated forest floor. It was concluded that there was a high correlation between results produced by field mesocosms and field data and that results from field mesocosms showed better agreement with field results than results obtained by laboratory microcosms.

The present subproject was closely coordinated with the subproject dealing with laboratory tests on Collembola and gamasid mites carried out at the Department of Terrestrial Ecology, The National Environmental Research Institute, Silkeborg (this report, chapters 4 and 5). Thus, the same soil, test animals and pesticides have been used in both laboratory and semi-field experiments.

Two progress reports (Gjelstrup and Petersen 1991, Petersen and Gjelstrup 1992) include some details of the work and results from the two first years of study which have not been repeated in the present report.

7.2 Material and methods

7.2.1 Field sites

The field site used for the preparatory experiments in 1990 was a spring barley field on loamy soil at the agricultural experimental station in Ødum, north of Århus. For practical reasons an experimental field, Stegeløkke, at the estate of Kalø belonging to the Forest and Nature Agency of the Danish Ministery of Environment was chosen for the continued experiments in 1991 and 1992. The soil of the two sites are similar (Krogh, Table 4.3). The particular part of the experimental field used at Kalø (Stegeløkke, parcel U12) had not been sprayed with pesticides since 1986. The field was sown with barley in April 1991 and 1992 and harvested August 30, 1991. In 1992 the field was harvested August 12 - 20 but the area used for the experiment was left unharvested.

Collembola fauna at Ødum and Kalø

The density of total Collembola at the two fields, Ødum and Kalø, differed by a factor 4 in early spring samples (unvegetated ploughed soil). The highest density was found in the Kalø field in July 1991 (*Table 7.1*). The composition of species differed somewhat. Thus, at Kalø *Tullbergia macrochaeta* was strongly dominant in July 1991, but not in March. The most dominant species at Ødum was *Isotoma notabilis* (66 %). *Folsomia fimetaria* which was used as the main test species (cf. section 7.2.4) was a little more abundant at Kalø than at Ødum.

Table 7.1

Population density and dominance percent of Collembola from spring barley fields at Ødum, 1990, and Kalø, 1991.

Species	Ødum, 20.02 1990, 10 units 0-10 cm depth		Kalø, 7.03 1991, 20 units 0-10 cm depth		Kalø, 30.07 1991, 25 units 2-12 cm depth	
	no.m^{-2}	percent	no.m^{-2}	percent	no.m^{-2}	percent
Hypogastrura succinea	700	4.1	510	10.7	0	0
H. denticulata	0	0			0	0
Willemia intermedia/spp.	100	0.5	180	3.8	1,020	4.2
Friesea mirabilis	100	0.5	6	0.1	0	0
Micranurida pygmaea	10	0.1	0	0	0	0
Onychiurus armatus s.l.	200	1.0	640	13.3	2,170	8.9
Tullbergia macrochaeta/spp	1,400	11.3	370	7.7	18,390	75.7
Anurophorus laricis	10	0.1	0	0	0	0
Folsomia quadrioculata/nana	10	0.1	80	1.6	0	0
F. fimetaria	300	1.7	530	10.9	770	3.2
Proisotoma minima	0	0	40	0.9	0	0
Isotomiella minor	0	0	6	0.1	0	0
Isotoma notabilis	10,900	65.7	840	17.6	640	2.6
I. anglicana	700	4.1	230	4.8	20	0.1
I. tigrina	1,200	7.2	260	5.4	0	0
Isotomurus palustris	400	2.3	120	2.5	0	0
Pseudosinella alba	30	0.2	70	1.4	550	2.2
Lepidocyrtus spp.	0	0	260	5.4	490	2.0
Heteromurus nitidus	0	0	6	0.1	0	0
Megalothorax minimus	30	0.2	60	1.2	0	0
Sminthurinus elegans	300	1.8	1,500	24	290	1.2
Bourletiella hortensis	300	1.7				
Sminthurus viridis	10	0.1				
Total Collembola	16,600	100	4,800	100	24,300	100

7.2.2 **Field microcosms (field enclosures)**

The starting point for development of the semi-field method was the type of enclosures used by one of us (Petersen 1971) for the study of body growth of ^{32}P tagged specimens of the collembolan *Onychiurus furcifer* in its natural habitat, i.e. litter and soil of a Danish beech forest. The enclosures were simply tubes open in both ends which were pressed into the forest floor to a depth of 5 cm. About a third of recently hatched speci-

mens of the collembolan was recovered and identified from the enclosures until 9 weeks after introduction. Considering possible losses due to damage by handling, extraction, predation, too weak radioactivity etc. it was concluded that the open tubes made up reasonably efficient confinements for the introduced specimens.

In the original experiment described above the soil inside the enclosures was practically undisturbed and contained the original microflora and fauna. The topical experiment aiming at examining population growth, however, requires that the start population of the test species is known and that immigration or emigration of that species do not occur during the experiment. Therefore, it was in practice neccessary to remove the microarthropod fauna from the soil substrate used in the enclosures by a defaunation process (see section 7.2.3) and subsequently introduce a known number of test specimens. Further, it was indispensable to test different types of enclosures or field microcosms in order to find a method which could satisfactorily isolate the test specimens from individuals of the same species in the surrounding field soil while differing so little as possible from the environment of an undisturbed soil.

Seven variants of microcosms

Seven different variants of field microcosms were tested during the three year study period. The high number of different variants tested was mainly due to unexpected difficulties of keeping the introduced experimental population isolated from the microarthropod populations of the surrounding soil.

a) High "open" cylindrical microcosm (inner diameter: 10.5 cm, length: 30 cm) with lateral holes covered by fine-meshed web. Open in both top and bottom. Inserted 27 cms into the soil. 27 cm layer of soil substrate inside microcosm. Rim of cylinder projecting 3 cms above soil surface. Divided into 3 x 9 cm strata during extraction. Tested 1990.

b) As *a*, but bottom closed with fine-meshed web. Tested 1990.

c) Low "closed" microcosm (inner diameter: 10.3 cm, length: 10 cm) with both top and bottom covered by fine-meshed web (Figure 7.1.B). Completely buried below 2 cm soil. Mesh-covered lid and soil cover removed during pesticide treatment. Tested 1991.

d) As *c*, but mesh-covered lid and soil not removed during pesticide treatment. Tested 1991.

e) High "open" cylindrical microcosm as *a* without lateral holes. Bottom closed with fine-meshed web. Inserted 20 cms into the soil. 20 cm

layer of soil inside microcosm. Rim of cylinder projecting 10 cms above soil surface. Divided into 2 x 10 cm strata during extraction. Tested 1991.

f) High "open" microcosm as *e*, but provided with a "collar-trap" containing benzoic acid around the projecting rim (Figure 7.1.A). Tested 1992.

g) Low "open" microcosm. As *f*, but inserted 10 cms into the soil and with only 10 cm layer of soil inside the microcosm. Tested 1992.

The fine-meshed web used for all microcosm types was a polyester monofilament web (Estal Mono 120T, Juhl A/S, Malling, Denmark) with a very exact meshwidth (45 microns) sticked on the surface of the microcosms by contactglue. The joinings were further secured by strong adhesive plastic-tape. The meshwidth was chosen to be narrower than the cross-section of the smallest Collembola. The head capsule width of newly hatched *Tullbergia macrochaeta* and *Folsomia fimetaria* was measured as 60 and 80 - 90 microns, respectively, and the diameter of eggs of the same species as 70 - 90 and 120 microns, respectively. In spite of the narrow mesh-width water can easily pass through this kind of web.

High "open" microcosm

re a and b. Both types of microcosms were 30 cm long pieces of PVC-tubes (drain pipes). In order to ensure the best possible exchange of humidity and microflora with the surrounding soil 12 holes (4 cm diameter) were drilled in the tube-wall at three levels and the whole surface of the tubes were covered with fine-meshed web. The bottom of the microcosms reached the compact soil of the ploughing sole which was believed to be difficult to pass for the soil microarthopod fauna. Defaunated soil was added subsequently to the level of the surrounding soil surface. In order to test for possible immigration or emigration a tape smeared with insect-glue was attached to the uppermost end of the microcosm. However, adhering dust soon made it impossible to distinguish the animals stuck in the glue.

"Closed" type microcosm

re c. The completely "closed" type of microcosm used for the 1991 experiments was constructed from plastic canisters with screw lid (Figure 7.1.B). The bottom part was cut off resulting in a 100 mm high microcosm. A 90 mm diameter hole in the lid and the cut off bottom of the canister was covered with the fine-meshed polyester web specified above. Great care was taken to ensure that the web was tightly sealed to the lid and canister by means of glue and strong adhesive plastic tape leaving no possible passages for the microarthropod fauna. A packing of dry elastic silicone glue was made on the inside of the lid in order to ensure a tight sealing between the lid and the upper edge of the canister.

The lid had to be unscrewed and the soil layer covering it had to be removed for a short moment while the plot was sprayed with pesticides because it was suspected that the polyester web might interfere with the downwards movement of the pesticides. Especially, screwing on the lid after spraying using safety clothes, gloves and face guard proved to be difficult and implies a risk of incomplete closure of the lid.

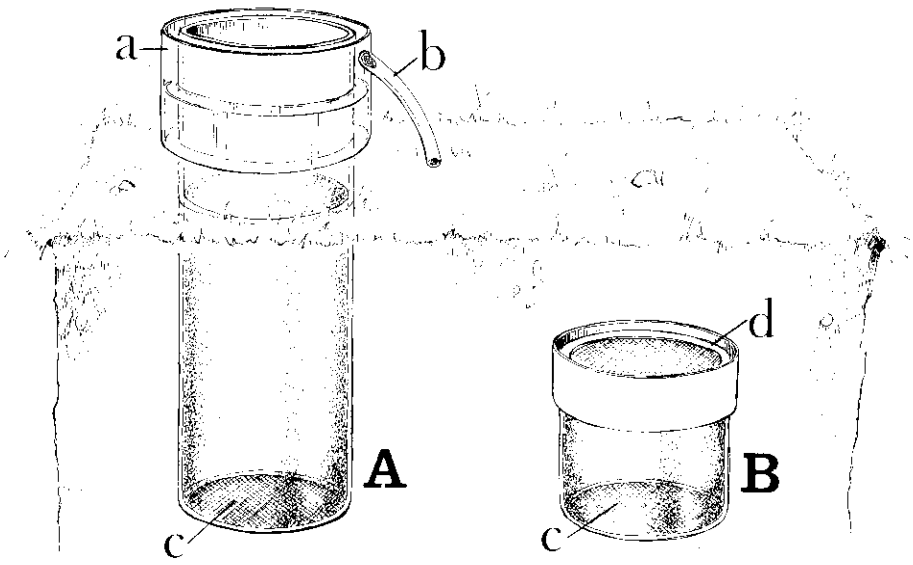


Figure 7.1

Two types of field microcosms. - A: high "open" type, B: low "closed" type. - a: "collar-trap" with benzoic acid solution, b: overflow drain closed with fine-meshed web, c: bottom covered with fine-meshed web, d: lid with hole covered fine-meshed web.

Ways of access for soil microarthropods

After examination of the fauna extracted from the "closed" type field microcosms it became apparent that specimens belonging to several collembolan and mite species other than the introduced test species were found in the microcosms in spite of the extreme care taken to prevent possible passages for the microarthropods. A test of the tightness of the "closed " type microcosms was made in order to trace possible ways of access for microarthropods (Petersen and Gjelstrup 1992).

Soil from the experimental field, beach sand and compost with its naturally occurring fauna was treated in the extractor under the following conditions (2 replicates for each condition): *1*) normal extraction without confinement, 2) confined in closed type enclosures without extra tape between canister and lid, *3*) as *2* but with tape between canister and lid, *4*) as *3* but inversed so the lid was turned down in the extractor, and *5*) confined in a carefully closed bag made of the 45 micron-mesh web.

Test for interception of pesticide by polyester web

re d. In order to test whether the pesticide effect on *Folsomia fimetaria* was lowered by the passage through a fine-meshed polyester web 6 microcosms of type *c* were exposed to spraying by dimethoate in 5 times recommended dosage without removing lids and overlying soil.

re e. It was believed that the higher wall separating the surface of the microcosm from the surrounding soil surface and the absence of lateral holes might improve the ability of the microcosm to keep the test population isolated from the microarthropods of the surrounding soil.

High and low "open" type microcosms with "collar-traps"

re f and g. The "collar-trap" (Figure 7.1.A) was made from canisters of soft PVC (bottom diameter: 12.5 cm, top diameter: 14 cm, height: 7.5 cm). A hole with the same diameter as the microcosm tube was cut in the bottom and the canister sealed tightly to the microcosm 7 cms from its upper edge by means of silicone glue. A narrow hole covered by fine-meshed web near the upper edge of the collar vessel connected to a 25 cm long flexible tube functioned as drain in case of high rainfall. The "collar-trap" was filled about 1/3 up with benzoic acid in aqueous solution. The content of the trap was emptied by means of a wash bottle.

7.2.3 **Preparation of defaunated soil**

Soil from the field site was treated in order to eliminate the soil microarthropods in a way which as far as possible does not disturb the physical and chemical properties of the soil and facilitate a fast recovery of the soil microflora. This goal was attempted by treating a sieved natural soil with alternating heating, freezing, drying and rewetting within limits which did not radically exceed extremes occurring in the nature. The method used in the three years was essentially the same and is similar to the one used by Krogh (section 5). The differences mainly concerned the duration of some of the treatments (see Gjelstrup and Petersen 1991, Petersen and Gjelstrup 1992). In 1990, in addition to the unamended soil from the Ødum field, soil amended with 1/6 defaunated horse manure and 1/6 defaunated commercial mould for flower pots was also used.

Direction for preparation of "defaunated" soil

The final direction followed in the 1992 experiments is as follows:

a) Sieve fresh soil through a coarse sieve (20 mm mesh width) and incubate at 20° C for 2 days.

b) Heat and dry the soil in metal trays (aluminum-foil baking tins) at 60° C for 4 - 6 days.

c) Moisten to about field capacity

d) Leave at room temperature (c. 20° C) for 3 - 6 days

e) Freeze to - 25° C for 2 - 6 days

f) Heat and dry the soil once more at 60° C for 2 - 6 days

g) Freeze to - 35° C for 5 - 8 days

h) Keep the soil until needed at - 18° C

Test of efficiency of defaunation

A test of the efficiency of defaunation was carried out in 1990 by comparison within 10 pairs of sample units from the experimental field at Ødum. One unit from each pair was defaunated using approximately the method described above but without the final freezing to - 35° C (drying/-heating in microarthropod extractor at 50° C for 2 days, at 60° C for 2 days, moistened and stored at 30° C for 3 days, freezed to -18° C for 2 days, heated to 40° C for 2 days, stored in freezer at -18° C until needed).

In 1991 a test was carried out by storing 5 "closed" type microcosms filled with defaunated soil in a thermostated room at about 10° C for 73 days (June 18 - August 30). The microcosms were kept moist through the period. In 1992 the test was repeated with 10 replicate microcosms stored for 5 weeks at room temperature.

7.2.4 **Choice of test animals**

Collembola and gamasid mites were chosen for the experiments because they are among the most dominant soil microarthropods in soil. Besides, Collembola were chosen as they are largely feeding on soil microflora, whereas gamasid mites are carnivores eating Collembola, other mites etc.

7.2.4.1 **Collembola**

4 species tested in 1990

It was decided to choose a species of Collembola common in Danish agricultural soils as the main test species. In a further set of experiments a test based on a Collembolan and a gamasid mite predator was explored. It was anticipated that only exclusively soil-living, i.e. eu-edaphic, species would be possible to confine within the types of open field microcosms proposed for the tests. Among the eu-edaphic collembolan species found in the exploratory sample at Ødum in March 1990 (Table 7.1) *Tullbergia macrochaeta*, *Folsomia fimetaria* and *Onychiurus armatus s.l.* were sufficiently abundant to be considered as ecological test animals. One further species, *Isotoma notabilis*, which was dominant in the Ødum field is normally found in the upper soil layer and litter and may as life-form be classified as intermediate between eu-edaphic and hemi-edaphic. These

four species which generally are among the most abundant species in Danish agricultural soils as well as in natural grassland etc. were chosen for the preparatory tests carried out in 1990.

T. macrochaeta and *I. notabilis* has the great advantage for experimental purposes that they reproduce parthenogenetically, i.e. all individuals are females which can reproduce. *O. armatus* and *F. fimetaria* reproduce bisexually with males constituting about 1/2 - 1/3 of natural populations (Petersen 1978 b). This is a problem because it is impossible or at least extremely difficult to identify the sex on living specimens. Thus, the number of females in a start population for reproductivity studies cannot be determined exactly and the start population has to be fairly large in order to ensure a representative number of sexes.

T. macrochaeta is a very small, slim species which is difficult to handle and proved especially difficult to confine in the microcosms. Further, the attempts to produce mass cultures were not successful. *O. armatus sensu lato* is a complex of species which are difficult to separate unless mounted on microscopic slides. They can be reared in mass cultures but have fairly long generation time and consequently fairly low productivity. Therefore, the specimens available for experiments were not sufficient.

Folsomia fimetaria
Isotoma notabilis

For these reasons only *F. fimetaria* and *I. notabilis* were used for the experiments in 1991. Mass cultures of *F. fimetaria* proved to provide a more reliable supply of specimens for the experiments than mass cultures of *I. notabilis*. Therefore, *F. fimetaria* was chosen as principal test Collembolan in 1991 and the only one used for the experiments in 1992.

7.2.4.2 **Gamasid mites**

A search for a suitable eu-edaphic gamasid mite focused at first on species belonging to the family *Rhodacaridae*. A search through 10 large soil sample units from the field at Ødum and 10 sample units from an ecological farm resulted in very few specimens. Sufficient number of specimens for initial feeding tests were obtained from heathland and grassland sites at the Mols Laboratory, i.e. soil which had not been cultivated for at least 50 years.

Laboratory experiments carried out in 1990 did not succeed in a method suited for rearing rhodacarid mites in culture and showed that these gamasids did not eat the above mentioned collembolan species. On the contrary, it was seen that they were strongly repelled from specimens of *I. notabilis* when touching them.

Hypoaspis aculeifer

It was attempted to culture several other species of gamasids without success. Only *Hypoaspis aculeifer* proved to be easy to rear and handle in the laboratory. It was observed that it willingly feed on the selected collembolan test species. The species is often found in Danish soils (woods, grassland ect.) and a few specimens were found in the agricultural field soil at Kalø. Therefore, it was chosen as test species.

In the microcosm experiments carried out in 1991 unidentified gamasid mites from cultures were supplemented with specimens extracted from the field (probably more than one species). In 1992 specimens from a culture of the gamasid *Hypoaspis aculeifer* was supplied by the Department of Terrestrial Ecology, The National Environmental Research Institute, Silkeborg.

7.2.5 **Development of culture techniques**

Several collembolan species can be reared in small plastic canisters with a bottom-layer of plaster of Paris mixed with powdered charcoal in the proportions 9:1 (Goto 1961, Petersen 1971). This method was used for culturing the test species using dryed baker's yeast as food source. It has the advantage that the cultured animals can be observed easily through a stereo-microscope. Thus, for instance, time of egg-laying and hatching, and number of eggs and hatched juveniles can be determined. Even-aged cultures were created by starting new cultures from juveniles hatched within a period of 1 - 5 days.

Creation and maintenance of cultures on plaster of Paris is very time consuming. It was hardly possible to obtain enough specimens for the microcosm experiments from using this method alone. Therefore, a method for easy mass culturing of Collembola and gamasid mites was developed:

Stock cultures in defaunated compost

Plastic boxes measuring 21 x 30 x 12 cm with a lid containing 2 holes (8 cm diameter) covered with fine-meshed polyester web were partly filled with defaunated compost. The compost was made from partly decomposed leaves, spruce needles, twigs, coffee grounds, cow manure, vegetable waste and a little lime. It was treated in a compost grinder and placed in a deep freezer at -18° C for three days. The compost was subsequently quickly heated and stored in a drying oven at 40° C for 2 days. The freezing was repeated and followed by a quick heating to room temperature.

100 - 300 specimens of the species in question were added to start the culture. The specimens added originated from the experimental fields but *O. armatus* and *T. macrochaeta* were supplemented with specimens from the area belonging to the Mols Laboratory. The cultures were stored at room temperature and normal light without direct sunlight until a dense

population had developed. Thereafter, they were transferred to a thermostated room at 5 - 10° C. During the first week the surface of the cultures became covered by mould which might become harmful for the Collembola. Addition of 20 - 25 woodlice (mostly *Oniscus asellus*) proved to solve this problem.

Mass cultures of *F. fimetaria* and *I. notabilis* increased rapidly during about two months to extremely dense, pure, i.e. consisting exclusively of individuals of the species introduced, or practically pure populations. Cultures of *O. armatus* developed more slowly and attempts to propagate *T. macrochaeta* by this method were not successful. Examination of the cultures after 6 - 10 months (1990 - 91) showed that the culture started with introduction of *F. fimetaria* in March 1990 contained a dense population of exclusively that species in January 1991, i.e. nearly 10 months after the start (Gjelstrup and Petersen 1991, Table 4).

On the contrary, a culture started with *I. notabilis* contained very few specimens after 10 months, probably because this culture partly dryed out during a period in the autumn 1990. On the other hand, a number of other cultures contained dense populations of *I. notabilis* in January 1991 suggesting ability of this species to migrate from one culture box to another in the same thermostated room (Gjelstrup and Petersen op.cit.).

Contamination by the Collembolan Folsomia candida

The collembolan species *Folsomia candida*, which was used in the laboratory tests (this report, chapter 4) was very easily cultured by the method described above. Like *I. notabilis* this species proved to be able to migrate from one culture box to the next. Thus, in the spring of 1992 a check of the mass cultures of *F. fimetaria* showed that they were contaminated by *F. candida*. This is very unfortunate because the appearance of the two species is very much alike so they can only be distinguished safely in a microscope at high magnification. The specimens needed for the microcosm experiments that year were instead supplied from the Department of Terrestrial Ecology, National Environmental Research Institute, Silkeborg.

7.2.6 **Preparation of field microcosms before placing**

1990: addition of test animals after placing in the field

In 1990 the empty microcosms were placed in the field 20 - 33 days before addition of soil. Just after filling the microcosms with defaunated soil 250 ml soil suspension sieved through 45 micron mesh polyester web was added to each microcosm in order to ensure a natural microflora. 5 grains of barley were sown in each microcosm. 20 test animals were added to each microcosm at four dates (May 23, May 30, June 6, June 21), i. e. 15 - 57 days after addition of soil.

1991 - 1992: addition of test animals in the laboratory

In 1991 and 1992 defaunated soil, soil suspension and test animals were added in the laboratory before they were placed in the field.

Addition of test animals in 1991

In 1991 Collembola specimens to be introduced to microcosms were extracted alive by means of simple Tullgren funnels from pure stock cultures cultivated in defaunated compost as described above (7.2.5.). Several specimens of uniform size, i.e. small/young adults or preadults were sorted out from the extracted population. From this selected population 20 *Folsomia fimetaria* or 10 *Isotoma notabilis* were sucked up into a small plastic canister with a moist bottom layer of plaster of Paris and charcoal and from there released into the microcosms. 20 *Folsomia* were added to all microcosms. 10 *Isotoma* were moreover added to 75 of the "closed" type microcosms and to all the "open" type microcosms. Finally 5 Gamasida (unidentified, probably more than one species) were added to 10 microcosms, and 5 Gamasida + 10 *Isotoma* were added to 5 microcosms.

Before introduction of test animals the microcosms had been filled half up with defaunated soil which was then remoistened to about field capacity. The test specimens were released at the centre of the soil surface. Thereafter, the animals were allowed to disperse into the soil for about 24 hours before the microcosms were filled up with defaunated soil.

Addition of soil suspension

Finally, in order to ensure the recovery of a natural microflora, 50 ml of a soil suspension was added to each microcosm. The soil suspension was made by shaking 2.5 kg of soil from the experimental field with 5 l of destilled water. The suspension was left for 30 minutes and then shaken again and left for 10 minutes. Finally the suspension was filtered through a sieve with 45 micron mesh width.

Addition of test animals in 1992

In 1992 the test animals, i.e. the Collembolan *Folsomia fimetaria* and the gamasid mite *Hypoaspis aculeifer*, were provided by the Department of Terrestial Ecology, National Environmental Research Institute. The *Folsomia* specimens were all about the same age (16 -23 days), i. e. young adults. 30 specimens of *Folsomia* were added to each of 190 microcosms in two increments. 15 were added on June 1 - 3 and 15 more on June 9 - 10. 5 adult *Hypoaspis* (exact age unknown) were added to 50 of the 190 microcosms on June 16.

The microcosms were filled with defaunated soil to 2/3 of the total height of the soil column. The surface was scratched to facilitate the penetration of the animals into the soil. Like in 1991 the test specimens were sucked up 5 at a time into a small suction flask, their number checked under stereo-microscope and finally forced from the flask to the soil surface by slightly tapping the side of the suction flask. After the last addition of test

animals the microcosms were cautiously replenished with the rest of the defaunated soil.

7.2.7 **Placement of microcosms in the experimental field**

Placement of microcosms 1990

In 1990 128 microcosms were placed in a rectangular plot (15 x 19.5 m^2), i.e. in 12 rows with 10 and 1 row with 8 microcosms. The distance between rows and between microcosms within rows was 1.5 m. Microcosms with and without fine-meshed web in the bottom and with amended and non-amended soil were placed in a regularly alternating pattern. The 4 test species were distributed equally in all four combinations of microcosm type and soil substrate type. 29 microcosms were left without addition of animals. This plot was not sprayed with pesticides.

2 other rectangular plots (10 x 45 m^2) were reserved for preliminary tests with the insecticide dimethoate. Each af these plots were divided into 5 pairs of adjacent 5 x 5 m^2 squares, one for pesticide treatment and one to serve as control. The relative position of squares within each pair was alternately to one and the other side. One microcosm was placed in each square. 20 specimens of *F. fimetaria* were added to 6 pairs of microcosms and 20 specimens of *I. notabilis* to 4 pairs of microcosms.

Experimental design 1991 - 92

In 1991 and 1992 rectangular areas measuring 30 x 60 m^2 were measured out in the experimental field Stegeløkke at Kalø (Figure 7.2). The areas used in the two years were parallel to each other. Fifty 2 x 2 m squares were placed in 10 rows (blocks). The distance between rows varied between 2 and 6 m. Squares within rows were separated by 2 m. Within each block the squares were randomly selected for 5 treatments: Control, i.e. no treatment (C), dimethoate, 5 times recommended dosage for agricultural use (D), dimethoate, recommended dosage (d), pirimicarb, 5 times recommended dosage (P), and fenpropimorph (= Corbel), 5 times normal dosage (F).

Placing of microcosms 1991

In 1991 3 - 6 "closed" and "open" type (cf. 7.2.2.c,d,e) microcosms were buried in the central part of each square. The allocation of microcosms is shown in Table 7.2. Microcosms with only Collembola were placed in the field 8 -9 days before spraying (June 12 - 13), those with mixed Collembola and Gamasida were placed 2 days before spraying (June 19).

Placing of microcosms 1992

In 1992 3 - 5 "open" type microcosms were placed in the central part of each square. The high microcosms with *Folsomia* only were placed on June 11 - 12, those with *Folsomia* and *Hypoaspis* and the low microcosms were placed on June 17. The allocation of microcosms in the blocks is shown in Table 7.3. Because of a long drought period before these dates it was neccessary to drill the holes needed for inserting the microcosms by a

motor-driven posthole digger. After insertion of the microcosms into the holes the gaps were packed with loose soil, the lids removed, and benzoic acid solution poured into the "collar-traps".

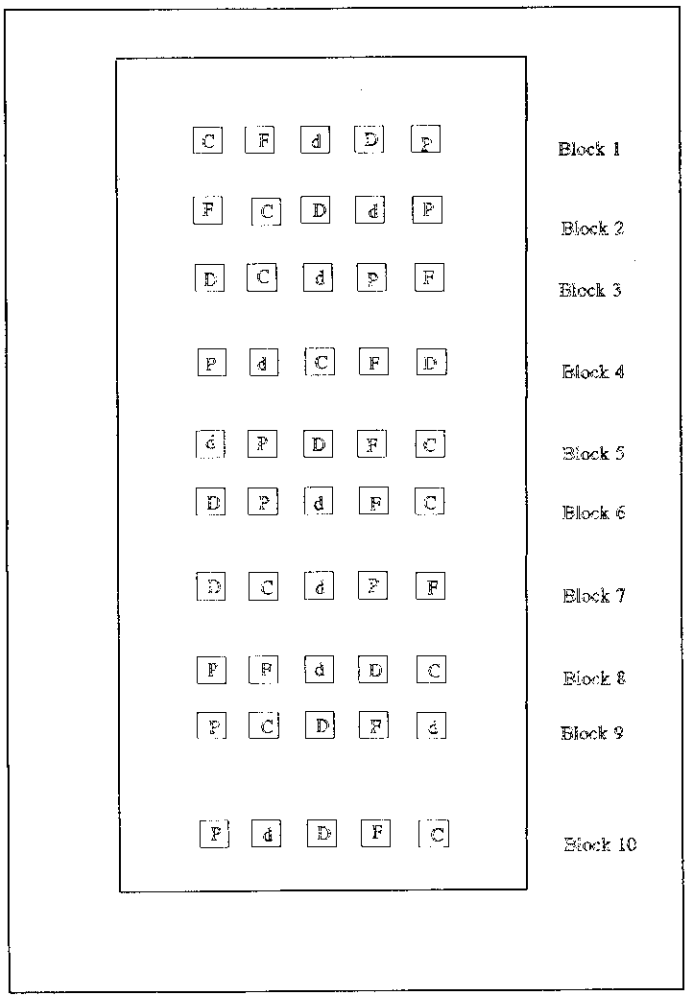


Figure 7.2
Experimental design 1991 and 1992. 50 2 x 2 m^2 plots arranged in 10 blocks x 5 rows. C: Control, i.e. no treatment, D: Dimethoate, 5 times recommended dosage, d: Dimethoate, recommended dosage, P: Pirimicarb, 5 times recommended dosage, F: Fenpropimorph, 5 times recommended dosage.

7.2.8 **Pesticide treatment**

Spraying method

The pesticide treatment was carried out by Ødum Agricultural Experimental Station by means of a boom-sprayer method used for small experimental plots at Danish agricultural experimental stations. The equipment includes a 2.5 m long spraying boom which is carried between two men walking in a standard pace along the plot to be treated. The dosage per unit area is determined by the concentration of pesticide solution, the pressure in the tank containing the spray, and the pace of movement of the spraying boom. The boom was provided with Hardi fan nozzles (type 4110-14) spaced at 25 cm intervals. The boom was carried about 55 cm above the ground. Thus, a double overlap of spray was ensured. The pressure was 1.1 - 1.2 Bar and the walking pace 1 $m.sek^{-1}$. The combination of nozzle type, boom height and pressure should ensure the best possible distribution of spray (Permin 1983, Permin, Jørgensen and Persson 1992).

Table 7.2

Microcosm experimental plan 1991. C: Control, D-5: Dimethoate, 5 times recommended dosage, D-1: Dimethoate, recommended dosage, P-5: Pirimicarb, 5 times recommended dosage, F-5: Fenproprimorph, 5 times recommended dosage. - F: Folsomia fimetaria, I: Isotoma notabilis, G: Gamasida.

Treatment	Days in field after treatment	block numbers				
		low "closed" type microcosm				high "open" microcosm F+I
		F	F+I	F+G	F+I+G	
C	3	2.4.7.8.10	1.3.5.6.9.			
	39	2.4.7.8.10	1.3.5.6.9.			
	59					all
	70	2.4.7.8.10	1.3.5.6.9.	5.6.7.8.	1.2.3.4.	
D-5	3	2.4.7.8.10	1.3.5.6.9.			
	39	2.4.7.8.10	1.3.5.6.9.			
	59					all
	70	2.4.7.8.10	1.3.5.6.9.	4.5.6.7.	1.2.2.3.	
D-1	3	2.4.7.8.10	1.3.5.6.9.			
	39	2.4.7.8.10	1.3.5.6.9.			
	70	2.4.7.8.10	1.3.5.6.9.	5.6.	1.1.2.2.3.4.	
P-5	3	2.4.7.8.10	1.3.5.6.9.			
	39	2.4.7.8.10	1.3.5.6.9.			
	70	2.4.7.8.10	1.3.5.6.9.			
F-5	3	2.4.7.8.10	1.3.5.6.9.			
	39	2.4.7.8.10	1.3.5.6.9.			
	70	2.4.7.8.10	1.3.5.6.9.			

In 1990 the squares within pairs marked for pesticide treatment were sprayed with a normal recommended dosage of dimethoate (DLG 1991) in week 26 (June 25 - 29).

Spraying of treatment squares

In 1991 and 1992 each 2 x 2 m^2 square was treated according to the experimental design described above (section 7.2.7.) and shown in Figure 7.2. The pesticides were applied in water solution. The planned spray dosage, content of active ingredients and concentration of active ingredient in the spray solution is shown in Table 7.4.

Spraying of each square was started 1/2 m outside one of the edges. At this spot some extra spray was applied while adjusting the pressure. When the correct pressure level was attained the two men walked 3 m with the spraying beam centrered over the squares. The area sprayed at each square was consequently 2.5 m broad and 3 m long, i.e. 7.5 m^2, and thus a little larger than the 2 x 2 m^2 squares.

Table 7.3

Microcosm experimental plan 1992. C: Control, D-5: Dimethoate, 5 times recommended dosage, D-1: Dimethoate, recommended dosage, P-5: Pirimicarb, 5 times recommended dosage, F-5: Fenproprimorph, 5 times recommended dosage. - F: Folsomia fimetaria, G: Gamasida: Hypoaspis aculeifer.

treatment	days in field after pesticide treatment	block numbers		
		high "open" type microcosm		low "open" type
		F	F+G	F
C	0	all		
C	14	2.4.7.8.10		
D-5				
D-1	38	all		
P-5				
F-5	59	1.3.5.6.9.		
C	70		all	all
D-5				
D-1				
P-5	70		all	
F-5				

Exposure of "closed" type microcosms to spray

In 1991 the upper surfaces of the "closed" type microcosms had to be exposed at spraying: Immediately before spraying a particular square the soil covering the upper part of the "closed" type microcosms was removed and the lids unscrewed. Immediately after spraying the lids were replaced and covered with soil again. Safety masks, rubber gloves and waterproof clothes were used as safety measures.

Table 7.4

Spray dosage of pesticides (cf. DLG 1991).

Pesticide	Dimethoate-5x	Dimethoate-1x	Pirimicarb-5x	Fenpropimorph-5x
Recommended dose (ha^{-1})	1 l	1 l	0.250 kg*	1 l
Active ingredient in recommended dose (ha^{-1})	0.280 kg	0.280 kg	0.125 kg	0.750 kg
Planned dose (ha^{-1})	5 l	1 l	1.25 kg	5 l
Volume of water (ha^{-1})	300 l	300 l	300 l	300 l
Active ingredient (ha^{-1})	1.4 kg	0.28 kg	0.625 kg	3.75 kg
Concentration (kg active ingredient/l water) · 100	0.467%	0.093%	0.208%	1.250%

* Highest recommended dose

Test of pesticide passage through polyester web

In order to test the effect of pesticides after passage through the fine-meshed polyester web 6 supplementary "closed" type microcosms to which 20 *Folsomia fimetaria* had been added were treated with dimethoate (5 times recommended dosage) without removing the cover of soil and unscrewing the lid.

Pesticide treatments were carried out on June 21, 1991 and June 19, 1992. On both dates the weather was clear and sunny and with only a light wind.

7.2.9 **Measurement of pesticide deposition**

Filter paper method

In 1991 and 1992 it was attempted to measure the amount of pesticide reaching the soil surface and the variability between these amounts by means of small pieces of filter paper placed on the ground in the squares during spraying. Pieces of filter paper (1991: 10 x 10 cm^2, 1992: 72 cm^2) were placed in small plastic bags with zip-locks and weighed with an accuracy of 1 mg. Immediately before spraying a square a piece of filter paper was taken out of its plastic bag and placed in a horizontal position on a dry plastic lid. Immediately after spraying the filter paper was again placed in the air-tight plastic bag. After return to the laboratory all plastic bags were placed at 4° C until weighing the next day. In 1992 2 pieces of filter paper were used in each square. One filter paper was placed on a plastic lid within a 10 cm high cylinder with the same diameter as the microcosms thus simulating a position on the soil surface inside the microcosms. The other was placed exposed on a plastic lid outside the cylinder.

7.2.10 **Tending of microcosms, watering, harvest**

In 1990 the barley plants growing up inside the microcosms and within the close surroundings were removed because of the risk that animals may immigrate to the microcosms via the plants. Half of the microcosms in the untreated plot at Ødum were watered on August 8, 1990. The barley crop was harvested in the area outside the experimental plots at Ødum on August 9, 1990 and on August 13 the experimental plots were harvested with a small combine harvester which could cut the straw above the top of the microcosms remaining in the field at that time. Before harvesting the opening of the microcosms were covered with plastic-bags.

Watering because of draught in 1992

Because of the extreme draught in May - July 1992 each microcosm in the field was watered with 100 ml tap water (June 11, 12, 17), and 50 ml each of the dates June 18, June 23, June 30 and July 7. About 10 ml rain fell in each microcosm the 1st of July. The water was spayed on the soil surface within the microcosms by means of a garden pressure syringe. The total amount of water added through about one month was 310 ml corresponding to 36 mm precipitation which is a little less than the 30-year normal precipitation for June in Aarhus county, i.e. 49 mm.

7.2.11 **Sampling and extraction of field microcosms**

Extraction in high gradient funnel extractors (modified from the funnel extractors described by Petersen 1978 and illustrated in Gjelstrup and Petersen 1987) was started the same day or one day after sampling, or the samples were stored in a refrigerated room at 5 - 10° C for 10 - 24 days before start of extraction when the number of sample units exceeded the capacity of 50 available at the Mols Laboratory. The surface temperature of the samples during extraction was set at 30° C for 2 days, 40° C for 2 days, 50° C for 2 days and 60° C for 3 - 4 days.

After extraction the animals were separated from soil particles by filtering the extracted sample through a 45 micron mesh sieve followed by glycerol flotation of the material retained on the sieve (Gjelstrup and Petersen 1987).

7.2.12 **Soil samples in treatment plots**

A series of soil cores with the same diameter and height as the "closed" type microcosms was sampled on July 30 1991 from the 2 -12 cm horizon of soil. The samples were taken with a soil corer about 10 cm from the microcosms sampled the same day. They were stored at 10° C for 10 days before extraction with the same method and temperature regime as used for the microcosms.

Similarly, 5 cores each divided into two 10 cm high horizons (0 - 10 cm and 10 - 20 cm depth) were sampled next to the "open" type microcosms (August 19, 1991). These samples were extracted simultaneously with the microcosms.

Twenty-five cores representing all treatments in 5 blocks were sampled next to the high "open" microcosms July 13, 1992.

The soil fauna has until now been counted from half of the sample units from July 1991, i.e. 1 unit per treatment plot in 5 blocks.

7.2.13 **Measurements of environmental factors**

In order to compare the volume weight of soil and water content of the microcosms with that of the surrounding soil the soil in the microcosms and in the cores sampled next to the microcosms on July 30, 1991 ("closed" type microcosms) and August 19 ("open" type microcosms) was weighed before and after extraction.

Temperatures inside and outside microcosms were measured with a digital thermometer on three different dates: August 5, 1991 (clear, sunny), August 13, 1991 (sunny changing to overcast) and August 14, 1991 (cloudy, no sun at time of measurement).

7.3 **Results**

7.3.1 **Exchange with the field populations**

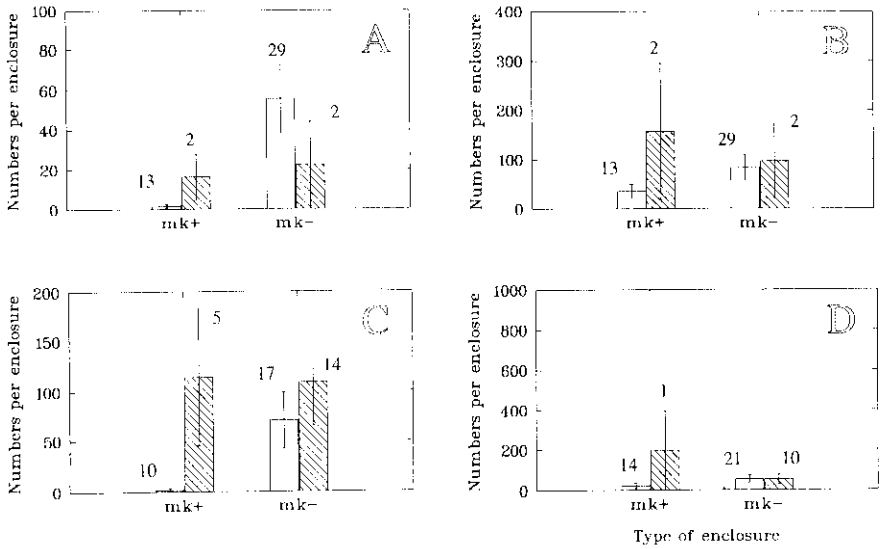
Other than introduced species present in microcosms

It became apparent that several collembolan and mite species other than the introduced test species were found in both the two "open" type microcosms used in 1990 and - to a lesser degree - the "closed" and "open" type microcosms used in 1991 (cf. section 7.2.2.) in spite of the great care taken for preventing possible passages for the microarthropods.

Exchange of specimens highest in open-bottom microcosms

In the microcosm types tested in 1990 exchange between the fauna inside and outside microcosms was most pronounced in the case of microcosms open in the bottom. Thus, initial introduction of the 4 species shown in Figure 7.3 (hatched columns) had positive effect on the final population size in microcosms with bottom closed by fine-meshed web (mk+), but not in microcosms with open bottom (mk-). Using a Mann-Whitney U-test the difference between closed-bottom microcosms with or without introduced test specimens was significant for *Folsomia fimetaria* ($P < 0.005$) and *Onychiurus armatus* ($P < 0.05$). The number of specimens extracted from microcosms open in the bottom was mostly higher than the numbers extracted from microcosms with bottom closed by web (Figure 7.4). This

was significant (Mann-Whitney U-test) for *O. armatus* in amended soil ($P < 0.01$) and *F. fimetaria* ($P = 0.05$).



Figur 7.3

Numbers of 4 collembolan species in microcosms with or without initial introduction of 20 specimens, 1990.- A: Onychiurus armatus, B: Tullbergia macrochaeta, C: Folsomia fimetaria, D: Isotoma notabilis - X-axis: mk+: top of microcosm open, bottom closed with fine-meshed web. mk-: both top and bottom of microcosm open. - Blank columns: No specimens introduced at start of the experiment, Hatched columns: 20 specimens introduced. Vertical lines: Standard error of the mean - N = 10-14 (mk+, blank), N = 1-5 (mk+, hatched), N = 17-29 (mk-, blank), N = 2-14 (mk-, hatched).

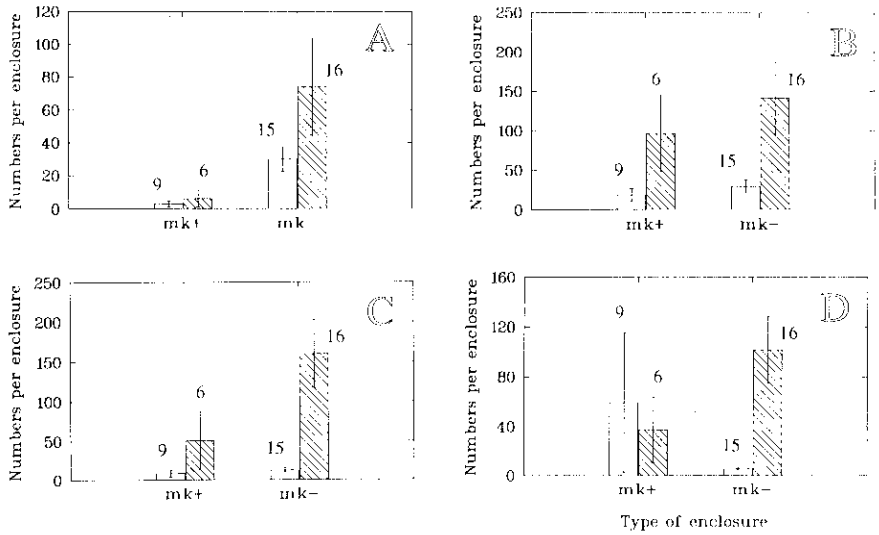
Consequently it was believed that emigrations and immigrations mainly occurred via the open bottom. The fact, however, that microcosms closed in the bottom with fine-meshed web also contained non-introduced species indicated that migrations via the exposed free upper end of the microcosms might occur. Alternatively, the access to the microcosms had to be through inconspicuous leaks at the joinings between web and plastic tube. Very small and slim species like *Tullbergia macrochaeta* might be able to squeeze themselves through the narrow meshes of the web.

Exchange with field population in "closed" type microcosms

It was concluded from the 1991 experiments that the "closed" type of microcosms could not completely prevent immigration of non-introduced specimens from the surrounding soil (Figure 7.5) and that the high "open" type microcosm tested that year was as efficient for isolating the test population as the "closed" type microcosm (Petersen and Gjelstrup 1992).

Test for access to microcosms

A test for tracing possible ways of access for the microarthropods from the surrounding soil to the "closed" type microcosms used in 1991 (cf. section 7.2.2. (re c)) showed that very small species belonging to the collembolan subfamily *Tullberginae* and the mite orders *Actinedida*, *Tarsonemida* and *Acaridida* seem to be able to penetrate the 45 micron-mesh web (Petersen and Gjelstrup 1992, Table 1). A few specimens of the larger species of Collembola (*Lepidocyrtus* sp.) and Gamasida were found after extraction of samples confined in "closed" microcosms. They are apparently able to pass through narrow leaks or cracks, most likely at the packing between the lid and the canister. An inspection through stereo-microscope showed indeed small irregularities in the surface of the packing which could explain the escape of that size of specimens. The test species *Folsomia fimetaria* and *Isotoma notabilis* were not found after extraction of samples confined in microcosms or in nylon mesh bags.



Figur 7.4

Effect of field microcosm type and soil substrate on 4 collembolan species, 1990.- A: Onychiurus armatus, B: Tullbergia macrochaeta, C: Folsomia fimetaria, D: Isotoma notabilis - X-axis: mk+: top of microcosm open, bottom closed with fine-meshed web. mk-: both top and bottom of microcosm open. - Blank columns: field soil, Hatched columns: field soil amended with organic matter. Vertical lines: standard error of the mean - N = 9 (mk+, blank), N = 6 (mk+, hatched), N = 15 (mk-, blank), N 0 16 (mk-, hatched).

Extractions from the "open" type microcosms with "collar-traps" employed in 1992 proved to contain very few individuals of other than the introduced test species (Figure 7.6).

7.3.2 **Effect of soil amendment**

In 1990 a comparison was made between microcosms with defaunated soil from the Ødum field and microcosms with the same soil amended with organic matter (cf. section 7.2.3.). Figure 7.4 shows that all 4 test species were favoured by the amendment. In microcosms with web-covered bottom (mk+), however, *Isotoma notabilis* was most numerous in non-amended soil.

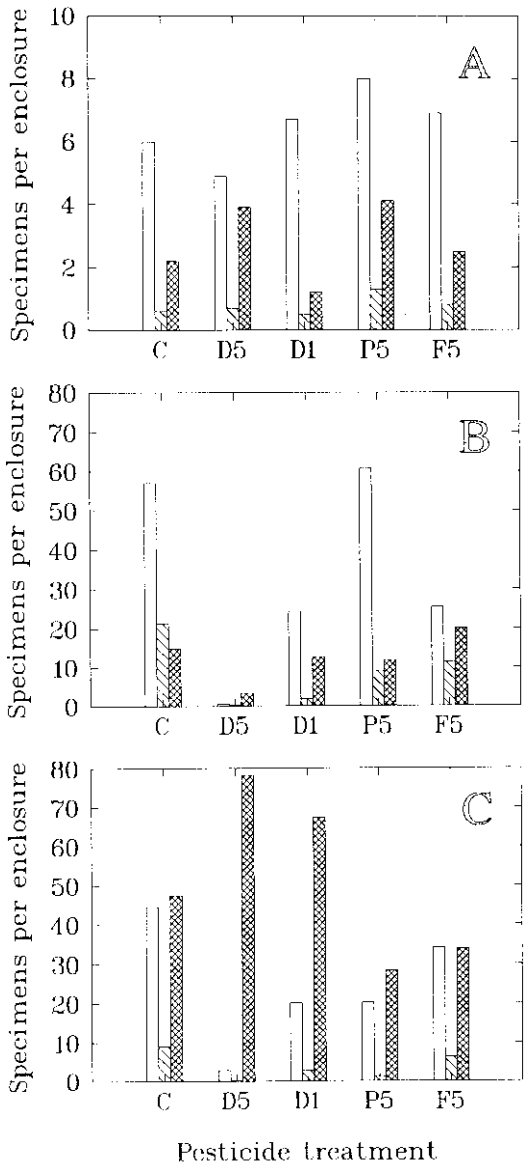


Figure 7.5
Numbers of Folsomia fimetaria and Isotoma notabilis compared to other collembolan species in "closed" field microcosms, 1991. - A: 3 days after treatment, B: 39 days after treatment, C: 70 days after treatment. - Blank columns: Folsomia, Hatched columns: Isotoma, Cross-hatched columns: other Collembola. N = 10.

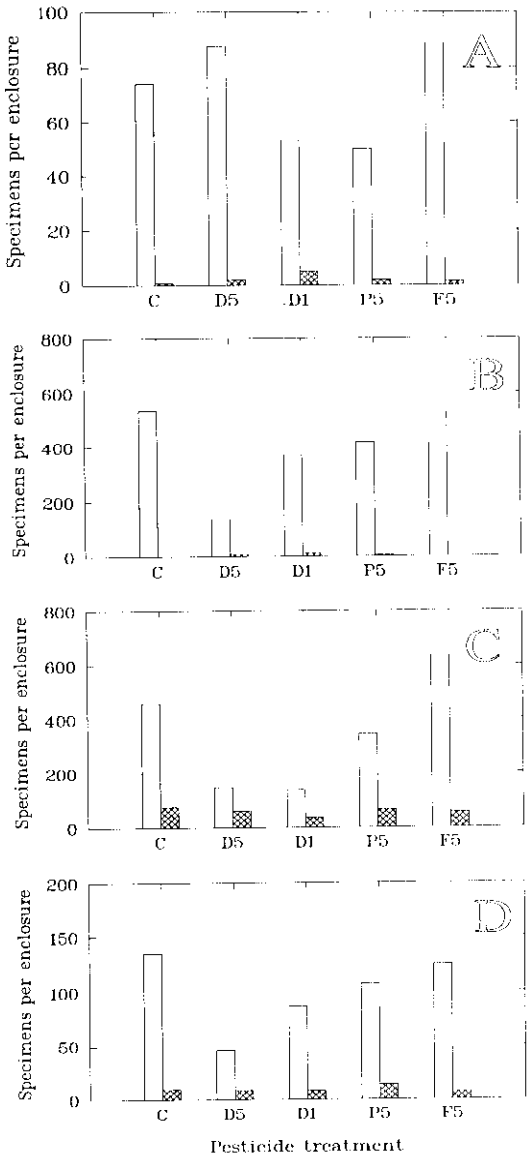


Figure 7.6

Numbers of Folsomia fimetaria compared to other collembolan species in high "open" field microcosms, 1992. - A: 14 days after treatment, B: 38 days after treatment, C: 59 days after treatment, D: 70 days after treatment, with the gamasid Hypoaspis aculeifer. - Blank columns: Folsomia, Shaded columns: other Collembola. N = 5 (A, C), 10 (B, D).

7.3.3 **Efficiency of defaunation**

Comparison of untreated sample units with units defaunated as described in section 7.2.3. but without the final freezing to -35°C showed that 4 of 10 defaunated units each contained 1 specimen of Collembola whereas the numbers of Collembola extracted from 10 untreated sample units varied between 38 and 90 (Gjelstrup and Petersen 1991, Table 5). The 4 specimens found in the defaunated sample units represented 3 species, i.e. *Tullbergia macrochaeta, Onychiurus armatus* s.l. and *Entomobrya albocincta* (possibly contamination from the extractor). 3 defaunated sample units contained *Acari* (1, 2 and 6 specimens) compared to 6 - 39 specimens in 10 untreated sample units. No other arthropods were extracted from the defaunated sample units. Subsequently freezing to -35°C was included in the defaunation procedure in order to make the defaunation more efficient.

No animals could be found in 4 samples of defaunated soil stored in "closed" type microcosms with adequate moisture at 10° C for 73 days in 1991 (cf. section 7.2.3) while 1 sample contained 1 single animal belonging to the collembolan subfamily *Tullberginae* (probably *T. macrochaeta*).

Defaunation process highly efficient for micro-arthropods

This should be compared to counts between 17 and 1,100 (mean = 148) specimens of *Tullberginae* extracted from 25 soil cores which were sampled next to the microcosms on July 30, 1991 (cf. section 7.2.12.) apart from a large number of other species of collembolans, mites and other soil arthropods. Only 2 of the 25 soil samples did not contain the test species *Folsomia fimetaria* while 23 samples contained between 1 and 22 specimens of that species.

One single Diptera larva, but no Collembola or mites were extracted from 10 replicate samples of defaunated soil stored for 5 weeks at room temperature in 1992.

It can be concluded that the defaunation procedure is highly efficient although occasionally a few microarthropods may survive the treatment or may have been hatched from eggs.

7.3.4 Environmental factors inside and outside microcosms

7.3.4.1 Water content of soil

Slightly higher water content inside microcosms

At the time of measurement (July 30, 1991) the mean water content of soil inside the "closed" type microcosms was about 2 % higher than that of the surrounding field soil (Figure 7.7). The soil inside the "open" type microcosms had about 3 % higher water content than the soil outside (August 19, 1991). The differences are statistically significant (Students t-test, $P < 0.001$). Thus, especially during draught situations, the microcosm may provide more favourable life conditions for the soil biota than the field.

7.3.4.2 Volume weight of soil

Higher volume weight of soil inside microcosms

Figure 7.8 shows that the soil inside the "closed" type microcosms after about 5 weeks in the field (July 30, 1991) had slightly higher volume weight than the soil of the field next to the microcosms. A t-test shows that the difference is statistically significant ($P < 0.001$). Probably, the soil in the microcosms was more densely packed with smaller pore volume than the field soil. Difference of the same magnitude is found between soil inside and outside the high "open" type microcosms sampled 3 weeks later (August 19, 1991). There was a general decrease in volume weight of the soil between the two dates.

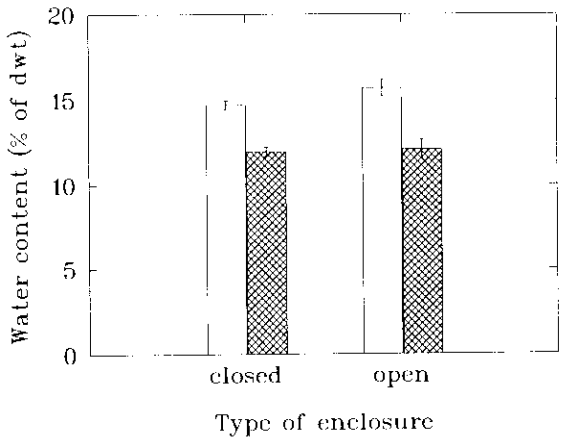


Figure 7.7

Water content in soil inside and outside two types of field microcosms (=enclosures), 1991. - Blank columns: soil inside microcosms, Filled columns: soil in the field outside microcosms. - Closed type: 39 days after treatment, Open type: 59 days after treatment. Vertical lines: Standard error of the mean. N = 50 (closed, inside), N = 25 (closed, outside), N = 20 (open, inside), N = 5 (open, outside).

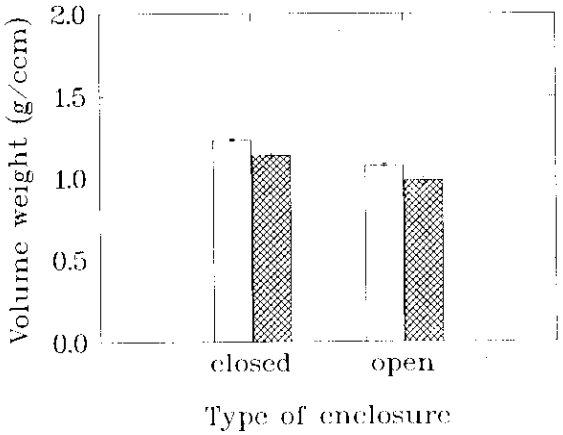


Figure 7.8

Volume weight of soil inside and outside two types of field microcosms (= enclosures), 1991. - Blank columns: soil inside microcosms, Filled columns: soil in the field outside microcosms. - Closed type: 39 days after treatment, Open type: 59 days after treatment. Vertical lines: Standard error of the mean. N = 50 (closed, inside), N = 25 (closed, outside), N = 20 (open, inside), N = 5 (open, outside).

7.3.4.3 **Temperature**

No difference in temperature

Temperature measurements made on three different dates and weather conditions in 1991 (Petersen and Gjelstrup 1992, Table 2) showed that the microcosms have little effect on the soil temperature.

7.3.5 **Pesticide deposition**

Based on the amount of pesticide solution absorbed on pieces of filter-paper placed on the ground (cf. section 7.2.9.) the effective dosages of active ingredients supplied to the microcosms in 1991 and 1992 were calculated (Table 7.5 and 7.6).

Variability of deposition within treatments

In spite of the standardized experimental equipment and professional execution of the treatment it appears that the variability of amounts of pesticide reaching the soil or the microcosms is great. Thus, within treatments maximum and minimum values most often differ by a factor 2 or more. The coefficient of variation within treatments ranged from 21.2 to 25.3 % in 1991 and from 19.1 to 32.8 % in 1992 (outside microcosms). Inside microcosms the coefficient of variation within treatments ranged from 11.5 to 28.4 % in 1992.

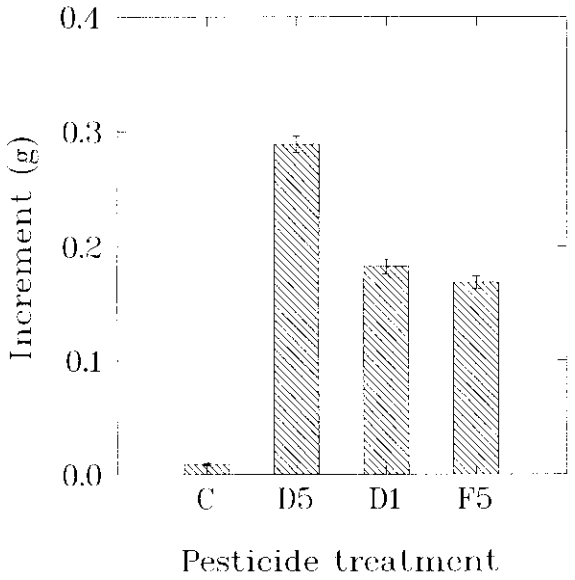


Figure 7.9

Weight increment of 100 cm² filter papers after spraying, 1991. - C: control, not sprayed - D5: Dimethoate, 5 times recommended dosage - D1: Dimethoate, recommended dosage - F5: Fenpropimorph. - Vertical lines: standard error of the mean. N = 10.

Table 7.5

Deposition of pesticides, 1991.

Pesticide		Dimethoate	Dimethoate	Pirimicarb	Fenpropimorph
Planned dose: times recommended		5	1	5	5
Solution absorbed on filterpaper	Mean.100 cm^{-2}	0.267 g	0.185 g	not measured	0.167 g
	Range (min. - max.)	0.175 - 0.401	0.124 - 0.241	not measured	0.119 - 0.237
Deposition: active ingredient.ha^{-1}		1.247 kg	0.172 kg	not measured	2.088 kg
Deposition: % of planned dose		89.1 %	61.4 %	not measured	55.7 %
Deposition: times recommd. dose		4.5	0.6	not measured	2.8

Table 7.6

Deposition of pesticides outside and inside field microcosms, 1992.

Pesticide			Dimethoate	Dimethoate	Pirimicarb	Fenpropimorph
Planned dose: times recommended			5	1	5	5
Outside microcosms	Solution absorbed on filterpaper	Mean. 72 cm^{-2}	0.217 g	0.203 g	0.200 g	0.169 g
		Range (min. - max.)	0.152 - 0.281	0.142 - 0.274	0.137 - 0.253	0.090 - 0.269
	Deposition: active ingredient (ha^{-1})		1.407 kg	0.262 kg	0.578 kg	0.2934 kg
	Deposition: % of planned dose		100.5%	93.6 %	92.5%	78.2 %
Inside microcosms	Solution absorbed on filterpaper	Mean. 72 cm^{-2}	0.146 g	0.140 g	0.158 g	0.117 g
		Range (min. - max.)	0.124 - 0.182	0.096 - 0.203	0.120 - 0.211	0.057 - 0.166
	Deposition: active ingredient (ha^{-1})		0.946 kg	0.180 kg	0.456 kg	2.031 kg
	Deposition: % of planned dose		67.6 %	64.3 %	73.0 %	54.2 %
	Deposition: times recommd. dose		3.4	0.6	3.6	2.7
Deposition: *inside/outside* microcosms (%)			67.3 %	69.0 %	79.0 %	69.2 %

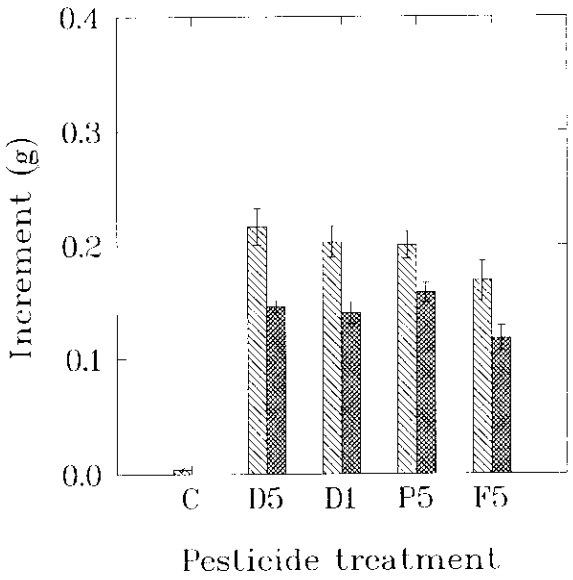


Figure 7.10

Weight increment of 72 cm^2 filter papers after spraying, 1992. - Hatched: outside microcosms, Cross-hatched: inside microcosms. - C: control, not sprayed - D5: Dimethoate, 5 times recommended dosage - D1: Dimethoate, recommended dosage - P5: Pirimicarb - F5: Fenpropimorph. - Vertical lines: standard error of the mean. N = 10.

Variability of deposition between treatments

Further, the mean amount of pesticide reaching the ground varied significantly between treatments. In 1991 the mean amounts of solution reaching the soil in the low- and high-dosage dimethoate treatment, respectively, were 10 % and 60 % higher than the mean deposition of fenpropimorph (cf. Figure 7.9). The deposition of dimethoate solution was also higher than the fenpropimorph deposition in 1992, especially outside the cylinders which simulated the above-ground part of the "open" type micro-

cosms (Figure 7.10). The difference was 20 % for low-dosage dimethoate solution and 28 % for high-dosage dimethoate solution.

Lower mean deposition inside than outside microcosms

The average amounts of pesticide solution absorbed were 21 - 33 % lower inside than outside the cylinders representing the above-ground part of microcosms (Figure 7.10). The deposition measured inside and outside the cylinders in the same plots were only weakly correlated (Figure 7.11).

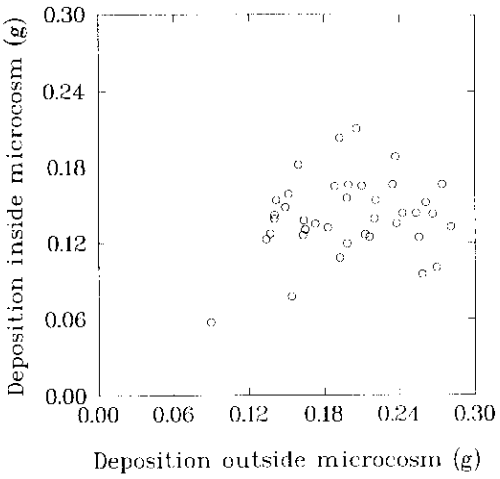


Figure 7.11
Relationship between pesticide spray deposition inside and outside high "open" microcosms.

"Shade" effect

In several cases the 10 cm high above-ground part of the "open" type microcosms obviously acts as protection against pesticide impacts on the soil surface. The horizontal distribution of deposition inside the microcosms was not measured but it may be assumed that the projecting wall of the microcosms creates a "shade" effect resulting in lower deposition in protected than exposed parts of the soil surface. This heterogeneity may greatly influence the conditions for survival and reproduction of the test populations in the microcosms compared with the soil outside.

The deposition on freely exposed filterpapers as percent of the planned dosage was higher in 1992 (78 - 100 %) than in 1991 (56 - 89 %). The deposition inside cylinders in 1992, however, was similar to the depositionon unprotected filterpapers in 1991. This means that the doses received by the two types of microcosms used in 1991 and 1992, respectively, were similar. Only the high-dosage dimethoate was significantly higher in 1991 than 1992.

Measured deposition of spray lower than planned dosages

The resulting dosages effective inside the microcosms consequently differed from the planned dosages. Thus, the high-dosage dimethoate treatment planned to be 5 times the dosage recommended for agricultural use was in fact 4.5 and 3.4 times the recommended dosage in 1991 and

1992, respectively. Dimethoate treatment planned as normal recommended dosage was 0.6 times the recommended dosage in both years. Pirimicarb and fenpropimorph treatments planned to use 5 times recommended dosages became 3.6 and 2.7 - 2.8, respectively.

Evaporation of water not measured

The method used for measurement of deposition does not consider the amount of water evaporated during spraying and storage until weighing. It is likely that the concentration of active ingredient was higher at weighing than in the original spray because of evaporation. Calculation of deposition from the amount of liquid deposited using the concentration in the original spray solution will therefore underestimate the amount of active ingredient deposited. However, the differences between deposition inside and outside microcosms and the heterogeneity between treatments and variability between microcosms with the same treatment are clearly demonstrated by the method.

7.3.6 **Effect of pesticides. Preliminary experiments**

No effect on collembola but negative effect on gamasids in 1990

No significant effect of dimethoate (recommended dosage) was found in 1990 for the 4 collembolan test species, *Onychiurus armatus*, *Tullbergia macrochaeta*, *Folsomia fimetaria* and *Isotoma notabilis* (Gjelstrup and Petersen 1991). However, dimethoate treated microcosms with non-amended soil had significantly lower numbers of gamasid mites than control microcosms (Mann-Whitney U-test: $P < 0.05$).

Tendencies to stimulation in organically amended soil

On the contrary, dimethoate treated microcosms with organically ameliorated soil showed a tendency to higher mean population size than non-treated microcosms for all taxons (Mann-Whitney U-test: $P < 0.1$ for *T. macrochaeta*, $P < 0.05$ for total Collembola).

The results from 1990 should be interpreted in the light of the very long period (98 - 159 days) from treatment to sampling and extraction.

7.3.7 **Effect of pesticides on *Folsomia fimetaria***

7.3.7.1 **Effects on whole microcosms**

The present section reports the results from the low "closed" type microcosms used in 1991 (cf. 7.2.2.,c) and the high "open" type microcosm used in 1991 and 1992 (cf. 7.2.2.,e,f). The high "open" microcosms were composed of two 10 cm deep horizons (0 - 10 cm and 10 - 20 cm depth) and contained twice as much soil as the "closed" type. This section treats the data obtained for whole microcosms, i.e. the sum of the two horizons in high "open" type microcosms.

The average effects of pesticide treatment on *Folsomia fimetaria* are shown in Figure 7.12 (1991, "closed" type microcosms) and 7.13 (1992, high "open" type microcosms). Each bar represents the mean number of specimens extracted per microcosm (N = 5 or 10) at three sampling dates (A, B, C) and 5 treatments (cf. Table 7.2 and 7.3).

Low recovery of introduced specimens soon after addition to microcosms

Three days after pesticide treatment in 1991 or 14 - 22 days after introduction of test specimens only about 1/4 of the 20 specimens introduced were recovered by extraction (Figure 7.12.A). No difference was found between treatments. Thus, the reduction of the introduced population cannot be explained as a result of acute toxicity.

No acute effect of pesticides

Similarly, 10 control microcosms sampled the same day as the pesticide treatment in 1992, i.e. 2 days after placing in the field and 9 - 18 days after introduction of test specimens, yielded only about 1/3 of the specimens of *Folsomia fimetaria* introduced (Figure 7.14).

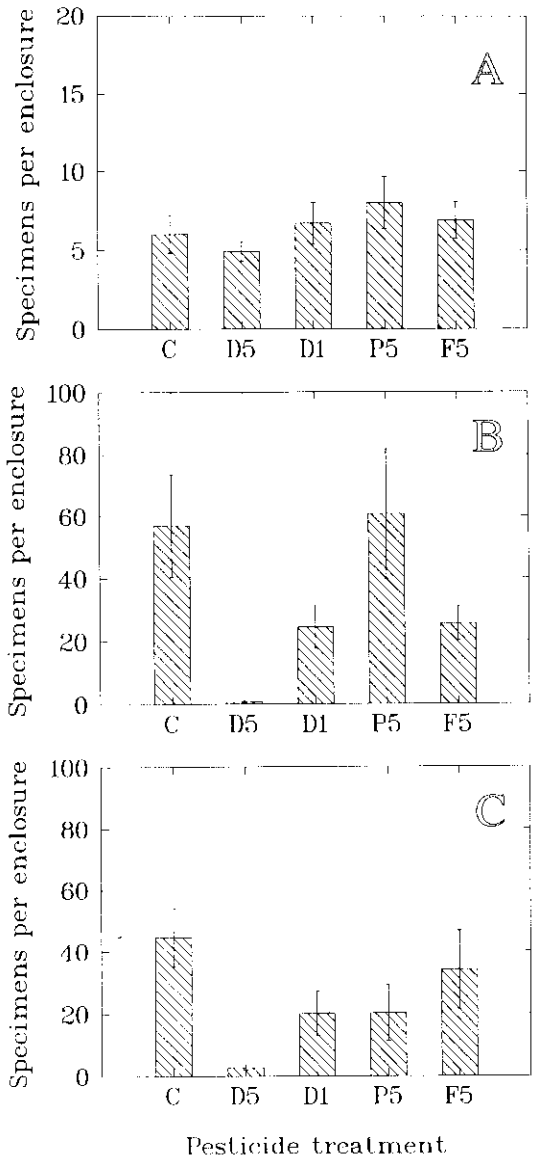
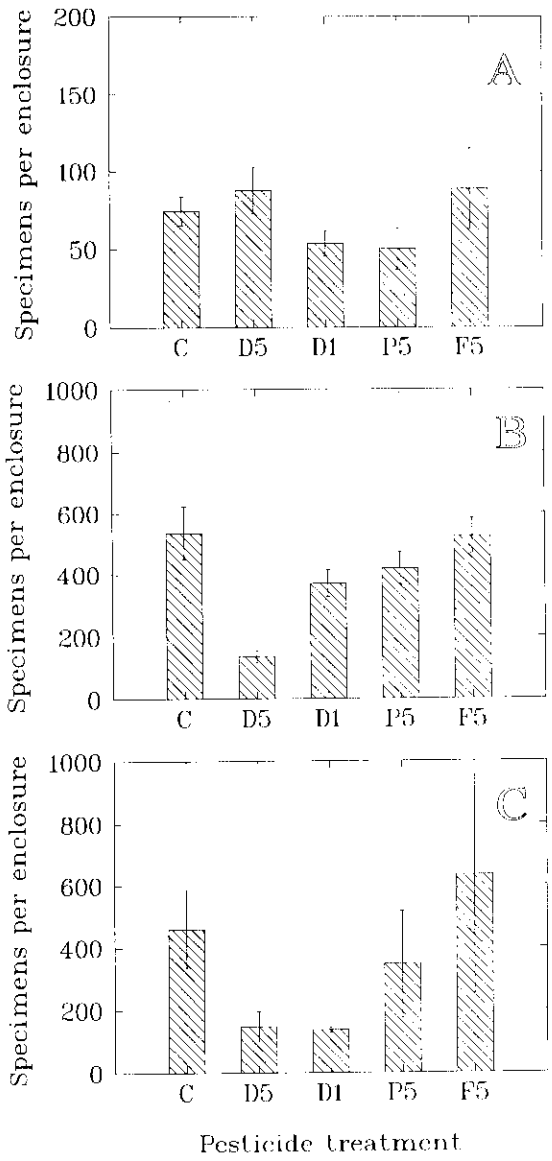


Figure 7.12
Effect of pesticide treatment on Folsomia fimetaria in "closed" field microcosms, 1991. -Time from treatment to collection of microcosms from the field: A: 3 days, B: 39 days, C: 70 days. X-axis: C: control, D5: Dimethoate, high dosage, D1: Dimethoate, recommended dosage, P5: Pirimicarb, F5: Fenpropimorph. Vertical lines: standard error of the mean. N = 10.

Figure 7.13

Effect of pesticide treatment on Folsomia fimetaria in high "open" field microcosms, 1992. - Time from treatment to collection of microcosms from the field: A: 14 days, B: 38 days, C: 59 days. X-axis: C: control, D5: Dimethoate, high dosage, D1: Dimethoate, recommended dosage, P5: Pirimicarb, F5: Fenpropimorph. Vertical lines: standard error of the mean. N = 5 (A, C), N = 10 ((B).

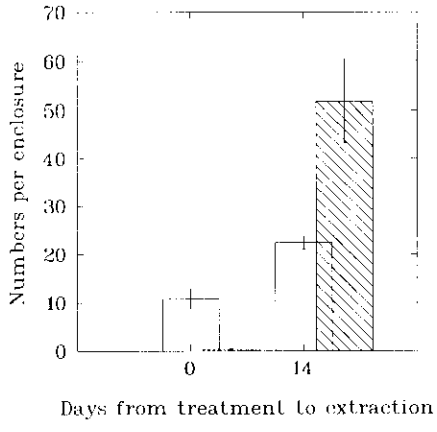


Reasonably good recovery 2 weeks after treatment

2 weeks later a mean number of 22 large specimens (length > 0.8 mm) were recovered in control microcosms from the 30 specimens introduced (blank column in Figure 7.14). These specimens were most probably the still surviving specimens originally introduced because the time would be too short for a new generation to reach that size.

Figure 7.14

Folsomia fimetaria, 1992. Population development in the control microcosms during the first 2 weeks. Open columns: Specimens > 0.8 mm Hatched: Specimens < 0.8 mm. Vertical lines: standard error of the mean. N = 10 (0 days), N = 5 (14 days).



No effect of pesticides 2 weeks after treatment

No significant treatment effects were observed 14 days after treatment in 1992 (Figure 7.13.A, Table 7.8 - 7.10).

39 days after pesticide treatment in 1991 the mean population of the control microcosms had increased to 3 times the number of specimens introduced in 1991 (Figure 7.12.B), but 38 days after pesticide treatment in 1992 the mean control population had grown to 536 specimens, i. e. nearly 20 times the originally introduced number of specimens (Figure 7.13.B). After this time the control populations decreased slightly in both years. The microcosms sampled 70 days after treatment in 1992 contained both *Folsomia fimetaria* and the gamasid *Hypoaspis aculeifer*. Thus, the lower population size may partly be due to a predation effect.

Negative effect of dimethoate 5 - 10 weeks after treatment

The high dosage of dimethoate (*D5*) had in both 1991 (Table 7.7., 7.10) and 1992 (Table 7.8., 7.9., 7.10.) a strong and statistically significant negative effect relative to the control 38 - 39 days after treatment. This effect was still significant 70 days after treatment in 1991 (Table 7.7.). In 1992 the effect was also significant 59 days after treatment (ANOVA) and 70 days after treatment for microcosms extracted immediately after sampling (Table 7.8.).

Low dosage of dimethoate (*D1*) resulted both years in lower mean populations than the control microcosms from 38/39 to 70 days after treatment. However, in 1991 the effect was only significant ($P < 0.05$) 70 days after treatment (Table 7.7.) and in 1992 38 days after treatment and after 59 days (Table 7.8). A Kolmogorov-Smirnov test supports the significant effect at day 59 but only in the upper 10 cm horizon (Table 7.10).

Dimethoate in 5 times recommended dosage (*D5*) had mostly a stronger effect than in the recommended dosage (*D1*). This was significant ($P < 0.05$) 70 days after treatment in 1991 and 38 and 70 days after treatment in 1992.

Effect of pirimicarb and fenpropimorph

The effects of pirimicarb and fenpropimorph are less convincing. However, Pirimicarb-treated microcosms had significantly lower number of specimens ($P < 0.05$) than the control microcosms 70 days after treatment in 1991, but not if "outliers" are removed (Table 7.7.). This is supported by a Kolmogorov-Smirnov test for microcosms with pure *Folsomia fimetaria* populations, i.e. without *Isotoma notabilis* and gamasids added (Table 7.10).

Table 7.7

Significance (P-values) of ANOVA-test and planned comparisons/contrasts (Wilkinson, 1990, Kirby, 1993) for Folsomia fimetaria, 1991. - a. low "closed" type microcosm. b. high "open" type microcosm. - C: Control, D5: Dimethoate, 5 times recommended dosage, D1: Dimethoate, recommended dosage, P5: Pirimicarb, 5 times recommended dosage, F5: Fenpropimorph, 5 times recommended dosage. - Hyphen: no data. - Parentheses: P-value, outliers included.

1991

Test	Factors/ comparisons	All sampling dates	Days in field after treatment			
			3[a]	39[a]	59[b]	70[a]
ANOVA	pesticide	**<0.001** (**<0.001**)	0.294	**0.008**	**0.011**	**0.002** (**0.005**)
	time	**<0.001** (**<0.001**)	-	-	-	-
	block	**0.026** (0.076)	**0.003**	0.391	0.475	0.557 (0.286)
	pesticide * time	**0.009** (**0.013**)	-	-	-	-
Pairwise comparisons	C - D5	**<0.001** (**<0.001**)	0.447	**0.003**	**0.011**	**<0.001** (**<0.001**)
	C - D1	**0.007** (**0.005**)	0.628	0.071	-	**0.027** (**0.013**)
	C - P5	0.606 (0.391)	0.171	0.834	-	0.095 (**0.037**)
	C - F5	**0.033** (0.057)	0.533	0.081	-	0.241 (0.335)
	D5 - D1	**0.022** (0.040)	0.216	0.183	-	**0.039** (0.272)

Table 7.8.

Significance (P-values) of ANOVA-test and planned comparisons/contrasts (Wilkinson, 1990, Kirby, 1993) for Folsomia fimetaria in high "open" microcosms, 1992. Data for whole microcosms - C: Control, D5: Dimethoate, 5 times recommended dosage, D1: Dimethoate, recommended dosage, P5: Pirimicarb, 5 times recommended dosage, F5: Fenpropimorph, 5 times recommended dosage. - Bold type: significant (P < .05) - Hyphen: No data. - Parentheses: P-value, outliers included.- [a] Microcosms with gamasid Hypoaspis aculeifer.

1992

Test	Factors/ comparisons	All sampling dates	Days in field after treatment + days stored				
			14	38	59	70 + 0[a]	70 + 13[a]
ANOVA	pesticide	**<0.001** (**<0.001**)	0.264	**<0.001** (**<0.001**)	**0.009** (0.315)	**0.014** (**0.027**)	0.627 (0.447)
	time	**<0.001** (**<0.001**)	-	-	-	-	-
	block	**0.023** (0.104)	0.314	0.055 (0.097)	0.118 (0.217)	0.464 (0.539)	0.477 (0.587)
	pesticide * time	**0.001** (0.066)	-	-	-	-	-
Pairwise comparisons	C - D5	**0.001** (**<0.001**)	0.544	**<0.001** (**0.001**)	**0.002** (0.253)	**0.038** (0.138)	0.185 (0.109)
	C - D1	**<0.001** (**0.015**)	0.350	0.132 (**0.031**)	**0.002** (0.239)	0.124 (0.279)	0.221 (0.118)
	C - P5	**0.002** (0.212)	0.276	0.465 (0.123)	**0.007** (0.672)	0.823 (0.878)	0.601 (0.454)
	C - F5	0.247 (0.456)	0.515	0.321 (0.885)	0.077 (0.515)	0.254 (0.056)	0.292 (0.313)
	D5 - D1	**0.028** (0.306)	0.133	**<0.001** (**0.003**)	0.909 (0.971)	**0.001** (**0.016**)	0.905 (0.966)

Table 7.9

Significance (P-values) of ANOVA-test and planned comparisons/contrasts (Wilkinson, 1990; Kirby 1993) for Folsomia fimetaria in two separate depths of high "open" microcosms, 1992. C: Control; D5: Dimethoate, 5 times recommended dosage; D1: Dimethoate, recommended dosage; P5: Pirimicarb, 5 times recommended dosage; F5: Fenpropimorph, 5 times recommended dosage; Bold type: significant (P < 0.05); Parentheses: P-value, outliers included.

A: 1992. ANOVA. All dates treated together.

Factors	Depth cm	Significance (P)
pesticide	0 - 10	**<0.001** (**<0.001**)
time		**<0.001** (**<0.001**)
block		**0.006** (0.071)
pesticide	10 - 20	**0.003** (**0.009**)
time		**<0.001** (**<0.001**)
block		0.440 (0.255)

B: 1992. Pairwise comparisons, all dates treated together.

comparisons	Significance (P)	
	0 - 10 cm	10 - 20 cm
C - D5	**<0.001**(**<0.001**)	**0.013**(**0.019**)
C - D1	**<0.001**(**0.007**)	0.720(0.954)
C - P5	**0.012**(0.095)	0.983(0.665)
C - F5	0.301(0.906)	0.153(0.242)
D5 - D1	0.111(0.315)	**0.004**(**0.022**)

C: 1992. ANOVA and planned pairwise comparisons. Sampling/extraction dates treated separately.

Test	Depth cm	Factors/ compa-risons	Days in field after treatment + days stored					
			14	38+0	38+10	59	70+0	70+13
ANOVA	0 - 10	pesticide	0.314	**0.015**	**0.004**	**0.009** (0.226)	**0.005** (**0.022**)	0.541 (0.365)
Pairwise compari-sons	0 - 10	C - D5	0.170	**0.003**	**0.003** (**0.001**)	**0.002** (0.195)	**0.049** (0.209)	0.522 (0.079)
		C - D1	0.579	0.163	**0.049** (**0.011**)	**0.002** (0.193)	**0.049** (0.209)	0.844 (0.091)
		C - P5	0.642	**0.050**	0.848 (0.216)	**0.009** (0.458)	0.440 (0.635)	0.963 (0.319)
		C - F5	0.821	0.925	0.726 (0.411)	0.079 (0.503)	0.106 (**0.029**)	0.290 (0.187)
		D5 - D1	0.062	0.059	0.098 (0.160)	0.989 (0.996)	**0.001** (**0.019**)	0.388 (0.938)
ANOVA	10 - 20	pesticide	0.595	**0.041**	**0.002** (0.304)	0.582 (0.501)	0.683 (0.584)	0.541 (0.515)
Pairwise compari-sons	10 - 20	C - D5	0.785	**0.038**	**0.006** (0.173)	0.931 (0.986)	0.236 (0.261)	0.522 (0.988)
		C - D1	0.500	0.494	0.538 (0.434)	0.409 (0.862)	0.297 (0.868)	0.844 (0.853)
		C - P5	0.363	0.824	0.761 (0.798)	0.155 (0.275)	0.492 (0.609)	0.963 (0.344)
		C - F5	0.566	0.276	0.221 (0.900)	0.660 (0.750)	0.795 (0.763)	0.290 (0.237)
		D5 - D1	0.686	0.137	**0.001** (**0.041**)	0.459 (0.876)	0.870 (0.200)	0.388 (0.865)

Table 7.10

Significance (P-values) of nonparametric test (Kolmogorov-Smirnov-2-sample test) for Folsomia fimetaria. C: Control; D5: Dimethoate, 5 times recommended dosage; D1: Dimethoate, recommended dosage; P5: Pirimicarb, 5 times recommended dosage; F5: Fenpropimorph, 5 times recommended dosage; Bold type: significant (P < 0.05); Hyphen: no data; [a]: low "closed" type microcosms; [b]: high "open" type microcosms; [y]: microcosms without I.notabilis.

1991

Comparisons	All sampling dates [a]	Days in field after treatment			
		3[a]	39[a]	59[b]	70[a]
C - D5	**<0.001**	0.962	**0.001**	**0.001**	**<0.001**
C - D1	**0.035**	0.962	0.112	-	**0.004**
C - P5	0.334	0.664	0.962	-	**0.048** [y]
C - F5	0.193	0.962	0.112	-	0.464
D5 - D1	**0.001**	0.309	**<0.001**	-	**0.013**

1992

Comparisons	Depth cm	All sampling dates[b]	Days in field after treatment + days stored					
			14[b]	38+0[b]	38+10[b]	59[b]	70+0[b]	70+13[b]
C - D5	0 - 20 whole microcosms	**<0.001**	0.664	**<0.001**	**0.001**	0.309	0.112	0.664
C - D1		0.120	0.664	0.112	0.309	0.112	0.309	0.664
C - P5		0.660	0.112	0.309	0.664	0.664	0.962	0.962
C - F5		0.925	0.962	0.309	0.664	0.962	0.309	0.962
D5 - D1		**0.005**	0.664	**0.001**	**0.006**	0.664	**0.001**	0.664
C - D5	0 - 10 upper horizon	**0.001**	0.820	**<0.001**	**<0.001**	0.320	0.080	0.600
C - D1		0.055	0.820	0.320	0.080	**<0.001**	0.320	0.600
C - P5		0.522	0.320	0.320	0.820	0.820	0.820	0.400
C - F5		0.740	0.820	0.820	0.820	0.820	0.320	0.400
D5 - D1		**0.004**	0.320	**<0.001**	0.080	0.320	**<0.001**	0.200
C - D5	10 - 20 lower horizon	0.197	0.820	**<0.001**	0.320	0.320	0.820	1.00
C - D1		0.921	0.820	0.080	0.320	0.320	0.320	0.820
C - P5		0.522	0.320	0.820	1.00	0.320	0.820	0.820
C - F5		0.921	0.820	0.320	1.00	1.00	0.320	0.820
D5 - D1		0.334	0.820	0.320	0.080	0.820	0.320	0.820

The effect of fenpropimorph was seen on the data for all sampling dates treated together in 1991 (Table 7.7.). In 1992 a significant effect was recorded 70 days after treatment in the upper 10 cm horizon, but not if "outliers" are removed.

Statistical analysis

The statistical analysis was carried out by the statistical- graphical programme package SYSTAT (Wilkinson 1990). The data were analyzed using the programmes MGLH (Multivariate General Linear Hypothesis) and NPAR (nonparametric statistics).

Increasing departure from normal distribution with time and treatment

The use of parametric statistics such as ANOVA is problematic because the conditions required, e.g. normality, homogeneity of variances etc. are rarely met. In the present case a Kolmogorov-Smirnov 1-sample test for normality using Lilliefors probability (Wilkinson op.cit.) on all combinations of sampling time and treatment showed a tendency to increasing departure from normality with increasing time and, except for one case (control 70 days after treatment in 1992), only for pesticide treated microcosms.

"Outliers"

Removal of a few of the most aberrant values ("outliers") changed in most cases the distributions so that they did not differ significantly from normal distributions and a number of comparisons became significant below the 0.05 level. The following values were considered as "outliers" (cf. fig. 7.15., 7.16): *1991: 70 days:* highest *C* and *F5*, - *1992: 38 days:* highest *C* and *D5*, - *59 days:* highest *P5* and *F5*, - *70 days:* highest *C* and *P5*, two highest *F5*. Based on normal distributions for the remaining values the probability is less than 0.001 that the "outliers" belong to the same population (more than 4 standard deviations larger than the mean).

Normal probability plots

Normal probability plots for the 4 treatments compared with the control at 3 sampling dates in 1991 (Figure 7.15) and 4 dates in 1992 (Figure 7.16) show that several plots for pesticide treated microcosms are characterized by a majority of low values while a few are comparable to the highest control values. Thus, the effect is unequally distributed among the microcosms. Because of large variances the statistical tests fail to show significance. Therefore a comparison of probability plots may be a useful complement to the conventional statistical tests.

7.3.7.2 **Pesticide effect and soil depth**

Interaction between pesticides and depth distribution

A high "open" type of microcosm containing a 20 cm deep column of soil was tested in 1991 and used as the principal type of microcosm in 1992. The soil column was divided into 2 horizons at extraction. Therefore, the interaction between pesticide effect and depth distribution of the test population could be analyzed.

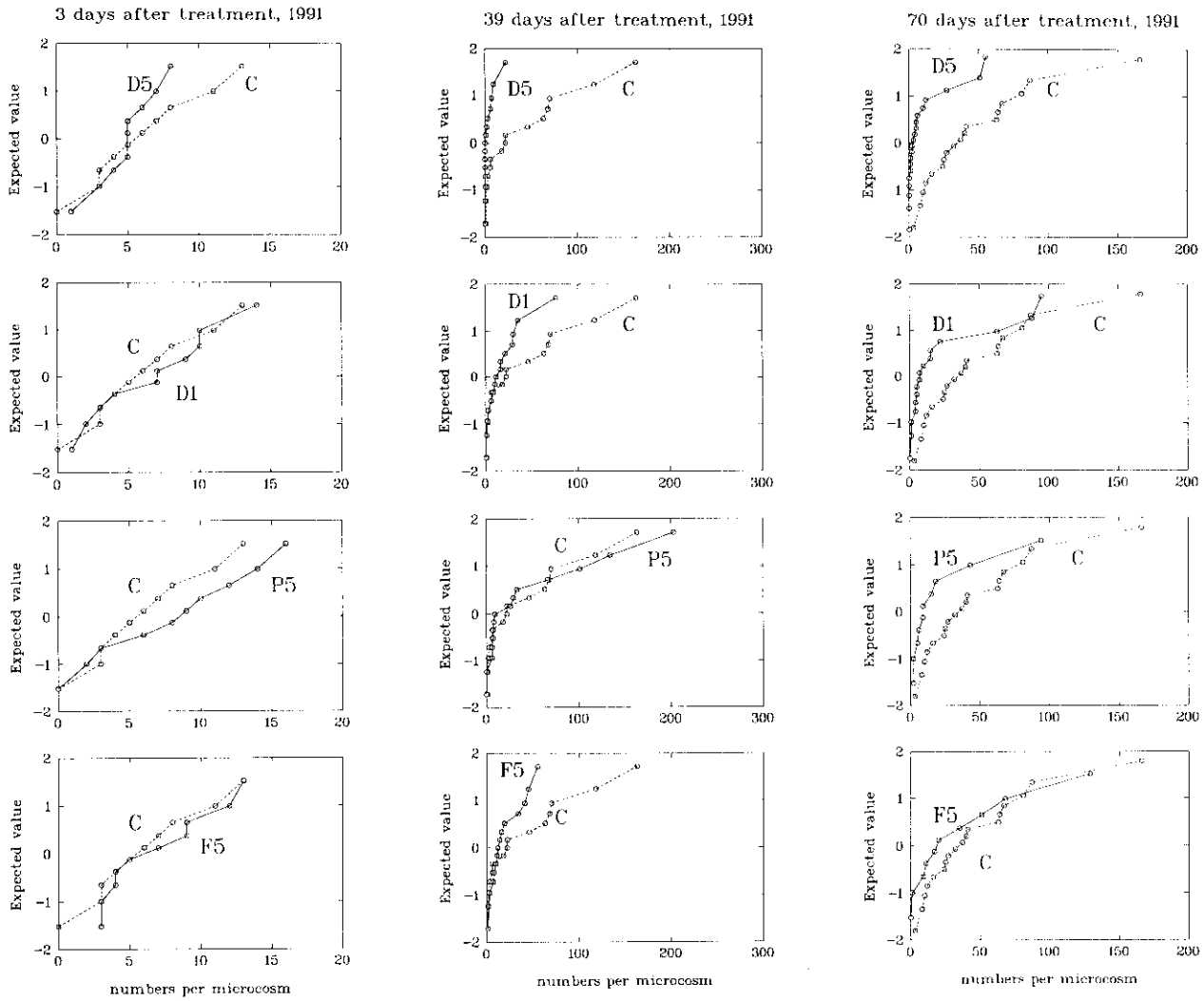


Figure 7.15

Normal probability plots of Folsomia fimetaria at 3 sampling dates, 1991. Comparisons between microcosms treated with pesticides and untreated microcosms. - C: control, D5: Dimethoate, high dosage, D1: Dimethoate, recommended dosage, P5: Pirimicarb, F5: Fenpropimorph.

Effect of high-dosage dimethoate in both horizons

In 1991 only dimethoate in 5 times recommended dosage was used for treatment of this type of microcosm. The *Folsomia fimetaria* population (Figure 7.17) was significantly depressed in both horizons (Wilcoxon signed ranks test: $P < 0.05$).

The depth distribution at 4 dates after pesticide treatment in 1992 is illustrated in Figure 7.18. Highest numbers were mostly found in the upper horizon but at the first date after treatment, i.e. 14 days, the deep horizon contained the largest population. It is likely that this was caused by the then prevailing draught. No effect of the pesticides on depth distribution could be noticed at that time.

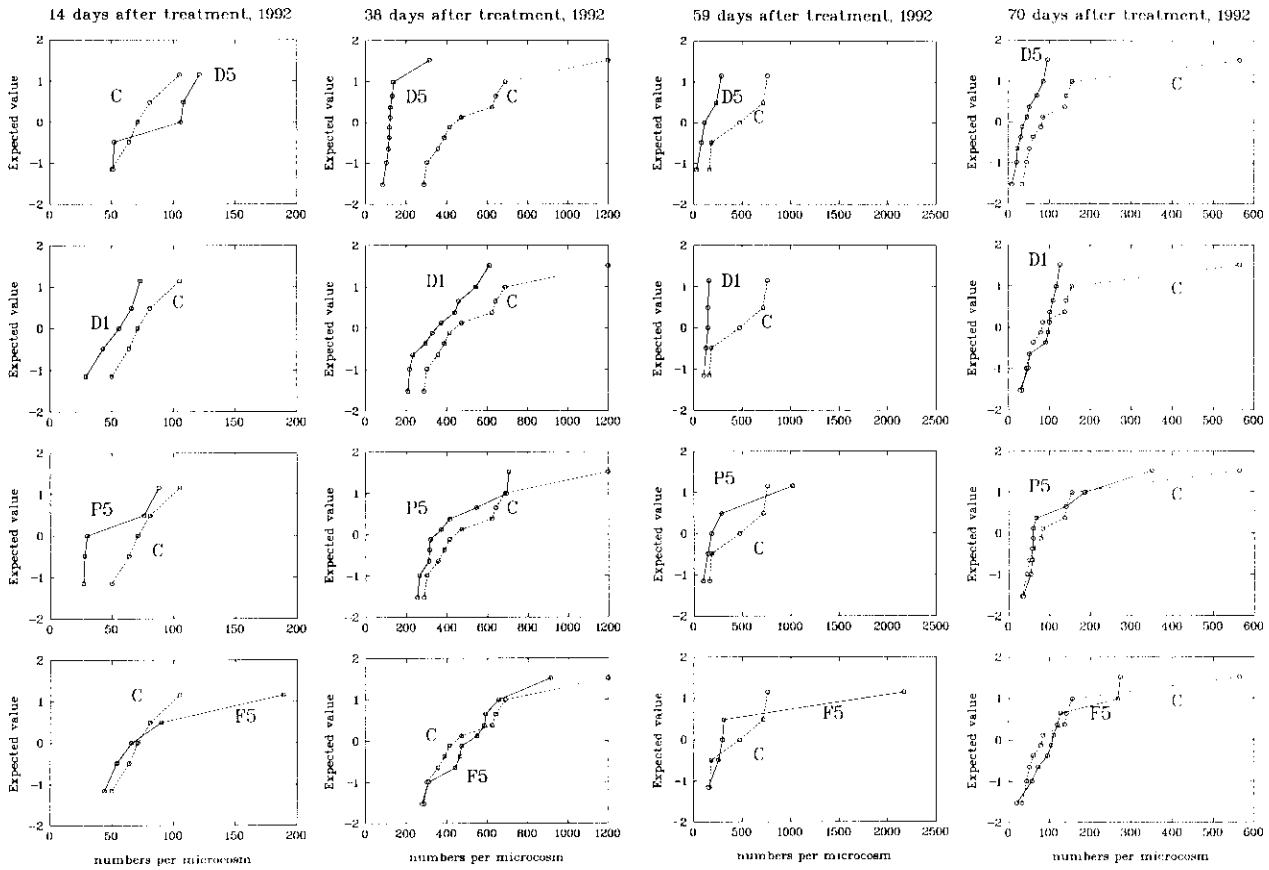


Figure 7.16

Normal probability plots for Folsomia fimetaria at 4 dates, 1992. Comparisons between microcosms treated with pesticides and untreated microcosms. - C: control, D5: Dimethoate, high dosage, D1: Dimethoate, recommended dosage, P5: Pirimicarb, F5: Fenpropimorph.

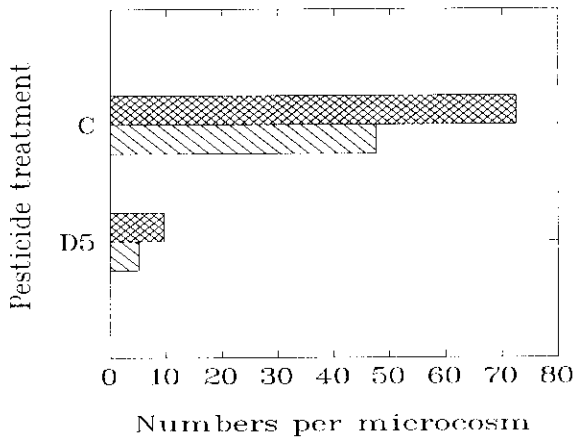


Figure 7.17

Depth distribution of Folsomia fimetaria in high "open" microcosms, 59 days after treatment, 1991. Cross-hatched: 0 - 10 cm depth. Hatched: 10 - 20 cm depth. - C: control, D5: Dimethoate, 5 times recommended dosage. N = 10.

38 days after treatment the numbers found in the upper horizon were higher than in the deep horizon except for the 2 dimethoate (D5, D1) treatments where the numbers were equal in the two horizons. This suggests a stronger effect of this pesticide on the surface population than on the deeper living specimens. The effect may be a combination of reduced population size and migration away from the source of toxicity. 59 days after treatment the depth distribution was strongly skewed towards the surface horizon by all treatments. 70 days after treatment the largest number of *Folsomia* was still found in the top horizon (microcosms with *Folsomia* + the gamasid *Hypoaspis*).

Mostly no treatment effect in the deep horizon

Except for the high dosage of dimethoate (D5) 38 days after treatment, the pesticide effect can only be seen in the upper horizon while in the deep horizon the mean numbers of sprayed microcosms did not differ from those of the control microcosms.

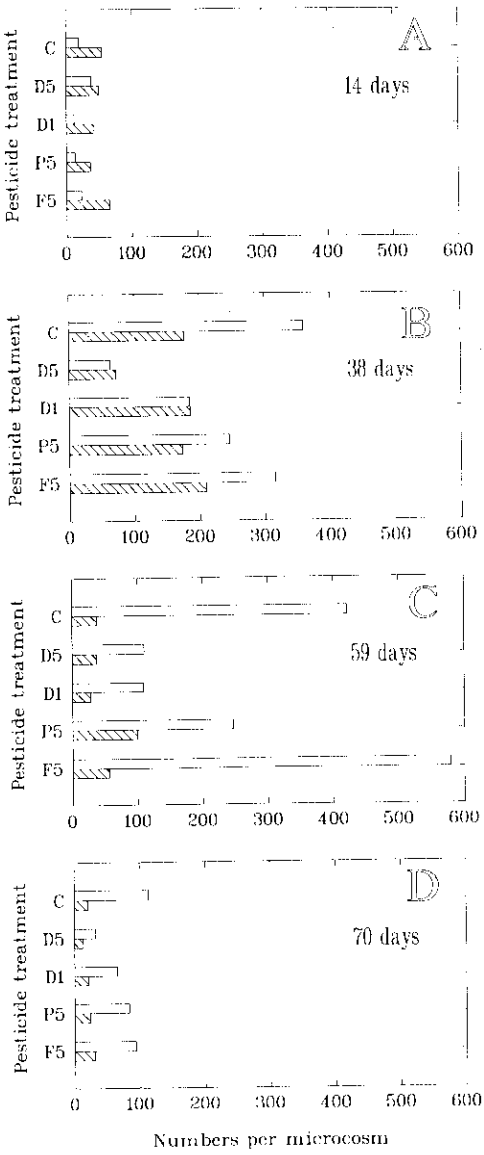


Figure 7.18
Depth distribution of Folsomia fimetaria in high "open" microcosms, 1992. - 4 collection times in days from pesticide treatment. 70 days: with gamasid (Hypoaspis) - Cross-hatched: 0 - 10 cm depth. Hatched: 10 - 20 cm depth. - Pesticide treatment: C: Control, D5: Dimethoate, 5 times recommended dosage, D1: Dimethoate, recommended dosage, P5: Pirimicarb, F5: Fenpropimorph.

The results of an analysis of variance based on separate data for the two horizons show that the pesticide*depth interaction term is significant, and that the pesticide factor is significant for both the upper and the lower 10 cm horizon when all sampling dates are joined (Table 7.9.A).

Planned pairwise comparisons or contrasts (Wilkinson, 1990, Kirby, 1993) for all dates joined (Table 7.9.B.) show significant effects relative to control microcosms in the upper 10 cm horizon for both high (D5) and low dosage dimethoate (D1), and for pirimicarb (P5). High dosage dimethoate (D5) differed in the upper horizon significantly from control 38 days after treatment (Table 7.9.C.). Both high and low dosage dimethoate differed significantly from the control 59 and 70 days after treatment. The effect of pirimicarb was significant in the upper 10 cm horizon 38 days after treatment and 59 days after treatment. Fenpropimorph had a statistically significant effect 70 days after treatment but not if "outliers" were removed.

In the 10 - 20 cm horizon the high dosage dimethoate (D5) had a statistically significant effect when all dates are joined (Table 7.9.B.) and 38 days after treatment (Table 7.9.C.). This was true both in comparison to control microcosms (C - D5) and to microcosms treated with low dosage dimethoate (D5 - D1). Otherwise no significant differences were found between pesticide treated microcosms and control microcosms in the deep horizon.

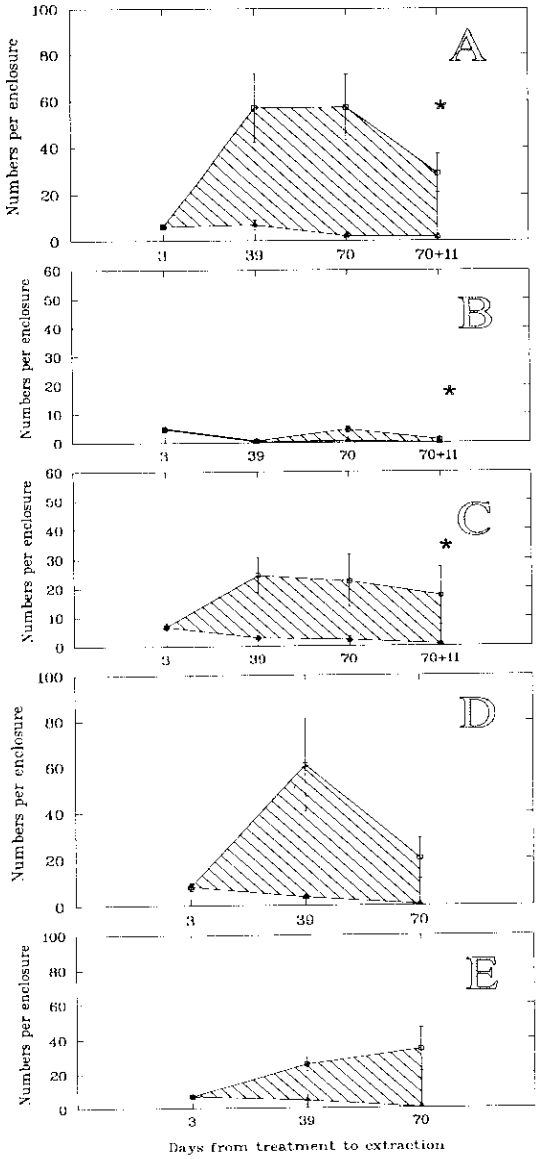
Population development 1992

7.3.7.3 **Population development. Adults and juveniles**

Folsomia fimetaria was counted in 2 size-classes, i.e. above or below 0.8 mm body length, which roughly corresponds to the distinction between juveniles and young adults and approximately equal to the size of test specimens introduced to the microcosms. Figure 7.20 indicates that the mean population size of both adults and juveniles in 1992 decreased from the 38th to the 59th day in the field in control microcosms as well as in microcosms which had been subjected to pesticide treatments. This is probably a result of a population dynamic cycle interacting with environmental factors. The exception was the high dosage dimethoate treatment which had the same or slightly increasing number of juveniles at the 2 dates. This might be explained as compensatory population growth which to some degree counteracts the still remaining toxicity. Suggestion of retarded population development in the pirimicarb and fenpropimorph treatments is largely due to aberrant results from one or two microcosms ("outliers", see above).

The specially low numbers found 70 days after treatment may be due to predation by the added gamasid mite *Hypoaspis aculeifer*.

Figure 7.19
*Folsomia fimetaria. Population development in "closed" field microcosms, 1991. - Hatched: specimens < 0.8 mm. - Unhatched: specimens > 0.8 mm. - A: control, B: Dimethoate, high dosage, C: Dimethoate, recommended dosage, D: Pirimicarb, E: Fenpropimorph. - * gamasids added. X-axis: days in the field + days stored at 5 - 10°C. Vertical lines: standard error of the mean. N = 10/8 (70 + 11 days).*



The pesticide treatments seem to have affected both adults and juveniles but at high dosage dimethoate the relative effect on juveniles was largest.

Population development, 1991

In 1991 the number of adults decreased throughout the period in both control and pesticide treatments (Figure 7.19). The mean control population (adults plus juveniles) did not decrease between the 39th and the 70th day in the field (Figure 7.19.A). The effect of pesticides on population development was generally more marked than in 1992. Thus, in the high dosage dimethoate treatment (Figure 7.19.B) both adults and juveniles nearly dissapeared and in the recommended dosage dimethoate treatment (Figure 7.19.C) the number of adults decreased while the number of juveniles was constantly low during the period between the 39th and the 70th day. Juveniles in pirimicarb treated microcosms (Figure 7.19.D) increased at the same rate as the control population but decreased strongly between the 39th and the 70th day. Finally, the number of juveniles slowly increased from the 3rd to the 70th day in fenpropimorph treated microcosms.

7.3.7.4 **Effect of storage before extraction**

Because of limited extraction capacity it was necessary to store part of the microcosms in a refrigerated room at 5 - 10°C for 10 - 24 days (cf. section 7.2.11) before extraction. Figure 7.20 demonstrates that a significant increase in numbers of both juveniles and adults occurred in most cases during storage. This is interpreted as a continuation of the population development before collection of microcosms from the field but in some cases the population development changed from decrease to growth during storage (Figure 7.20 A,B,D - 70 days). The storage did not change the difference between mean treatment effects, i.e. the relative population growth during storage was the same for all treatments (Figure 7.21).

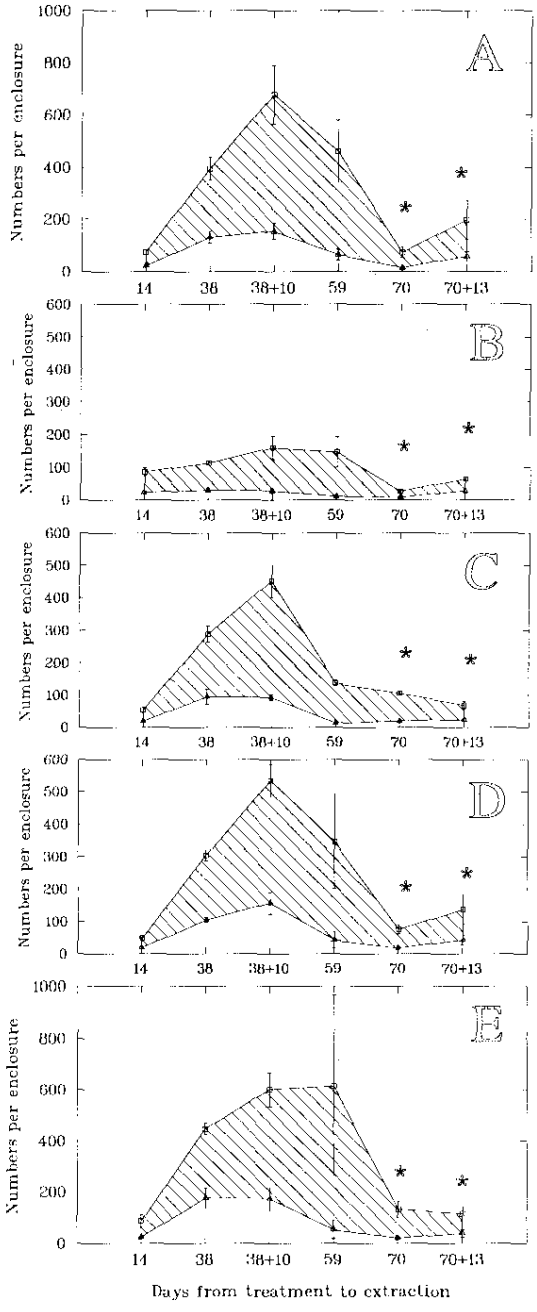


Figure 7.20

*Folsomia fimetaria. Population development in high, "open" field microcosms, 1992. - Hatched: specimens < 0.8 mm. Unhatched: specimens > 0.8 mm. - A: control, B: Dimethoate, high dosage, C: Dimethoate, recommended dosage, D: Pirimicarb, E: Fenpropimorph. - * gamasids added. - X-axis: days in the field + days stored at 5 - 10°C. Y-axis: Number of animals per mikrokosmos.*

However,the separate or joint handling of data from microcosms which were extracted immediately or stored before extraction influenced the possibility to show significant differences between pesticide treatments (In Tables 7.8 - 7.10 separate or joint handling, respectively, depended on which method showed the most significant treatment effect).

7.3.7.5 **Results from low "open" type microcosms**

The low "open" type microcosms tested in 1992 (section 7.2.2.,g) were treated with dimethoate in recommended and 5 times recommended dosage and collected from the field 70 days after pesticide treatment. Because of lack of extraction capacity they had to be stored for 24 days in a refrigerated room at 5 - 10°C. The result of extraction is illustrated in Figure 7.22 (hatched part).

Emigration from microcosms during storage.

Examination of the content of "collar-traps" of these microcosms revealed large numbers of *Folsomia fimetaria* (Figure 7.22, cross-hatched part). This species was only exceptionally found in "collar-traps" recovered from the field or stored after 38 days in the field. Therefore, it was concluded that the specimens found in the traps originated from the test populations within the microcosms which had attempted to emigrate during storage.

The soil in the microcosms collected 70 days after treatment was very moist and the microcosms gave off a rotten smell at the end of the long storage time. Thus, it seems likely that the emigration was caused by increasingly anoxic conditions in the pore space of the microcosm soil.

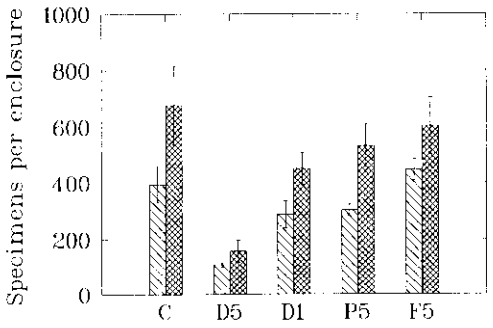


Figure 7.21

Effect of 10 days storage at 5 - 10°C on Folsomia fimetaria in microcosms recovered from the field 38 days after treatment, 1992. Hatched: No storage before extraction. - Cross-hatched: 10 days storage - X-axis: C: control, D5: Dimethoate, high dosage, D1: Dimethoate, recommended dosage, P5: Pirimicarb, F5: Fenpropimorph. - Vertical lines: standard error of the mean. N=5.

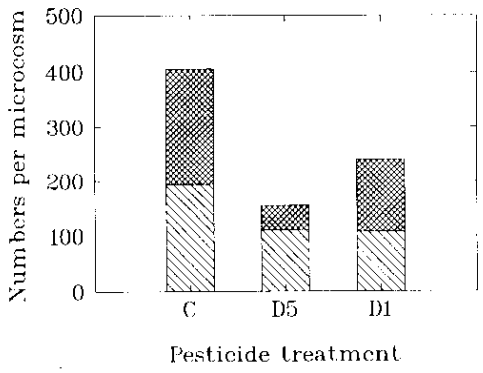


Figure 7.22

Numbers of Folsomia fimetaria in "collar-traps" and numbers extracted from low "open" field microcosms after 70 days in the field and 24 days in cold store, 1992. - Hatched: extracted from microcosms, Cross-hatched: found in "collar-traps" after storage for 24 days. - X-axis: C: control, D5: Dimethoate, high dosage, D1: Dimethoate, recommended dosage. N=10.

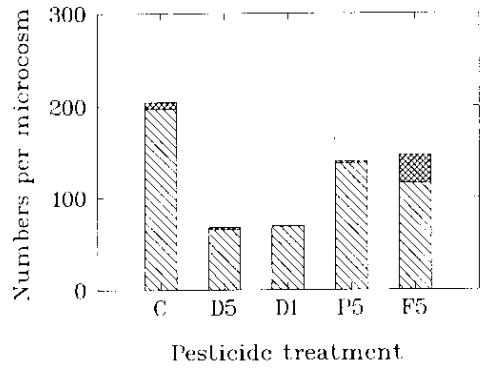


Figure 7.23

Numbers of Folsomia fimetaria in "collar-traps" compared with numbers extracted from high "open" field microcosms. Microcosms were recovered from the field 70 days after treatment and stored for 13 days at 5 - 10°C, 1992. Hatched: extracted from soil in microcosms, Cross-hatched: found in "collar-traps" after storage for 13 days. - X-axis: C: control, D5: Dimethoate, high dosage, D1: Dimethoate, recommended dosage, P5: Pirimicarb, F5: Fenpropimorph. N=5.

Effect of dimethoate in low "open" type microcosms

If based on the sum of specimens extracted and specimens found in the "collar-traps" the high dosage dimethoate treatment significantly reduced the numbers of *Folsomia fimetaria* (Kolmogorov-Smirnov 2-sample test: $P < 0.01$). The effect of the low dosage was not statistically significant.

7.3.7.6 Folsomia fimetaria in "collar-traps" of high "open" microcosms

The "collar-traps" of high "open" type microcosms stored for 13 days also contained specimens of *Folsomia fimetaria* but their numbers were small in comparison with the numbers extracted (Figure 7.23).

7.3.7.7 **Interaction with other Collembola**

No effect of Isotoma notabilis

In 5 of the 10 replicates of each treatment in 1991 10 specimens of *Isotoma notabilis* were added. Figure 7.24 compares the population size of *Folsomia fimetaria* in microcosms with and without *Isotoma* added. The differences were not statistically significant and it cannot be concluded that introduction of *Isotoma notabilis* had any influence on the population development of *Folsomia fimetaria* or on the effect of pesticide on that species.

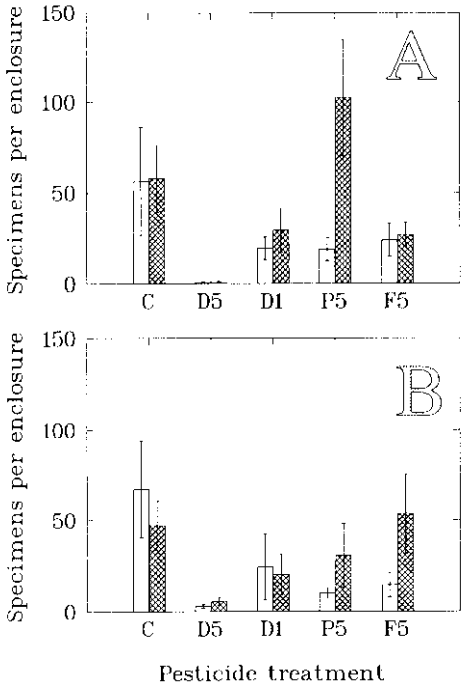


Figure 7.24

Effect of pesticide treatment on Folsomia fimetaria in "closed" field microcosms with and without addition of Isotoma notabilis , 1991. - A: 39 days after treatment, B: 70 days after treatment. - Blank columns: no Isotoma added, Filled columns: 10 Isotoma added. - X-axis: C: control, D5: Dimethoate, high dosage, D1: Dimethoate, recommended dosage, P5: Pirimicarb, F5: Fenpropimorph. Vertical lines: standard error of the mean. N=5.

7.3.7.8 **Interaction with Gamasida**

The mean population size of *Folsomia fimetaria* in 24 microcosms to which 5 gamasid mites had been added in 1991 was lower than in microcosms without gamasids 70 days after treatment (Figure 7.25) but the difference is not statistically significant. The effect of dimethoate on *Folsomia* was the same whether gamasids had been added or not.

Effect of predation by Hypoaspis

In the 1992 experiment the gamasid mite *Hypoaspis aculeifer* had been introduced to all high "open" type microcosms which were collected 70

days after treatment. Thus, the effect of gamasids on the collembolan population size cannot be evaluated properly based on identical conditions. However, the mean population size of *Folsomia fimetaria* in low "open" type microcosms (including the number of specimens found in "collar-traps") was about 3 times higher than the mean population size in the high "open" type microcosms with *Hypoaspis*. This may suggest a strong reduction of the collembolan population due to predation which is equally strong in dimethoate treated and untreated microcosms. The two types of microcosms were both 70 days in the field, but the low microcosms were stored for 24 days while half of the high microcosms were extracted immediately after collection and the other half were stored 13 days after collection. The population development during the 13 days of storage, however, was minimal (Figure 7.26).

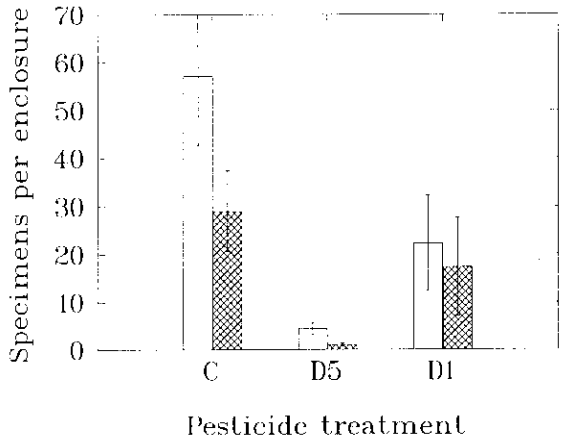


Figure 7.25

Numbers of Folsomia fimetaria in field microcosms with and without addition of gamasid mites. 70 days after treatment, 1991. - Blank columns: no gamasids added. (N=10). Filled columns: 5 gamasids added. (N=5). - X-axis: C: control, D5: Dimethoate, high dosage, D1: Dimethoate, recommended dosage. - Vertical lines: standard error of the mean.

Moderating effect of gamasids on pesticide effects

A negative effect of 5 times recommended dosage of dimethoate on the numbers of Folsomia fimetoria in these microcosms where gamasids were added was significant (Table 7.8, 70 + 0 days).

7.3.7.9 **Pesticide action through fine-meshed polyester web**

Passage through fine-meshed polyester web decreased effect of dimethoate

A main disadvantage of the "closed" type microcosms used in 1991 was the need to open the lids during pesticide application because it was feared that the 45 micron mesh polyester web covering the surface of the lids of the microcosms might prevent the pesticides from passing into the soil of the microcosms. Figure 7.27 shows much lower effect of high dosage dimethoate on *Folsomia fimetaria* in microcosms closed at treatment

(*D5+*) than in microcosms open at treatment (*D5-*) and not significantly different from non-treated microcosms (*C+*).

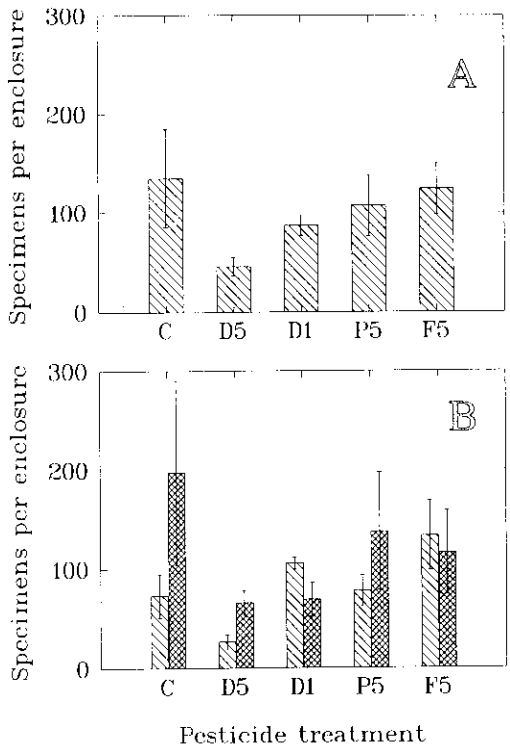


Figure 7.26

Numbers of Folsomia fimetaria in field microcosms with the gamasid Hypoaspis aculeifer 70 days after treatment, 1992. - A: All microcosms (N=10) , B: hatched: No storage, Cross-hatched: stored at 5 - 10°C for 13 days (N=5). - X-axis: C: control, D5: Dimethoate, high dosage, D1: Dimethoate, recommended dosage, P5: Pirimicarb, F5: Fenpropimorph. - Vertical lines: standard error of the mean.

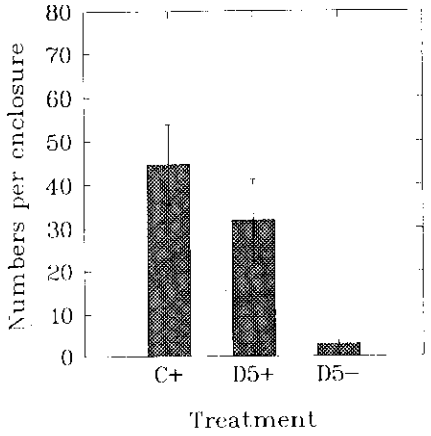


Figure 7.27

Numbers of Folsomia fimetaria in field microcosms with (+) or without (-) lid at treatment. - X-axis: C: control, D5: Dimethoate, high dosage. - Vertical lines: standard error of the mean. N=10 (C+, D5-), 6 (D5+).

7.3.7.10 **Effect of dimethoate dosage on *Folsomia fimetaria***

Dimethoate was sprayed in 2 different dosages, i.e. *low* dosage planned as equal to the dosage recommended for agricultural use and *high* dosage planned as 5 times the recommended dosage. The actual doses deposited on the surface soil within microcosms, however, were lower and varied much (cf. section 7.3.5.). These data on the effect of two different dosages and the control as zero dosage makes evaluation of models for the relationship between dosage and effect possible.

Exponentially decreasing dosage-effect model

In Figures 7.28 and 7.29 the values for actual deposition on filter paper in each treatment square were preferred for average planned dosage values because of the wider dispersion of dosage values. The arithmetic-logarithmic plot suggests that the observations at most dates of collection may be interpreted as an exponentially decreasing model:

$$y = c \cdot \exp (-k \cdot x) \qquad (1)$$

where y = population size (number of specimens), x = deposition of active substance, c = mean numbers in control microcosms and k = a constant (slope of regression line).

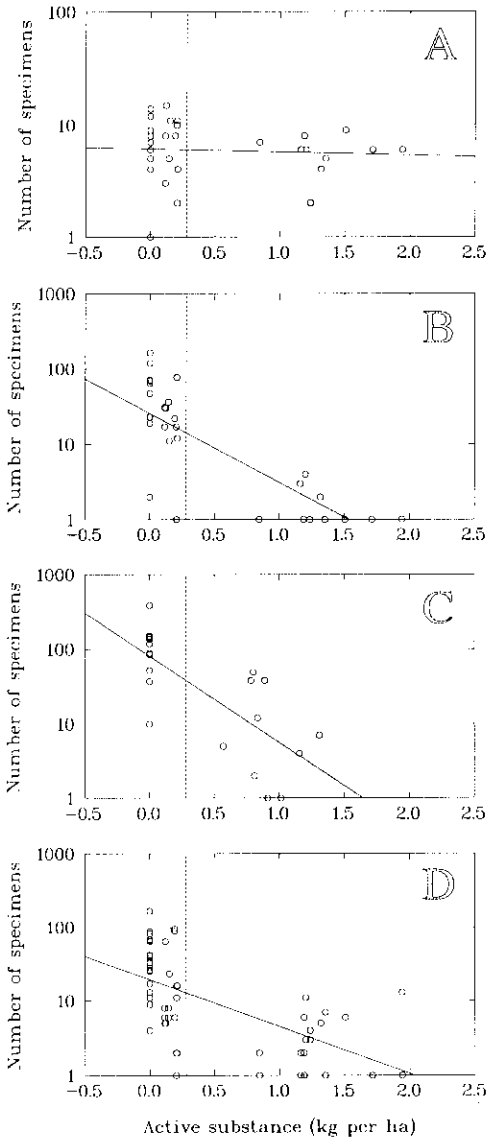
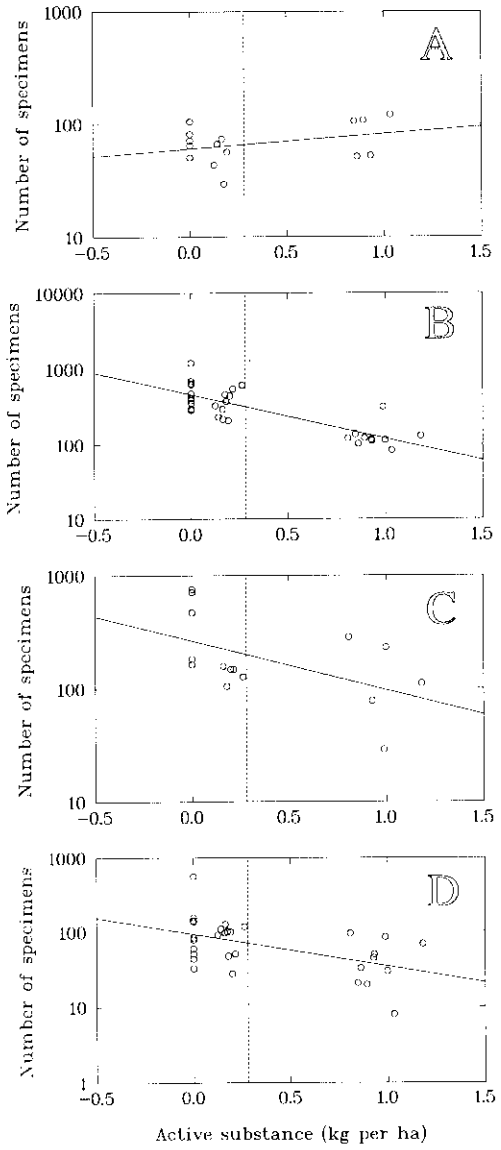


Figure 7.28
Effect of dimethoate-dosage on population size of Folsomia fimetaria in field microcosms, 1991. - Time from treatment to recovery from the field: A: 3 days, B: 39 days, C: 59 days, D: 70 days. - Vertical dotted line: recommended spraying dose. - A, B, D: "closed" microcosms, C: high "open" microcosms. - Y-axis: numbers + 1.

The slopes of the regression lines were statistically significant except for the first date of collection each year, i.e. 3 days after treatment in 1991 (Figure 7.28A) and 14 days after treatment in 1992 (Figure 7.29A). (Significance: $P < 0.001$, except $P < 0.05$ (59 days after treatment 1992), $P < 0.01$ (70 days after treatment 1992)). The position of all values from the low dosage treatment below the regression line in Figure 7.29C (59 days after treatment, 1992) may indicate that a decreasing exponential model does not provide the best fit for that set of data.

Figure 7.29
Effect of dimethoate-dosage on population size of Folsomia fimetaria in high "open" field microcosms, 1992. - Time from treatment to recovery from the field: A: 14 days, B: 38 days, C: 59 days, D: 70-days. - Vertical dotted line: recommended spraying dose. - D: Folsomia with gamasids (Hypoaspis aculeifer).



EC_{50} index

The EC_{50} values are measures of the effective concentration when the biological parameter, in this case the population size, is halved. These measures are useful for comparison of toxic effects.

Estimation of EC_{50} for semi-field data was done by fitting the following exponentially decreasing model to the data with the SAS procedure NLIN

(SAS Institute Inc. 1988). The model was developed by Paul Henning Krogh (pers.comm.) by calculating the k-constant after substitution of y = C/2 when x = EC_{50} in formula 1.

$$y = c \cdot \exp (-EC_{50}^{-1} \cdot \log_e 2 \cdot x) \qquad (2)$$

The minimum requirements for the model fitting procedure is the existence of data for controls (zero concentration) and at least two concentrations above the control. The concentrations should span the desired EC_X-value. The few data points in the present data set did not justify the use of more sophisticated models, but it cannot be excluded that more data points would lead to a choice of a different model.

Lowest EC_{50} after 8-10 weeks

Estimates for EC_{50} vary considerably between dates of collection from the field in the two years and between tests with or without other collembolan or gamasid mite species interacting with *Folsomia fimetaria* (Table 7.11).

EC_{50} below recommended dosage

The table suggests that the EC_{50} index without interaction or with interaction with the other collembolan *Isotoma notabilis* drops to the lowest values after 59 or 70 days. At that time the mean EC_{50}-values (0.21 and 0.22 kg.ha^{-1}, respectively) were below the dosage of active ingredient recommended for agricultural use in Denmark, i.e. 0.28 kg.ha^{-1}. In 1991 the mean value calculated for the 39th day after treatment (0.23 kg.ha^{-1}) was also below the recommended dosage. The high value for the 38th day after exposure calculated in 1992 may be due to weaker effect in the bottom layer of the high microcosm type used that year.

Table 7.11.

Estimates of EC_{50} for Folsomia fimetaria (only positive estimates included). - C.I.: Confidence interval. - Asterisk: Average for each time interval after spraying.

year	days after treatment	interaction	N	EC_{50}		95 % C.I.	
						lower	upper
1991	3	*I.notabilis*	15	1.760	1.76*	0.0	3.820
1992	38	none	30	0.476	0.31*	0.170	0.780
1991	39	none	15	0.182		0.0	0.524
1991	39	*I.notabilis*	15	0.280		0.0	0.668
1992	59	none	15	0.115	0.21*	0.0	0.267
1991	59	*I.notabilis*	10	0.311		0.0	0.674
1991	70	none	15	0.193	0.22*	0.0	0.520
1991	70	*I.notabilis*	15	0.247		0.0	0.591
1992	70	*Gamasida*	30	0.560	0.53*	0.0	1.300
1991	70	*I.notabilis* +*Gamasida*	12	0.505		0.0	1.778
mean (excl. time 3 days)				0.319			

The 1991 values from microcosms containing *I. notabilis* but without gamasids were at all dates higher than than those from microcosms with pure *F. fimetaria* populations. EC_{50}-values were in both years higher in experiments with than without gamasid mites.

7.3.7.11 **Effect of pirimicarb and fenpropimorph dosage**

Decreasing exponential function not significant for pirimicarb and fenpropimorph

Although only data from untreated (dosage = 0) microcosms and microcosms treated with one planned dosage, i.e. 5 times recommended dosage, the spread of amounts of active ingredients of pirimicarb and fenpropimorph actually deposited (cf. section 7.3.5) suggest for some dates of collection a decreasing exponential relationship between amount of active substance deposited and population size (Figures 7.30, 7.31, 7.32). The regression lines, however, are not statistically significant.

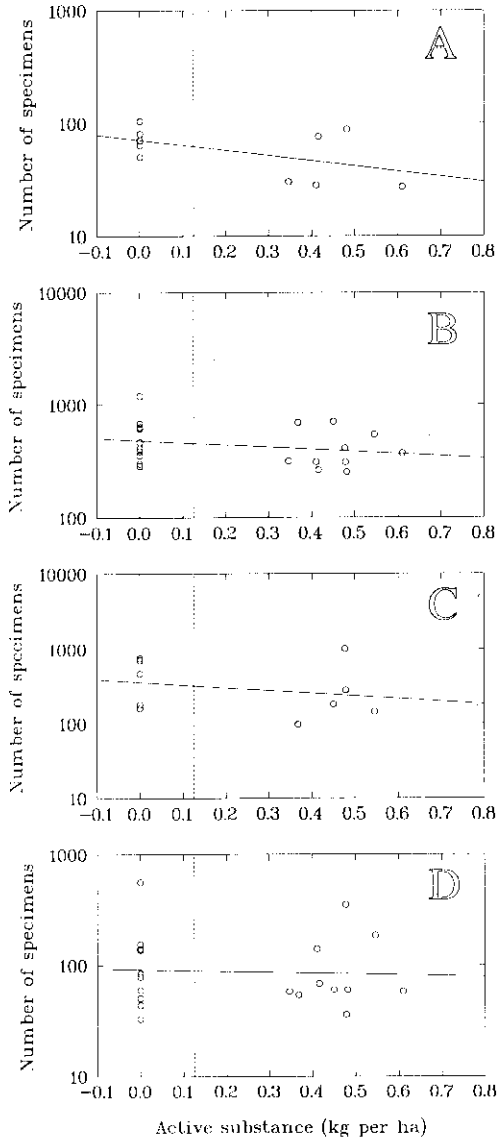


Figure 7.30
Effect of pirimicarb-dosage on population size of Folsomia fimetaria in high "open" field microcosms, 1992. - Time from treatment to recovery from the field: A: 14 days, B: 38 days, C: 59 days, D: 70 days. - Vertical dotted line: recommended spraying dose. - D: Folsomia with gamasids (Hypoaspis aculeifer).

7.3.8 **Effect of pesticides on *Isotoma notabilis***

I. notabilis: Negative effect of dimethoate 39 days after treatment

The effect of pesticide treatment on introduced *Isotoma notabilis* populations observed 3 and 39 days after treatment in 1991 (Figures 7.33A and 7.33B) was similar to that found for *Folsomia fimetaria*. Thus, the recovery of introduced specimens was poor from all treatments including the control at the first sampling occasion and there were no statistically significant acute effects of the pesticides. 39 days after treatment both dimethoate treatments (recommended and 5 times recommended dosage) showed significantly lower numbers of specimens than the control (Table 7.12).

Table 7.12

Significance (P-values) of ANOVA and nonparametric test (Kolmogorov-Smirnov-2-sample test) for Isotoma notabilis, 1991. - a. low "closed" type microcosm. b. high "open" type microcosm. - C: Control, D5: Dimethoate, 5 times recommended dosage, D1: Dimethoate, recommended dosage, P5: Pirimicarb, 5 times recommended dosage, F5: Fenpropimorph, 5 times recommended dosage. - Hyphen: no data. - Bold type: Significant (P < 0.05).

A: ANOVA and planned comparisons/contrasts (Wilkinson, 1990, Kirby, 1993)

Test	Factors/ comparisons	All sampling dates	Days in field after treatment			
			3[a]	39[a]	59[b]	70[a]
ANOVA	pesticide	**0.004**	0.634	**0.039**	**0.004**	0.204
	time	**0.003**	-	-	-	-
	block	**0.021**	**0.009**	0.098	0.483	0.599
	pesticide* time	0.210	-	-	-	-
Pairwise comparisons	C - D5	**0.001**	0.856	**0.005**	**0.004**	**0.034**
	C - D1	**0.002**	0.856	**0.009**	-	0.085
	C - P5	**0.032**	0.210	0.093	-	0.117
	C - F5	0.167	0.717	0.169	-	0.601
	D5 - D1	0.701	0.717	0.834	-	0.759

B: Nonparametric test (*Kolmogorov-Smirnov-2-sample test*)

Comparisons	Days in field after treatment			
	3[a]	39[a]	59[b]	70[a]
C - D5	1.00	**<0.001**	**<0.001**	0.32
C - D1	0.82	**<0.001**	-	0.82
C - P5	0.82	0.32	-	0.32
C - F5	1.00	0.32	-	0.82
D5 - D1	1.00	0.82	-	1.00

70 days after treatment the negative effects of high dosage dimethoate and pirimicarb (Figure 7.33C) were not statistically significant but a Wilcoxon signed-ranks test indicates that the fenpropimorph treated microcosms contained larger mean number of specimens than untreated microcosms ($P < 0.05$).

I. notabilis: more superficial distribution than F. fimetaria

Figure 7.34 shows that *Isotoma notabilis* was nearly exclusively found in the upper 10 cm layer of high "open" microcosms collected from the field 59 days after treatment (cf. the different depth distribution of *Folsomia fimetaria* on the same date (Figure 7.17)). This distribution may explain why *I. notabilis* is more sensible to dimethoate treatment than *F. fimetaria*.

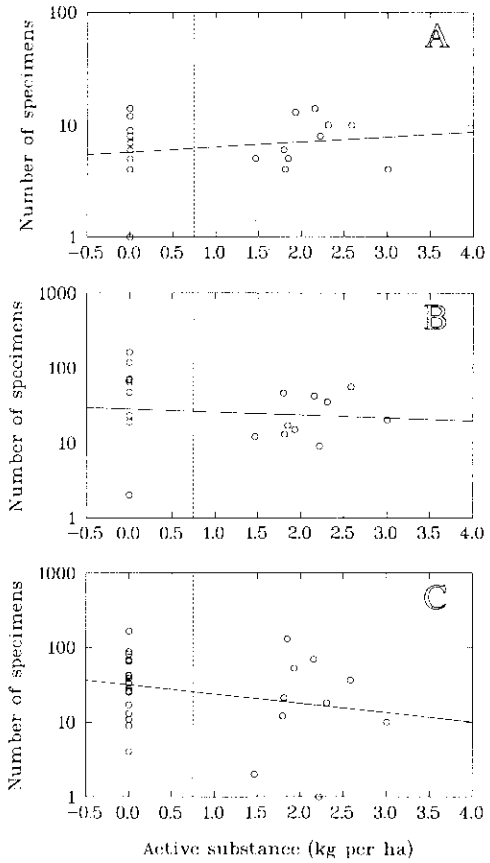


Figure 7.31
Effect of fenpropimorph-dosage on population size of Folsomia fimetaria in "closed" field microcosms, 1991. - Time from treatment to recovery from the field: A: 3 days, B: 39 days, C: 70 days. - Vertical dotted line: recommended spraying dose. - Y-axis: numbers + 1.

Table 7.13
Estimates of EC_{50} for Isotoma notabilis (only positive estimates included). - C.I.: Confidence interval.

Year	Days after treatment	Interaction	EC_{50}	95 % C.I.	
				Lower	Upper
1991	39	*F. fimetaria*	0.070	0.0	0.240
1991	59	*F. fimetaria*	0.173	0.0	1.116
1991	70	*F. fimetaria*	0.510	0.0	2.160
1991	70	*F. fimetaria* + *Gamasida*	0.077	0.0	0.383
Mean			0.208		

Exponentially decreasing dosage-effect model significant 39 and 59 days after treatment

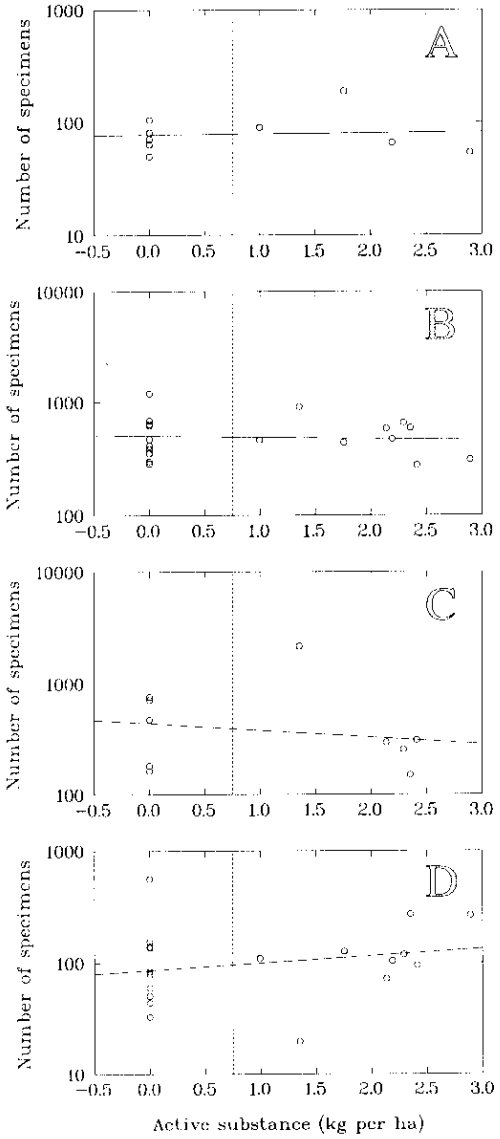
Regressions between deposited dimethoate dosage and population size of *Isotoma notabilis* are shown in Figure 7.35. The regressions at day 39 (B) and 59 (C) are statistically significant ($P < 0.05$ and $P < 0.001$, respectively).

EC_{50} below recommended dosage

EC_{50}-values for dimethoate calculated on the assumption of a decreasing exponential model are shown in Table 7.13 (cf. section 7.3.7.9) The EC_{50}-values 39 and 59 days after treatment were well below the dose recommended in Denmark for agricultural use.

The regressions between fenpropimorph dosage and population size (Figure 7.36) are not statistically significant.

Figure 7.32
Effect of fenpropimorph-dosage on population size of Folsomia fimetaria in high "open" field microcosms, 1992. - Time from treatment to recovery from the field: A: 14 days, B: 38 days, C: 59 days, D: 70 days. - Vertical dotted line: recommended spraying dose. - D: Folsomia with gamasids (Hypoaspis aculeifer).



7.3.9 **Effects of pesticides on Gamasida**

Reduction of gamasids by dimethoate in 1991

Treatment of microcosms to which gamasid mites had been introduced with the high dimethoate dosage (Figure 7.37) resulted in a statistically

significant reduction of the gamasid population 70 days after treatment in 1991 (Kolmogorov-Smirnov 2-sample test: $P < 0.001$).

No pesticide effect on Hypoaspis in 1992

In 1992 the gamasid *Hypoaspis aculeifer* had been introduced to all microcosms that were collected from the field 70 days after treatment. The results from that year (Figure 7.38) showed no significant differences between treatments. About the same mean numbers were found in the upper and lower layer of the microcosms (Figure 7.39) and no effect of pesticide treatment on the depth distribution was demonstrated.

7.3.10 **Comparison with the surrounding field soil**

Lower population density in the field soil

The result of a preliminary census of *Folsomia fimetaria* and *Isotoma notabilis* in the field soil next to the microcosms which was done 5 weeks after pesticide treatment in 1991 (cf. section 7.2.12.) indicates much lower population densities in the field soil than inside the microcosms (Table 7.1). Further, the effects due to pesticide treatment found for the enclosed populations did not appear in the field soil.

No treatment effects observed for the field population

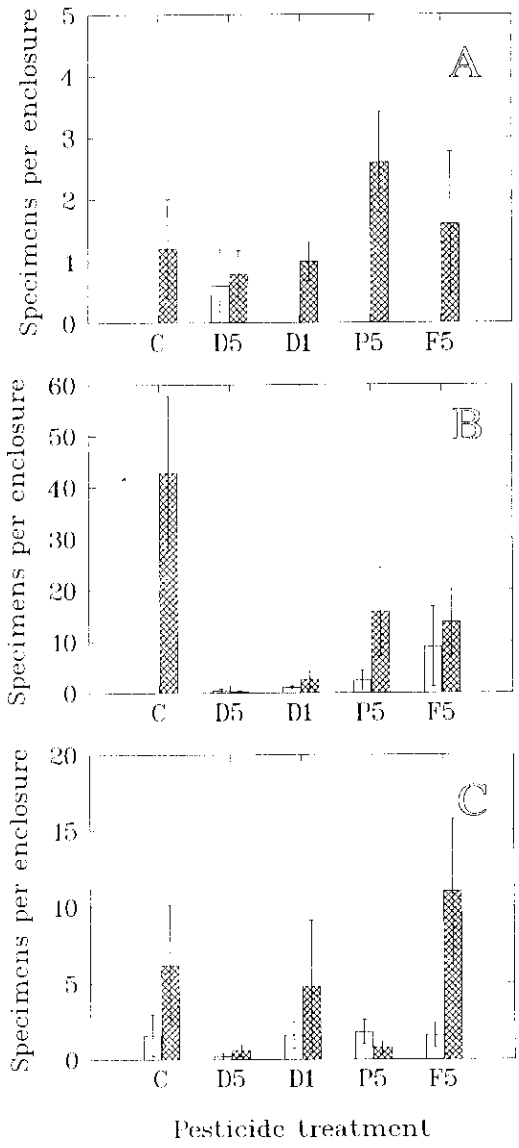


Fig. 7.33
Numbers of Isotoma notabilis in "closed" field microcosms, 1991. -Time from treatment to recovery of microcosms from the field: A: 3 days, B: 39 days, C: 70 days. - Blank columns: No I.notabilis added. - Filled columns: 10 I.notabilis added. - X-axis: C: control, D5: Dimethoate, high dosage, D1: Dimethoate, recommended dosage, P5: Pirimicarb, F5: Fenpropimorph. - Vertical lines: standard error of the mean. N = 5.

Fig. 7.34
Depth distribution of Isotoma notabilis in high "open" microcosms, 1991. - Cross-hatched: 0 - 10 cm depth. Hatched: 10 - 20 cm depth. - C: control, D5: Dimethoate, 5 times recommended dosage. N = 10.

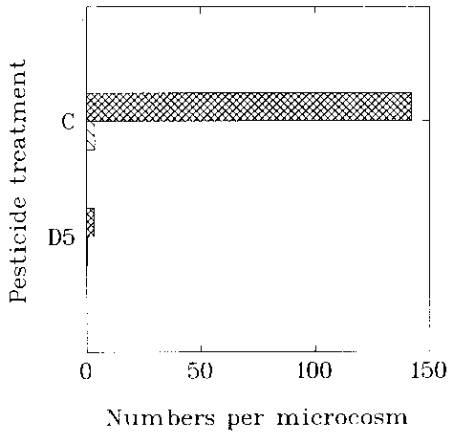
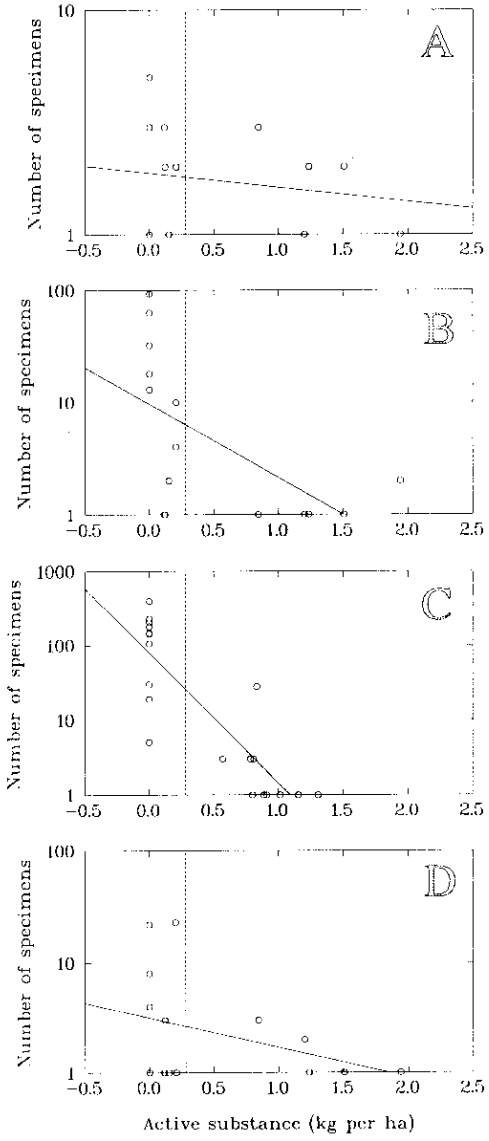


Fig. 7.35
Effect of dimethoate-dosage on population size of Isotoma notabilis in field microcosms, 1991. - Time from treatment to recovery from the field: A: 3 days, B: 39 days, C: 59 days, D: 70 days. - Vertical dotted line: recommended spraying dose. - A, B, D: "closed" microcosms, C: high "open" microcosms. - Y-axis: numbers + 1.



The results of a more complete census of the field populations in the treatment squares outside the microcosms which was carried out in 1992 will be described in a subsequent report.

7.4 Discussion and conclusions

7.4.1 Field microcosms

Tests of seven types of microcosms

Seven different types of field microcosms containing defaunated soil were tested during the study in order to find a technique suitable for confining a test population of introduced specimens in a habitat which differs as little as possible from an un-manipulated field soil. If the start population of the test species is controlled and exchange with the surrounding field population can be avoided a study of population development under close to natural conditions would be possible.

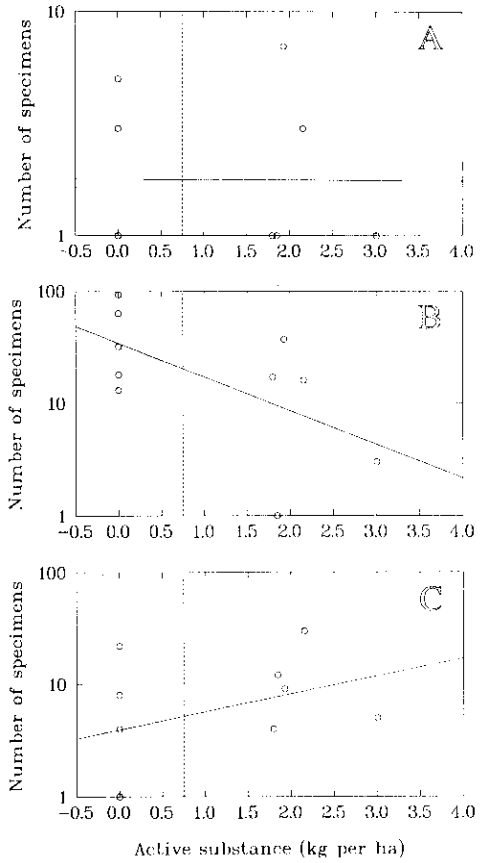


Fig. 7.36

Effect of fenpropimorph-dosage on population size of Isotoma notabilis in field microcosms, 1991. - Time from treatment to recovery from the field: A: 3 days, B: 39 days, C: 70 days. -Vertical dotted line: recommended spraying dose. -Y-axis: numbers + 1.

Suitability of the microcosm types for rearing Collembola and gamasids

The attempt to keep and rear populations of the collembolan species *Folsomia fimetaria* and *Isotoma notabilis* and the gamasid *Hypoaspis aculeifer* in the microcosm types tested was generally successful. 19 of 174 "closed" type microcosms with introduced test populations of *F. fimetaria* extracted in 1991 contained no specimens of the test species. Most of these microcosms had been treated with pesticides but the test

population was also missing in one af the control microcosms. In 1992, however, the mean population size of *F. fimetaria* extracted from the "open" type microcosms used that year was at most sampling dates about 10 times as high as the mean population size found in 1991 and the test species was present in all but one of the microcosms, i.e. a low type microcosm treated with dimethoate.

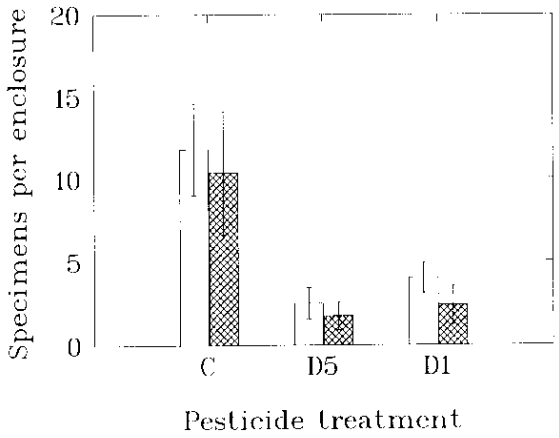


Figure 7.37

Numbers of gamasid mites in field microcosms, 70 days after treatment 1991. - Blank columns: no gamasids added (N = 10). Filled columns: 5 gamasids added (N = 8). - X-axis: C: control, D5: Dimethoate, high dosage, D1: Dimethoate, recommended dosage. Vertical lines: standard error of the mean.

Greater rearing success in 1992 than in 1991

The greater success with the *Folsomia* test population in 1992 than in 1991 may have several causes:

a) In 1991 20 specimens of approximately the same size selected from cultures with specimens of mixed age were added to each microcosm. In 1992 30 specimens sampled from even-aged cultures were introduced to each microcosm in two increments. Thus, in 1992, the higher probability of adding about equal numbers of males and females of the same age together with the higher start population without doubt improved the chance for population growth in the microcosms.

b) The larger depth of the high "open" type microcosms used in 1992 offered better possibility for the test population to find protection from for instance draught or pesticide impact by moving to deeper soil layers.

c) Watering of the microcosms during the draught period in June - July 1992. The amount of water added did not exceed the normal precipitation during the period in question but it was more evenly distributed over time than the usual rainfall.

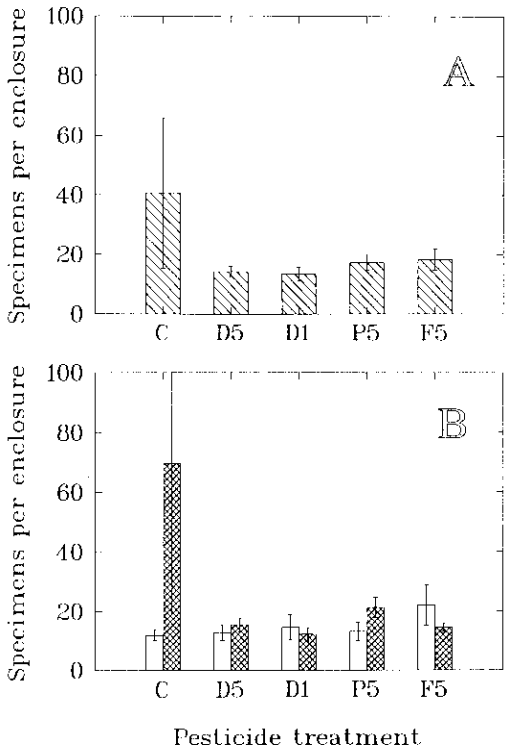


Figure 7.38

Numbers of Hypoaspis aculeifer in field microcosms 70 days after treatment, 1992. - A: All microcosms, B: Blank: No storage, Cross-hatched: stored at 5 -10°C for 13 days. - X-axis: C: control, D5: Dimetho-ate, high dosage, D1: Dimethoate, recommended do-sage, P5: Pirimicarb, F5: Fenpropimorph. - Vertical lines: standard error of the mean. N = 10 (A), N = 5 (B).

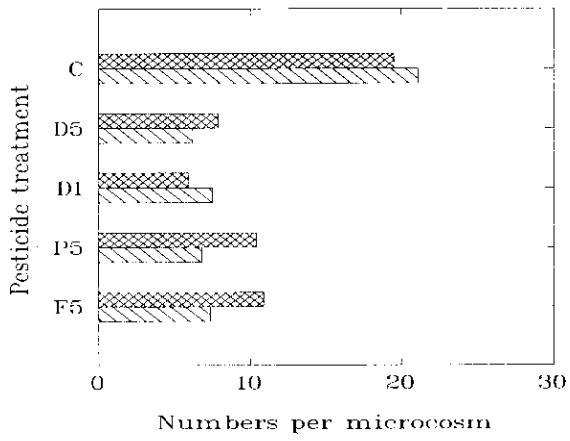


Figure 7.39

Depth distribution of the gamasid Hypoaspis aculeifer in high "open" microcosms, 70 days after treatment 1992. - Cross-hatched: 0 - 10 cm depth. Hatched: 10 - 20 cm depth. - Pesticide treatment: C: Control, D5: Dimethoate, 5 times recommended dosage, D1: Dimethoate, recommended dosage, P5: Pirimicarb, F5: Fenpropimorph. N = 10.

Tightness of microcosms

It proved to be very difficult to make the microcosms tight enough to ensure the desired isolation from the field populations. This contradicts the experience gained from experiments with simple cylindrical enclosures without bottom used in a forest floor (Petersen 1971). The explanation may be that the possibility for vertical movement of soil Collembola is improved by the soil preparation of agricultural fields which agrees with the generally deeper and more equalized depth distribution in agricultural as compared to natural soils.

Even microcosms covered with fine-meshed polyester web in both ends ("closed" type microcosms) proved to be penetrable for e.g. some species of Collembola and gamasid mites. A test showed that some very small species are probably able to squeeze themselves through the 45 micron-mesh web. Larger species probably made their way through inconspicuous gaps between the lid and the edge of the microcosms left after unscrewing and rescrewing the lid at spraying. The exposure of the soil surface within microcosms at pesticide treatment was necessary because the polyester web impedes the percolation of pestides. This was demonstrated by a strongly reduced effect of dimethoate treatment on *Folsomia fimetaria* when the lids of microcosms were not removed during spraying.

Best isolation from field population in "open" type microcosms with "collar-traps"

The best and nearly complete isolation of the test population was obtained with the two "open" types of microcosms, i.e. high and low, used in 1992 with fine-meshed web in the bottom but open in the upper end. A "collar-trap" prevented escape or immigration of animals and made possible a registration of specimens attempting to do so.

Shade effect for spray deposition caused by "open" type microcosms

One disadvantage of that type of microcosm was that the 10 cm high upper end projecting above the soil surface interfered with the distribution of the spray by causing a shade effect so that some parts of the soil surface within microcosms received a lower dosage than the soil surface of the field outside microcosms. The mean deposition of spray inside microcosms was measured to be only 67 to 79 % of the mean deposition outside microcosms. Thus, the conditions for survival and reproduction may be much better below the shaded parts within microcosms than in the field soil as a whole.

High vs. low "open" microcosms

The high "open" type microcosm containing a 20 cm deep layer of defaunated soil was chosen as the principal microcosmos type for the experiments in 1992 because the relatively deep soil horizon approximately corresponds to the depth of vertical distribution of the soil microarthropod fauna and permits the vertical movement of the test animals in response to microclimate, food and other environmental impacts including the action of pesticides. Thus, effects of such environmental factors measured on the populations in this high type of microcosm are believed to be similar to

the effects which could be observed on the unconfined field populations in their total vertical distribution.

The content of the high microcosms was divided into two 10 cm horizons which were extracted and counted separately. The distribution of animals between the lower and higher horizon indicated a stronger effect of pesticides in the upper than in the lower horizon but it does not tell to which degree the specimens in the upper part had moved down in order to escape the pesticide action or moved upwards from the lower horizon to replace a reduced population in the upper horizon. Populations confined to the uppermost part of the soil without possibility for escaping to or recruiting new specimens from greater depths would certainly be more strongly affected than deeper living populations. Thus, tests using lower field microcosms placed in the soil surface would without doubt be more sensitive to pesticides but also more vulnerable towards other environmental events such as drought.

A test of low "open" type microcosms in 1992 showed that they were suitable for rearing *Folsomia fimetaria*. Populations of that species were found in all but one of this type microcosm after 70 days in the field. Effect of high dosage dimethoate was found but none of this type of microcosms were recovered from the field at shorter intervals of time after pesticide treatment than 70 days. Thus, a comparison of pesticide effects between high and low microcosms was not possible after shorter exposure times when the pesticide effect was expected to be stronger.

A practical and resource-wise drawback of the high microcosms is the need to divide the soil column in two at extraction. This means a demand for double extraction capacity as compared to the low type microcosms. A consequence of the maximum capacity of 50 units available at the Mols Laboratory was that half of some collections of microcosms from the field had to be stored in a refrigerated room for 10 - 14 days before extraction and that the low type of microcosms was stored for 24 days before extraction. (They had to be removed from the field 70 days after treatment because of the harvest).

Effect of storage

Comparison between microcosms extracted immediately after collection from the field 39 or 70 days after pesticide treatment and microcosms collected the same dates but stored before extraction showed distinct changes in population size and structure during the storage time. This may be interpreted as a continuation of the population development started when the microcosms were placed in the field but may also partly be an artifact caused by the sudden decrease of temperature. Thus, it is possible that the increase of juveniles observed was due to accellerated reproduc-

tion corresponding to the population peak recorded in field populations in the autumn (e.g. Wallwork, 1970).

It is also possible that the population growth during storage found in microcosms collected 70 days after treatment was due to a lower activity of the gamasid mites present in this set of microcosms.

After storage and especially after the long-time storage of the low type microcosms in 1992 several specimens of the test species *Folsomia fimetaria* were found in the "collar-traps" of the microcosms. This indicates that the animals attempted to emigrate from the microcosms. The soil of the microcosms was very wet at collection after recent rainfall. Resulting anoxic conditions in the soil pore system may explain the attempt of the animals to escape from the microcosms during storage.

7.4.2 **Defaunation**

Comparison with other defaunation methods

The soil was treated with alternating heating, freezing, drying and rewetting in order to obtain a soil devoid of microarthropods. The term "defaunation" has been used although nematodes, protozoans and other microfauna groups without doubt survived the treatment. The relatively gentle treatment was chosen in order to disturb microflora and soil properties as little as possible. Huhta, Wright and Coleman (1989) obtained a more complete defaunation by treating the soil in a microwave-oven or freezing to 80°C followed by drying. However, soil properties such as water-holding capacity, leaching of NH_4^+ and NO_3 were rather strongly affected.

Tests showed that the mild defaunation method proposed here is highly efficient for elimination of microarthropods. No specimens of the test species chosen were found in the tests. The presence of microfauna is not viewed as a disadvantage except perhaps in the mixed Collembola - Gamasida experiments. Some gamasids are nematode feeders and it cannot be excluded that some consume both kinds of animals. Thus, presence of nematodes may interfere with the collembolan-gamasid interaction and influence the result of the pesticide tests.

7.4.3 **Environmental factors**

Soil in microcosms more compact than field soils

Volume weight of soil, water content and temperatures inside microcosms were compared with the same factors in the field soil outside microcosms. The volume weight of soil indicated a slightly more compact soil inside than outside microcosms. The microcosm soil was well mixed and therefore probably more homogeneous than the field soil. Earthworms and other macrofauna were missing in the microcosms. Therefore, macropores caused by these animals would be missing. The high water content inside

Better protection against dessication

the microcosm may be correlated with the volume weight of soil but may also partly be explained by the protection of the microcosm walls against drainage and evaporation. Temperatures inside and outside microcosms did not differ at the moments of measurement.

Restoration of microflora

A soil suspension was added to the microcosms in order to facilitate the restoration of a natural microflora. The similarity between microflora composition and biomass inside and outside the microcosms was not checked. However, the vigorous population growth of the microbivorous test Collembola suggests that the microbial production was high.

7.4.4 **Choice of test species**

Criteria for choice of species

The choice of collembolan test species was based on the following criteriae: a) common occurrence in Danish agricultural fields including the fields chosen for the topical experiments, - b) specificity for the true soil environment, i.e. species living in the soil pore system (eu-edaphic species) were preferred while surface living (epedaphic) species were avoided, - c) short generation time, - d) high reproductive potential, and - e) ease of culturing in the laboratory.

Four species were tested during the first year but only two, i.e. *Folsomia fimetaria* and *Isotoma notabilis* were chosen for the experiments in 1991. *Isotoma notabilis* has the special advantage for this kind of experimental purposes that it is normally parthenogenetic, i.e. all individuals are females able to reproduce without fertilization. It also showed high sensitivity against dimethoate treatment. However, the laboratory cultures of that species were less productive and reliable than those of *Folsomia fimetaria* and it proved difficult to get enough specimens for the start populations of the microcosms. *Folsomia fimetaria* was therefore chosen as the principal species in 1991 and the only collembolan species used in the final experiments of 1992. *Isotoma notabilis*, however, is still a promising test species and further efforts should be done in order to find a method for culturing it.

It proved difficult to find a gamasid mite which could be reared on cultures of the selected test collembolans. However, *Hypoaspis aculeifer*, a species common in grassland and woodland soils but also occurring in agricultural soils, was able to multiply on a diet of *Folsomia fimetaria* and was therefore chosen as predator in the mixed Collembola - Gamasida tests.

Collembola communities in agricultural fields

The two test Collembola are common in Danish agricultural fields and the cultures of these species were started from specimens extracted from the experimental fields. Animal communities of agricultural fields may be

considered as empoverished remnants of the much more diverse communities characteristic of natural grasslands or forests. Similarly, collembolan communities in agricultural soils are remnants of the richer collembolan communities found in undisturbed soils. It is likely that only those species which are most resistant against agricultural practices including pesticide treatment have been able to exist in this environment. It is therefore likely that species which do not or rarely occur in agricultural soils are more sensible against for instance pesticides than species common in agricultural fields. A relatively high resistance against pesticide treatment shown by test species common in agricultural soils is therefore not indicative of low effect on a fully developed soil fauna community.

Adaptation of Collembola populations to human activity

It is also very probable that populations of species occurring in agricultural fields have adapted through selection to the special conditions of agricultural fields and therefore are genetically different from populations of the same species living in natural soils. Such adaptation of animal populations to the effect of human activity, e.g. deposition of heavy metals, has recently been demonstrated for Collembola (Posthuma 1990).

7.4.5 **Heterogeneity of pesticide deposition**

It was shown by weighing pieces of filter paper before and after spraying that the amount of spray deposited on the soil surface differed much between treatments and between individual squares receiving the same treatment. Thus, within treatments the deposition in individual squares differed by a factor 2 or more. The coefficient of variation between squares varied between 19 and 33 %. This is within the range of coefficients from measurements for several combinations of nozzle type, pressure and height published by Tønnesen (1981) and Permin (1983). However, a coefficient of variation about 10 % would be expected for the actual combination of nozzle type, boom height and pressure employed (Permin, op.cit.) and coefficients above 20 are characterized as "unsatisfactory" by Tønnesen (op.cit.).

Several factors may influence the distribution of spray:

a) The type of nozzle determines the spray pattern and the interaction between nozzle type and spray pressure determines the size of droplets (Tønnesen 1981).

b) The height above the ground seems to be very important (Tønnesen 1981, Permin 1983) which is due to different degrees of overlapping between spray-cones. The boom height in the present experiment was about 55 cm.

c) Possible differences in surface tension of the different spray solutions may cause differences in the size of droplets and thus explain the heterogeneity between treatments observed in the present study. In 1991 it was actually remarked that the dimethoate droplets absorbed on filter paper were larger than the fenpropimorph droplets.

d) Another possible cause of variation may be the small 2.5 x 3 m^2 areas sprayed at a time instead of the much larger plots usually treated in agricultural field experiments. It may in practice be impossible to adjust the pressure in the tank containing the spray and the walking pace sufficiently exactly to ensure that all squares receives the same dosage.

The amount of spray solution deposited was 54 - 89 % of the planned dosage. The loss may be due to drift and interception by plants. However, the amount of water evaporated during spraying and storage until weighing is not known. Therefore, the deposition of active ingredient may be closer to the planned dosage.

7.4.6 **Pesticide effects on Collembola and gamasids**

Statistical treatment

The statistical analysis was based on data from microcosms treated as a whole or, in the case of high "open" type microcosms, also as separate horizons. It was shown that the requirement of normality was not fulfilled for all combinations of dates and treatments. It appeared that departure from normality was found nearly exclu-sively in data for pesticide treated microcosms and that the tendency increased with time from the date of spraying.

Normal probability plots show that some pesticides at certain dates after treatment had decreased the collembolan population size considerably in the majority of microcosms while one or a few microcosms treated with the same pesticide contained as high as or even higher number of specimens than the untreated control microcosms. This variability may be the result of the uneven deposition of pesticides discussed above but may also be due to differences in exposure of the animals to the pesticide depending on the distribution and movements of specimens within the microcosms during the time between treatment and extraction (c.f. Fábián and Petersen 1994).

Finally, the success of the introduced animals to establish reproductive populations in the microcosms may have varied due to a number of experimental factors, e.g. the quality of the soil and microflora, the vitality of the animal cultures used, and the proportion of males and females among the introduced animals. Thus, at a few sampling dates even the

data sets for control microcosms included anomalous values. In 1992 the introduced test populations initially showed successful reproduction in all microcosms whereas in 1991 some of the test populations including those in some of the control microcosms failed to reproduce and grow for the reasons discussed above. Thus, some of the variability found that year was evidently caused by this experimental error.

Pesticide effects on F. fimetaria and I. notabilis

Evaluation of the statistical analysis for the two years 1991 and 1992 based on normal probability plots, analysis of variance and non-parametric tests clearly demonstrated negative effects of dimethoate on the collembolan species *Folsomia fimetaria* and *Isotoma notabilis* and on gamasid mites (unidentified species) in 1991. The strongest effect traceable over the longest span of time was shown by dimethoate in high dosage, i.e. planned to be 5 times the dosage recommended for agricultural use in Denmark (D.L.G. 1991) but also dimethothate in a planned dosage equal to the recommended dosage affected the populations of the two species significantly at some dates after treatment.

The high dosage dimethoate had significant negative effect on the gamasid mites tested in 1991 (un-identified species) but no significant effect was observed on the gamasid mite *Hypoaspis aculeifer* in 1992.

Negative effects of pirimicarb and fenpropimorph (planned dosage: 5 times that recommended for agricultural use) on *Folsomia fimetaria* were suggested at some sampling dates in both years by statistically significant differences from the control microcosms or by probability plots but more experiments will be required in order to demonstrate the effect of these pesticides convincingly.

Low initial recovery of introduced specimens

It was characteristic for both years that very few specimens, i.e. only about 1/3 or 1/4 of the test specimens introduced, were extracted from microcosms collected and extracted on the date of spraying or up to a few days after whereas in 1992 3/4 of the introduced adult specimens were recovered 2 weeks after treatment.

It is possible that the low recovery of introduced species shortly after introduction and placing in the field may not be due to high mortality but rather to disturbances which induce moulting and egg-laying. This might in turn inactivate the Collembola and decrease their mobility for some time.

No effect first 2 weeks after treatment

No statistically significant effect of treatment on *Folsomia fimetaria* and *Isotoma notabilis* was observed during the first 2 weeks after pesticide treatment. Thus, it can be concluded that no acute effect can be demonstrated by the microcosm techniques tested.

Pesticide effects 5 - 10 weeks after treatment

A negative effect of both high and low dosage dimethoate on the two collembolan species, *Folsomia fimetaria* and *Isotoma notabilis*, was significant 5 - 6 weeks (38 - 39 days) after treatment. The effect was also significant 8 weeks (59 days) after treatment and persisted in 1991 for *F. fimetaria* at least until 10 weeks after treatment. In 1992 the effect on *F. fimetaria* 10 weeks after treatment was more uncertain. This may be due to interaction with the gamasid *Hypoaspis aculeifer* which was introduced to all microcosms collected at that date.

Negative effects of pirimicarb on *Folsomia fimetaria* were suggested 70 days after treatment in 1991 and 59 days after treatment in 1992. In the case of the fenpropimorph treatment negative effects on *Folsomia fimetaria* were suggested 39 days after treatment in 1991 and 70 days after treatment in 1992. A statistically significant stimulating effect of fenpropimorph on *Isotoma notabilis* was found 70 days after treatment in 1991.

Population development

The general pattern of population development observed was growth in numbers until the 5th - 6th week and thereafter more or less stable numbers until 8 or 10 weeks after treatment (Figures 7.19 - 7.20). The population was dominated by juveniles (specimens < 0.8 mm). The number of adults decreased throughout the period in 1991 but increased until 5 - 6 weeks after treatment in 1992 and decreased thereafter. The strong decrease observed between the 8th and the 10th week in 1992 may be due to predation by gamasid mites present in the microcosms collected 10 weeks after treatment. Low "open" type microcosms with pure *F. fimetaria* population collected at the same date contained about 3 times as many specimens per microcosm than the high type microcosms containing both gamasids and Collembola.

The time for egg development of *Folsomia fimetaria* was found to be 10 - 12 days and the development time from hatching to maturity 19 -20 days at 20° C (Krogh and Holmstrup, in prep.). Thus, the generation time is about 30 days at 20° C. It is therefore possible that 3 generations were hatched during the time of the experiment.

Pesticide effects and soil depth

At the start of the experiments the test specimens were introduced in the middle (1991) or about 7 cm from the soil surface (1992) of the microcosms (cf. section 7.2.6.). 2 weeks later in 1992 the majority of the *Folsomia fimetaria* test population was found in the deep horizon of the high "open" type microcosms. Thus, most Collembola had moved to a greater depth probably because of the prevailing draught.

Repellent effect of dimethoate

The possibility that the movement was due to the pesticide treatment was not likely because the depth distribution at that date was the same in untreated and treated microcosms. At later collection dates the distribution

had changed so that the majority of the population was in the upper 10 cm layer. However, 5 - 6 weeks after treatment the distribution of *F. fimetaria* was deeper in dimethoate treated microcosms than in the other treatments. This may suggest that dimethoate has a repellent effect on the collembolan but may also be the effect of higher rates of mortality or immobili-zation (Fábián and Petersen, 1994).

Strongest pesticide effect in upper soil layer

The effects of pesticide treatments were mainly observed in the upper horizon of the high "open" type microcosms. Only the high dosage dimethoate treatment had a statistically significant effect in the deep horizon. Otherwise, the mean numbers of *Folsomia fimetaria* found in the deep horizon were not significantly different from the control.

It is concluded that although the whole population was probably subjected to the pesticide action because of vertical movements in the soil core of the microcosms the effect was nevertheless dampened with depth.

Isotoma notabilis had a more superficial distribution in the microcosms than *Folsomia fimetaria* and would therefore be more exposed to the pesticide action. Some of the results obtained actually suggest that *I. notabilis* reacted more strongly against the pesticide treatments.

The gamasid *Hypoaspis aculeifer* was about equally abundant in the upper and lower horizon. This species is very mobile and it is likely that it moves frequently between the two horizons.

Interaction with other animals

There was no clear effect on the *Folsomia fimetaria* populations or on the effect of pesticides attributable to the presence of the other collembolan *Isotoma notabilis*. However, EC_{50}-values calculated for *F. fimetaria* interacting with *I. notabilis* were higher than values based on pure *F. fimetaria* populations at all dates in 1991. Only little is known about the precise ecological niches of the two species so it is not possible to deduce to which extent these species compete for the same ressources.

Although based on two different sizes of microcosms collected 70 days after treatment in 1992 the comparison of numbers of *Folsomia fimetaria* with or without the presence of the gamasid mite *Hypoaspis aculeifer* suggests a strong negative effect of the gamasid mite on the collembolan population irrespective of treatment. The lack of pesticide effects on *F. fimetaria* found at that occasion might be explained as a result of reduced predation compensating for the negative effect of the pesticides. However, the lack of negative effects of dimethoate on the number of *H. aculeifer* seems to contradict that explanation, unless the reduced predation is due to lower predation rate per gamasid specimen.

Effect of dosage

An EC_{50}-index for dimethoate corresponding to the dosage when the population size is 50 % of the untreated population size was calculated for *Folsomia fimetaria* and *Isotoma notabilis* at each date in the two years (Table 7.11, 7.14). The index was based on the assumption of a decreasing exponential function between population size and pesticide dosage. Great variation was found between EC_{50}-values on different dates and depending on presence or absence of interaction with *I. notabilis* and gamasid mites. EC_{50} values for *F. fimetaria* were well below the recommended dimethoate dosis 5 - 6 weeks after treatment in 1991 and 8 - 10 weeks after treatment in 1992. Thus, if the effect in microcosms corresponds to the effect on the non-confined field populations it can be concluded that use of dimethoate in the recommended dosage has severe effect on populations of the test species.

Persistence of dimethoate and time of effect

Dimethoate is a cholinesterase inhibiting insecticide with a half-life of 4.8-9.7 days measured in three different soils at 20° C (Kolbe et al. 1991). With such short persistence the toxicity would be very low, i.e. less than 1/16 of the toxicity at spraying after 40 days and less than 1/128 after 70 days. The late effect observed on the collembolan populations suggests a secondary lag effect which cannot be attributed to a still persistent toxicity of dimethoate. Several possible reasons may explain why the effect is expressed so late while no effect was observed during the first weeks after treatment:

a) The effect of the pesticide is largely not lethal to the soil animals but has sublethal effects which are finally expressed as a decrease of reproductive rate.

b) At first the pesticide was still concentrated in the surface layer of the soil. The possibility of the animals for getting into contact with the pesticide was low at that time. Later the pesticide becomes more dispersed in the soil column.

c) The pesticide may be repellant to the animals. In that case the animals would escape the pesticide by moving away. Such reaction of *Folsomia fimetaria* against dimethoate was observed by Fábián and Petersen (1994) in two dimensional laboratory experiments whereas the reaction in the threedimensional soil system is less clear.

d) Collembola have a strongly hydrophobic cuticle. Therefore an immediate effect through direct contact with the pesticide is not possible. It is most probable that the pesticides have to be ingested to have an effect on the animals.

7.4.7 **Comparison with the field populations**

The numbers of soil samples analysed were not sufficient for detecting pesticide effects on the field populations outside the microcosms but the field population size of the two test Collembola was much lower than the population size within microcosms. A discussion of this will be postponed to a subsequent report on a study in progress.

7.4.8 **Proposals for improvements and further studies**

Improvements of the microcosm method

The interference with deposition of spray (shade effect) caused by the 10 cm high top end projecting above the soil surface was a serious shortcoming of the "open" type microcosms used in 1992. A microcosm type with only 2 - 3 cm of the upper end projecting above the soil would probably solve the problem. If still provided with a "collar-trap" the danger of exchange with the field population would be minimized or could at least be checked. Another possibility is that the above-ground part of the microcosm including the "collar-trap" is removable during spraying. A completely tight fitting af the above-ground and below-ground part of the microcosm might be secured by smearing the joint with silicone-fat.

Checking and improving the spraying method

The variability of spray deposition observed suggests that the method used is not ideal for spraying the small areas used as treatment units in the present study. It is not clear, however, whether the variability was caused by differences in amount of spray applied to each square, i.e. due to lack of accuracy in adjusting the pressure and pace, or whether the distribution of spray within squares was uneven, e.g. caused by inaccurate spray nozzles or air movements during spraying. A test of these questions using the filter paper method would be advisable. The amount of water evaporated during spraying and storage until weighing should be measured.

Comparison between high and low "open" type microcosms

In order to obtain more information about the pesticide effect on test populations in different depths of the soil a comparison between pesticide effects in "open" type microcosms with soil cores of different depths, e.g. 20, 10, and 5 cm, is recommended. The low "open" type microcosm with 10 cm deep soil proved satisfactory as regards survival and reproduction of the test collembolan *Folsomia fimetaria* but because of limited capacity of extraction the test of that type of microcosm was not satisfactory. In a further test the period from treatment to recovery from the field should be shorter, for instance 6 weeks, and storage of the microcosms before extraction should be avoided.

Tests with other species

The collembolan *Isotoma notabilis* proved to be a promising test species candidate. The present work has provided some evidence for a higher sensitivity of this species than *Folsomia fimetaria*. Further tests should aim

at exploring this question and the culture technique should be improved to fit the requirements of that species.

EC_{50}

The relationship between pesticide dosage and population size of the test species should be studied more carefully by employing a broader range of dosages. In this way the validity of the decreasing exponential model assumed for the calculation of EC_{50} values in the present work could be tested.

7.5 Recommendations for a toxicity test based on field microcosms

Proposal for test procedure

Based on the results of the study described in the present section of the report the following test procedure can be proposed:

a) Microcosms of the high or low "open" type provided with "collar-traps" (section 7.2.2.:f,g) but modified according to the proposals in section 7.4.9. are filled to 2/3 of the planned soil volume with "defaunated" soil produced following the procedure described in section 7.2.3. The surface of the soil is scratched up in the centre of the surface in order to facilitate the entrance of test animals into the soil pore system.

b) 30 young adult specimens (age: 16 - 23 days at room temperature) of *Folsomia fimetaria* sampled from a culture of even-aged specimens are added at the centre of the soil surface in two increments (section 7.2.6.).

c) After 24 hours the microcosms are cautiously replenished with defaunated soil and stored in a refrigerated room at 5 - 10°C for 1-7 days until placing in the field.

d) The microcosms are buried in the field in squares (treatment plots) arranged in a randomized block's design with 10 replications of each treatment. If a simpler experimental design is used attention should be paid to possible gradient effects, for instance moisture gradients, which could affect the environment within microcosms.

e) Pesticide treatment should be carried out using experimental equipment for spraying small plots. The variability of spray doses deposited within and between treatment plots should be minimized (cf. section 7.4.9.).

f) After placing the microcosms in the field the "collar-traps" are emptied and refilled weekly.

g) Collection of microcosms from the field depends on the pesticide but it can be recommended to collect microcosms 2 weeks after treatment for acute effects and 6 - 10 weeks after treatment for prolonged effects is done 6 - 7 weeks after treatment when the effect on the test population is expected to be most pronounced.

h) The soil cores of the microcosms are placed in high gradient types of microarthropod extractors without storage, i.e. not more than 2 days after collection. Thermoregulated heating of the extractor starts at 30°C and is gradually changed to a final temperature of 60°C after 6 days. The extraction ends after 8 - 10 days.

i) The extracted animals are prepared in glycerol in small petri-dishes (diameter: 5 cm) and counted using a grid. A separation in size-classes may be useful for demonstration of pesticide effects on the population dynamics.

7.6 **Summary and conclusions**

Microcosms

Among 7 different types or variants of field microcosms tested a cylindrical microcosm type closed in the bottom with 45 micron mesh polyester web and provided with a "collar-trap" surrounding the open upper end proved to be highly efficient for isolating the test populations of eu- and hemiedaphic species. Moisture content and volume weight of the "defaunated" soil inside microcosms to which the test specimens were added was a little higher than the surrounding soil while no difference in temperature was measured. A high version of the "open" type of microcosm was used as the standard method in 1992 while a low "closed" type covered with 2 cm soil was used as standard in 1991. The soil of the high "open" type microcosm was divided into 2 horizons during extraction of the microarthropods.

Defaunation

A relatively gentle defaunation method using alternately freezing, heating, drying and rewetting proved to eliminate the microarthropods efficiently. A filtered soil suspension was added in order to facilitate the recovery of the soil microflora.

Test species

The collembolan *Folsomia fimetaria* and the gamasid mite *Hypoaspis aculeifer* were chosen as principal test species, but in 1991 the collembolan *Isotoma notabilis* was also tested. The gamasids used in 1991 were not identified. The choices were based on experience on distribution and

behaviour in the soil, generation time, ease of culturing and success of population development in microcosms.

Establishment of test population

20 - 30 collembolan specimens of equal size/age were added to each microcosm in the laboratory. 5 specimens of gamasid mites were further added to a selection of the microcosms.

Experimental design

The microcosms were placed in the experimental field according to a randomized block's design with 10 replications. Treatments included control, dimethoate (recommended dosage for agricultural use), dimethoate (5 times recommended dosage), pirimicarb (5 times recommended dosage), and fenpropimorph (5 times recommended dosage).

Spraying

Spraying was done by means of an experimental boom sprayer. Measurement of deposition of pesticides showed significant differences between treatments and between squares within treatments. The deposition was lower inside than outside the projecting upper end of the "open" type microcosms.

No acute effect

No effect of pesticides was observed 3 or 14 days after treatment. Thus, it can be concluded that spraying the soil with the 3 pesticides tested had no acute toxic effect on the soil living test species populations.

Effect of dimethoate

The treatment with the recommended dosage of dimethoate caused lower numbers than in control microcosms. The differences were statistically significant 38-39 days after treatment for *F. fimetaria* and 39 days after treatment for *I. notabilis*. In microcosms sprayed with 5 times recommended dosage of dimethoate, significantly lower numbers of *F. fimetaria* were observed in both years compared to untreated control microcosms at the 3 sampling dates 38-39 days (about 5 weeks), 59 days (about 8 weeks), and 70 days (10 weeks) after treatment. In 1991 that treatment had also significant negative effect on *I. notabilis* 39, 59, and 70 days after treatment, and on gamasids (unidentified species) 70 days after treatment. Measured 70 days after treatment in 1992 no effect was observed on *H. aculeifer*.

Effect of pirimicarb

Documentation of effects of pirimicarb and fenpropimorph on the test species is less convincing than the effects of dimethoate. However, statistical tests for *F. fimetaria* from microcosms recovered from the field 38 days after spraying in 1992 and 70 days after spraying in 1991 suggest negative effect of pirimicarb.

Effect of fenpropimorph

A negative effect of fenpropimorph on *F. fimetaria* was suggested by statistical tests 39 days after treatment (1991) while a positive effect on *I. notabilis* was suggested 70 days after treatment (1991).

Delayed indirect effects

The short persistence time of dimethoate (half-life: 4.8 - 9.7 days at 20° C) makes it plausible that the effects recorded 5 - 10 weeks after treatment are delayed indirect effects due to reduced reproduction of the originally introduced test animals and the first generation hatched in the microcosms.

Vertical distribution

Irrespectively of the treatment, the majority of the *F. fimetaria* test population had moved down to the 10 - 20 cm horizon 2 weeks after treatment in 1992. At later sampling dates the general vertical distribution had changed so that most of the population was in the upper 10 cm horizon. This was true except for dimethoate treated microcosms recovered from the field 38 days after treatment. In these microcosms the number of specimens was about equal in the two horizons thus indicating a deeper distribution than in the other treatments. This could be the result of a repellent effect or higher rates of mortality or immobilization in the upper than in the lower soil horizon.

Pesticide effect mostly restricted to upper 10 cm horizon

Pesticide effects on the number of test specimens compared to the control population were mostly restricted to the upper 10 cm of the high "open" type microcosms but the population size in the deep horizon was significantly smaller in microcosms treated with the high dosage dimethoate than in control microcosms 59 days after treatment in 1991 and 38 days after treatment in 1992.

I. notabilis showed a more superficial distribution in the high "open" type microcosms than *F. fimetaria*. The gamasid mite *H. aculeifer* was found in about equal numbers in the two soil horizons within the microcosms.

Effect of interaction with other species

No significant changes of the pesticide effect on *F. fimetaria* were observed when another collembolan species *I. notabilis* was added. However, the EC_{50} values for dimethoate dosage effect on *F. fimetaria* were higher for microcosms with *I. notabilis* than without this additional species. Addition of gamasid mites appeared to decrease the population size of *F. fimetaria* in all treatments. Presence of gamasids did not alter the effect of pesticides on *F. fimetaria* in 1991, but the results from 1992 suggest that interaction with the gamasid *H. aculeifer* can reduce the effect of pesticides.

EC_{50} for dimethoate is lower than the recommended dosage

EC_{50} values for dimethoate dosage effect on *F. fimetaria* calculated on the assumption of a decreasing exponential relationship were lower than the recommended dosage for agricultural use in Denmark 39 days after treatment in 1991 and 59 and 70 days after treatment in 1992.

Comparison with field samples

A preliminary census of *F. fimetaria* and *I. notabilis* in the field soil next to the microcosms done simultaneously with the recovery of microcosms from the field 39 days after treatment in 1991 showed that the test popula-

tion inside the microcosms was much higher than the field population. No pesticide effects could be demonstrated from this field sample.

Proposal for a semi-field test-procedure

A semi-field test-procedure is proposed based on the high or low "open" type microcosm with "collar-trap" placed in the field for 2 weeks after treatment for acute effects and 6 - 10 weeks for prolonged effects.

8 Extrapolation from the laboratory to the field

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8.1 Introduction

In ecotoxicology the extrapolation of test results from the laboratory to large scale field situations is one of the key tasks. Especially, there is a need to understand and assess the uncertainty of the extrapolation procedures. However, there is currently no agreed basis on which to predict events in the field on the basis of laboratory tests. The present study focused on the validation of laboratory tests compared with semi-field tests, and on the assessment of "safe concentrations" in the field by use of a statistical extrapolation method.

The two different approaches to validation and extrapolation were applied to predict effects in the field by use of the same standardized soil types, test compounds, application rates and test species. To facilitate the final comparison, the laboratory and semi-field studies were conducted in parallel.

8.2 Comparison of laboratory and semi-field results

The results from the laboratory and from the field showed effects caused by dosages 5 times that recommended for agricultural practise by the insecticide pirimicarb and by the fungicide fenpropimorph. At the recommended field dosage of pirimicarb and of fenpropimorph the field experiments resulted in significant effects at single sampling dates. Treatment with the insecticide dimethoate at recommended dosage showed severe effects on the test species.

Effects of dimethoate

The results obtained from laboratory tests on the collembolan species *Folsomia candida* and *Folsomia fimetaria* are compared with the collembolan species *Folsomia fimetaria* and *Isotoma notabilis* from the corresponding semi-field microcosm experiments. The results are shown in Figure 8.1. The 95% confidence limits are indicated.

From Figure 8.1 it is seen that the EC_{50} effects level were comparable in the laboratory and field microcosms. Thus, the present study indicates that the environmental factors in the semi-field microcosms may be simulated in the laboratory. However, large variations were observed in the field and in several cases the semi-field studies showed lower EC_{50} values compared with the laboratory experiments. In addition, the adverse effects appeared after a longer time lag in the semi-field studies.

No clear statistical significant effects were observed in the surrounding field areas on the same species which were exposed to the same chemical treatment.

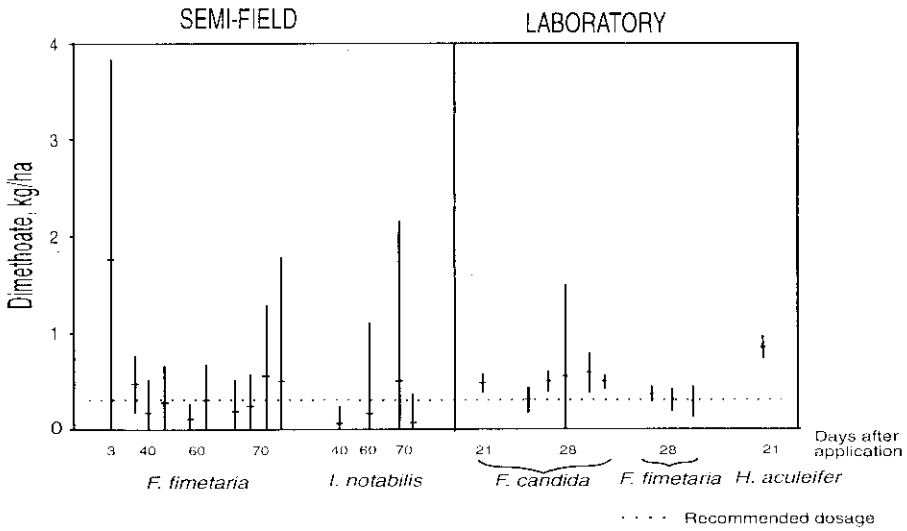

Figure 8.1

EC_{50} values for dimethoate from the semi-field and the laboratory tests with microarthropods. The vertical lines indicate the span of the 95% confidence intervals. For the laboratory results, the dashed line shows the normal dosage level assuming that 100% hits the soil surface and penetrates 5 cm into the soil with homogeneous concentration. The number of replicates are 4 for the laboratory and 10 for the semi-field experiments.

The microarthropod fauna in the field microcosms differed from that in the field situation by controlled and extremely simple species composition, higher and more uniform number of individuals per square unit, and prevention of migration. The collected field data will be studied more closely and compared with in an ongoing project which will be finished by 1994.

Effects of pirimicarb and fenpropimorph

In the laboratory, pirimicarb caused no significant effects at recommended dosage rates. The fungicide fenpropimorph was the more toxic of the three pesticides to protozoa, but significant effects were only observed at dosage rates much higher than the recommended. These findings were consistent with the results from the field microcosms which showed effects by treatments with pirimicarb and fenpropimorph at five times the recommended

dosage rate. In general, almost the same degree of toxicity was found in the field and in the laboratory for the three test compounds: dimethoate>-pirimicarb>fenpropimorph.

Conclusions of the comparison of laboratory and semi-field testing

The results obtained in the laboratory with collembolan species and a predatory mite simulated or reflected actual effects in the semi-field tests. Apparently, laboratory testing with these species is less sensitive compared with the semi-field test procedure. Therefore, by use of a safety factor, laboratory tests may be feasible for extrapolation from the laboratory to the field and thereby for hazard and risk assessment of the impact of pesticides on these species in the semi-field situation. However, the gap between the semi-field scale and the larger field scale situation needs further studies to make an appropriate extrapolation possible.

8.3 Assessment of "safe concentrations" by statistical extrapolation using dimethoate as model compound

Extrapolation was performed with the procedure of Wagner & Løkke (1991) on dimethoate which showed a sufficient dataset for the calculations. The results from the different laboratory test methods on species of ciliates, amoebae, flagellates, enchytraeids, Collembola and mites were normalized to the same standard soil for statistical extrapolation using the method of Løkke (1992) and Løkke et al. (1994). The extrapolation method based on the log-normal distribution was applied on EC_{10} values calculated on an area based unit ($kg \cdot ha^{-1}$). This method offers the advantage that results from a whole battery of test methods can be utilized and expressed as a single number, e.g. as "safe concentrations" for 95% of all species in an ecosystem assessed with a certain probability, e.g. 95%.

All results were normalized to a standard soil with 1.7% organic carbon (OC), a water fraction of w = 0.15 (dry weight basis) and a density d = 1.44 kg dm^{-3}. The soil adsorption coefficient $K_d = 0.46\ dm^3 \cdot kg^{-1}$ was used. Under the assumption that the bioavailable amount of pesticide is present in the soil pore water fraction, results from toxicity test performed in water solution were transformed to soil concentrations (c_t $mg \cdot kg^{-1}$, dry weight basis) by calculation from water concentrations (c_w $mg \cdot l^{-1}$) by the expression (cf Section 2.3):

$$c_t = c_w \cdot (K_d + w)$$

The effective concentrations $EC_{x,y}$ were inserted in the equation

$$EC_{x,soil} = EC_{x,water} \cdot (K_d + w)$$

The normalized data set for dimethoate is shown in Table 8.1.

The dataset was tested for log-normality by use of SAS UNIVARIATE (SAS Institute, 1988B). The procedure produces a test statistic for the null hypothesis that the input data values are a random sample from a log-normal distribution. The Shapiro-Wilk statistic, W, was computed. W is greater than zero and less than or equal to one, with small values of W leading to rejection of the null hypothesis. The present dataset resulted in W = 0.84. The associated probability, PROB<W, testing the hypothesis that the data come from a log-normal distribution was estimated to be 0.10. Values of PROB<W smaller than 0.05 leads to rejection of the null hypothesis. The data are shown on Fig. 8.2 as normal scores versus $\log_{10}$ (EC_{10}).

Table 8.1

EC_{10} values for the sublethal effect of dimethoate on seven species. Data are normalized to a standard soil.

Species	EC_{10} (mg · kg^{-1})
Cercomonas sp.	50
Acanthamoebae sp.	130
Colpoda sp.	70
E. bigeminus	37
F. fimetaria	0.1
F. candida	0.3
H. aculeifer	0.7

As shown by Figure 8.2 and by the W value the dataset hardly fulfilled the requirement of log-normality. However, to demonstrate the procedure the calculations were accomplished. In this case a relatively low "safe concentration" is anticipated due to the large standard deviation.

Statistical extrapolation of the measured single species data

By the Wagner and Løkke extrapolation method (Wagner & Løkke, 1991) the lower statistic tolerance limit K_p was determined so that with the probability 95% no more than 5% of all species in the soil community (p = 0.05) have an EC_{10} value smaller than K_p. The geometric mean of the measured EC_{10} values, $\exp(\bar{x}_m)$, and the sample standard deviation of *m* $\log(EC_{10})$ values were calculated. K_p is defined by

$$K_p = \exp(\bar{x}_m - s_m \cdot k) = \exp(\bar{x}_m)/T$$

where $T = \exp(s_m \cdot k)$ can be regarded as a safety factor with which the geometric mean of the measured EC_{10} values ($\exp(\bar{x}_m)$ should be divided. The safety factor T is dependent on the sample standard deviation of *m* $\log(EC_{10})$ values, and of the model constant k which depends upon the number *m* of $\log(EC_{10})$ values, upon the chosen degreee of confidence, and upon the chosen percentage of species to be protected. The values of k are tabulated in Wagner & Løkke (1991). For *m* = 7, 95%-confidence, and protection of 95% of the species in the soil community, k = 3.401.

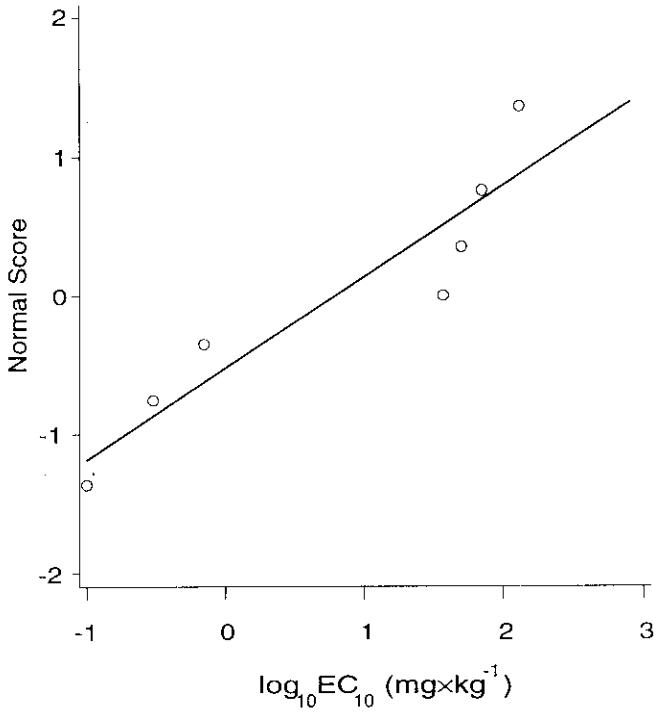

Figure 8.2

Log_{10}-normality plot and regression lines of data from the present study (Løkke et al., 1994).

Scenarios with varying soil penetration depth and deposition rates on the soil.

The data were transformed to the area based unit g · ha^{-1}. K_p was calculated for different scenarios: The amount of dimethoate which hits the soil surface (D%) was varied from 10 to 100% of the recommend field dosage at 280 g · ha^{-1}. The penetration depth into the soil (L) was varied from 1 to 10 cm under the assumption that dimethoate was homogeneously mixed into the soil, and that the exposure of the animals was at maximum. The field value of K_p is called $K_{p,field}$ and was estimated by multiplying K_p with the soil density d = 1.44 [kg·dm^3], the soil penetration depth L [dm], and the deposition fraction of dimethoate on the soil surface, and with 10^{-3} [g · mg^{-1}] and 10^6 [dm^3 · ha^{-1}].

Results

The results are shown in Table 8.2 for soil depth at 1, 2,5, 5, and 10 cm, and for deposition of 100, 50, 25, and 10% of the field dosage of dimethoate, respectively.

It is a feature of logaritmic extrapolation models that the standard deviation s_m is independent upon transformation of the dataset by multiplication of the EC_{10}s or the K_p value with a constant such as the soil depth L, or the deposition fraction of dimethoate on the soil surface. As seen from the Table 8.2, the K_p value (protection of 95% of the species (p = 0.05) with 95% confidence) for the sublethal effects of dimethoate on soil dwelling organisms is proportional with the soil depth L and the inverse fraktion of deposition by the following simple equation:

$$K_{p=0.05,95\%,dimethoate} = 3.46 \cdot L/deposition(\%)$$

In Table 8.2 the K_p values are also given as the percentage of the recommended field dosage of dimethoate at 280 g · ha^{-1}. Preliminary data from this study has been used for the development of an indicator for the impact of pesticides on the flora and fauna in the agricultural area. By combining EC_{10} values from the present study with NOEC values for other species of microfauna and arthropods, birds and mammals, a K_p value at 0.35 g · ha^{-1} was obtained. It was assumed that by the spraying, the pesticide was distributed with 85% on the plants, 10% on the soil with 0.5 dm penetration into the soil, and 5% was lost by spray drift. The corresponding K_p value from the present study is 1.73 g · ha^{-1}. Taking into consideration that this value only includes soil dwelling organisms and that the data were poorly fitted to the log-normal distribution, there is a reasonable proportion between the two values.

Table 8.2

Extrapolated "safe dosages" of dimethoate for 95% of the animal species in the soil community determined with 95% confidence. The values are calculated for different percentages of the applicated field surface hitting the soil surface, and for different penetration depth into the soil assuming homogeneous concentration of dimethoate and maximum exposure of the animals.

"Safe dosages" $K_{p,field}$ given in g · ha^{-1} or as % of the recommended dosage at 280 g · ha^{-1} (in brackets)		Percentages of field applicated dimethoate which hit the soil surface (D%)			
		100%	50%	25%	10%
Penetation of dimethoate into the soil (cm)	1	0.04 (0.01)	0.09 (0.03)	0.17 (0.06)	0.43 (0.15)
	2.5	0.09 (0.03)	0.17 (0.06)	0.35 (0.13)	0.86 (0.31)
	5	0.17 (0.06)	0.35 (0.13)	0.69 (0.25)	1.73 (0.61)
	10	0.35 (0.13)	0.69 (0.25)	1.38 (0.49)	3.5 (1.25)

8.4 **Conclusion**

Comparison of laboratory and field data

Toxicity outcomes of the laboratory and semi-field tests with microarthropods were highly correlated: The EC_{50} values were of the same order of magnitude. The laboratory and semi-field results await final validation

from a true field study which is conducted in a continuation of the semi-field test project.

Statistical extrapolation

The dataset obtained in the present study was poorly fitted to the log-normal distribution. However, to demonstrate the procedure the calculations were accomplished. The statistical extrapolation of single species laboratory data to the field was done by varying the leaching depth of dimethoate from 1 to 10 cm. Further, the fraction of dimethoate which was deposited on the soil surface was varied from 10 to 100%. If the deposition on the soil was 100%, and the leaching depth was 1 cm, the "safe dosage" for 95% of the soil dwelling species was estimated with 95% confidence to be 0.01% of the recommended field dosage at 280 g · ha^{-1}. By changing the deposition to 10% of the field dosage, and the leaching depth to 10 cm, the "safe dosage" was 1.25% of the recommended field dosage. At 10% deposition, and 5 cm leaching, the "safe dosage" was estimated to be 0.61% of the recommended field dosage. The values should be regarded as premature due to the lack of proper log-normality of the data. By analysing a larger dataset, Løkke et al. (1994) estimated a "safe dosage" of dimethoate at 0.35 g · ha^{-1} corresponding to 0.125% of the recommended dosage assuming for soil-dwelling species that 10% of the spray hitted the soil, and that the leaching depth was 5 cm. This value is lower than the corresponding estimate from the present study. However, the larger study included species living on the soil and in the vegetation where they are more exposed to pesticides than the soil dwelling organisms.

9 Conclusions

The conclusions of each subproject are summarised at the end of each chapter. All the laboratory tests presented have successfully met the demand for both realism and feasibility; the tests are easy to perform and last less than a month.

All the tests need further improvements to simulate realistic bioavailability and exposure routes, taking into account the natural habitat of each particular species.

Digital image processing has proved to be a promising tool in the characterization of microarthropods. It is being further developed in an EEC project to optimize the counting of unaggregated objects.

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Toxicity outcomes of the laboratory and semi-field tests with microarthropods were highly correlated: The EC_{50} values were of the same order of magnitude. The laboratory and semi-field results await final validation from a true field study which is conducted in a continuation of the semi-field test project.

Statistical extrapolation

The dataset obtained in the present study was poorly fitted to the log-normal distribution. However, to demonstrate the procedure the calculations were accomplished. The statistical extrapolation of single species laboratory data to the field was done by varying the leaching depth of dimethoate from 1 to 10 cm. Further, the fraction of dimethoate which was deposited on the soil surface was varied from 10 to 100%. If the deposition on the soil was 100%, and the leaching depth was 1 cm, the "safe dosage" for 95% of the soil dwelling species was estimated with 95% confidence to be 0.01% of the recommended field dosage at 280 g · ha^{-1}. By changing the deposition to 10% of the field dosage, and the leaching depth to 10 cm, the "safe dosage" was 1.25% of the recommended field dosage. At 10% deposition, and 5 cm leaching, the "safe dosage" was estimated to be 0.61% of the recommended field dosage. The values should be regarded as premature due to the lack of proper log-normality of the data. By analysing a larger dataset, Løkke et al. (1994) estimated a "safe dosage" of dimethoate at 0.35 g · ha^{-1} corresponding to 0.125% of the recommended dosage assuming for soil-dwelling species that 10% of the spray hitted the soil, and that the leaching depth was 5 cm. This value is lower than the corresponding estimate from the present study. However, the larger study included species living on the soil and in the vegetation where they are more exposed to pesticides than the soil dwelling organisms.

It may be suggested to supplement the presented single species tests with laboratory multi-species tests in a natural medium, thereby obtaining more realistic estimations of toxicity. Such systems take into account the species interactions neglected in the single species tests. However, the quality, manpower and realism in such a test should be compared to true field tests.

The procedures should be incorporated in decision procedures for approval of pesticides.

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Annex I

Approaches to the use of soil protozoa as test organisms for evaluating the side effects of pesticides

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Abstract

Three pesticides Dimethoat, Pirimor and Corbel had a negative impact on the colonisation of a sterilised soil by a natural population of soil protozoa. Fenpropimorph had an effect at a concentration of 25 mg/l corresponding to 1% of the concentration normally used for spraying on agricultural fields. Dimethoat and fenpropimorph reduced the soil respiration for about 10 days after application. The pesticides were also tested on single species cultures of soil protozoa in liquid media. The flagellate *Cercomonas sp.* turned out to be a better test organism than the ciliate, *Colpoda sp.*, and the amoeba *Acanthamoeba sp.*, with respect to interpretation of the dose response curve and ease of observation.

1 Introduction

Soil protozoa are in a key position in the soil ecosystem as important consumers of bacteria and to a smaller extent of fungi, and as food for larger organisms e.g. nematodes (Hunt et al. 1987). A limited number of protozoa can be grown axenically, and many toxicity experiments have been carried out under these conditions. Test organisms commonly used for toxicity research in axenic culture are the ciliate *Tetrahymena pyriformis* and the amoeba *Acanthamoeba castellanii* (Dive et al. 1984, Lord & Wright 1984, Otsuka et al. 1988, Prescott et al. 1977).

Several pesticide experiments have been carried out on ciliates grown on a particular strain of bacteria as food source e.g. *Colpidium campylum* (Dive

et al. 1980), Stylonychia notophora (Lal & Saxena 1980) and *Paramecium primaurelia* (Komala 1984). A few experiments have been carried out on amoebae grown on bacteria. Pons & Pussard (1980) tested 6 herbicides on 23 strains of amoebae. Dive et al. (1984) tested the toxicity of the fungicide thiram on 33 different strains of amoebae, which encompassed at least 29 species. Small naked amoebae and heterotrophic flagellates are the absolutely dominant groups of soil protozoa in agricultural soil (Clarholm 1983, Christensen et al. 1992). Among the organisms mentioned above Acanthamoeba is the only one commonly encountered in soil.

Two parameters often used for evaluation of drug effects on pure cultures are minimal inhibitory concentration and lethal concentration (Steinberg et al. 1990). Other parameters have also been used e.g. mortality (Persoone & Dive 1978) and cell size (Persoone & Dive 1978, Lal & Saxena 1982). The study of the effects of different toxins on the colonisation of an artificial substrate by protozoa in freshwater (Cairns Jr. et al. 1986) is one of the few studies of pesticide effects on protozoan communities.

Very few experiments have been carried out on the effect of pesticides on soil protozoa in situ. MacRae & Vinckx (1973) investigated the effect of DDT and Lindane on the number of protozoa in air dried garden soil after rewetting, and found significant negative effects some of which lasted for several months. Steinberg et al. (1990) found that addition of PCB had a significant effect on the grazing on an added bacterial strain (*Azospirillum lipoferum*) by indigenous soil amoebae to fresh non sterilised soil. Later liquid culture experiments showed that PCB made the amoebae lyse while their cysts were unaffected.

The purpose of this investigation is to study the effect of commonly used pesticides on soil protozoa by comparing protozoan activity during recolonisation of sterilised soil with liquid cultures of single protozoa as influenced by the pesticides.

2 **Materials and methods**

Three commercial formulations of pesticides were tested: the fungicide Corbel, Schering (active component: fenpropimorph) and the two insecticides Dimethoat 28, DLG (active component: dimethoate) and Pirimor, c, (active component: pirimicarb). Commercially available products were used. All concentrations of pesticides are expressed as mg active compound/l. Commonly used concentrations (active compound) of the three pesticides are: Dimethoate 930 mg/l, Pirimor 500 mg/l, and Corbel 2500 mg/l.

2.1 Microcosm experiments

2.1.1 Soil

The soil was a coarse sandy soil from the Danish National Agricultural Research station at Jyndevad (Table 1).

Table 1

Soil characteristics

clay[a]	(%)	3.9
silt[a]	(%)	4.1
fine sand[a]	(%)	12.2
coarse sand[a]	(%)	76.8
humus[a]	(%)	3.0
soil water capacity#	(%)	34.0
pH	(%)	6.5

[a]: Data from Hansen (1976).

#:Container Capacity determined according to Cassel & Nielsen (1986).

The soil was air dried, and sieved twice through a 1 mm mesh. The number of protozoa in the air dried soil was determined by the most probable number (MPN) method (Darbyshire et al. 1974), using 1/100 Tryptic Soy Broth (Difco) as nutrient media. Microcosms were prepared by adding 5,0 g of soil to 116 ml serum bottles followed by dry sterilisation (18 h 180°C). Two colonisation experiments were carried out in the microcosms. In the first experiment all three pesticides were tested, whereas one pesticide at four different concentrations was tested in experiment 2.

2.1.2 Effects of Dimethoat 28, Pirimor, and Corbel

Protozoan inocula and pesticides were added to the sterilised microcosms as slurries, prepared from air dried, but unsterilised soil. Each microcosm contained 34% water (container capacity), and was inoculated with an amount of unsterilised soil corresponding to 10% of the sterilised soil. The final concentrations of Dimethoat, Pirimor and Corbel were 930, 500 and 250 mg a.i.l^{-1}, corresponding to 316, 170, and 85 mg/kg soil, respectively. The bottles were sealed with airtight rubber-stoppers and incubated at 15°C in darkness. The number of protozoa was determined by the MPN method (Darbyshire et al. 1974) three times during a three week incubation period by destructively sampling three replicate microcosms. During the incubation the carbon dioxide content in three microcosms of each treatment (Dimethoat 28, Pirimor, Corbel and control) was determined five times by analysing 1 ml headspace air from the microcosms on a gas chromatograph with thermal conductivity detector. Carbon dioxide was measured on the microcosm systems that were subsequently used for enumeration of protozoa.

2.1.3 **Effect of Corbel in different concentrations**

This experiment was carried out in the same way as above with a few exceptions. The temperature was lowered to 10 °C, and the humidity of the soil was only at 25% water in order to get a slower breakdown of the pesticide and a less fluctuating protozoan community. Corbel was tested in concentrations of 25, 74, 250 and 740 mg/l corresponding to 6.3, 18.5, 63, and 185 mg/kg soil, respectively. Enumeration of protozoa by destructively sampling microcosms took place six times during a seven week incubation. Carbon dioxide was measured on nine occasions during the incubation.

2.2 **Liquid culture experiments**

Liquid culture experiments were carried out on three common indigenous soil organisms isolated a few weeks before the present experiment, representing the three major groups of protozoa inhabiting agricultural soils: a flagellate of the genus *Cercomonas*, an amoebae of the genus *Acanthamoeba*, and a ciliate of the genus Colpoda. *Acanthamoeba sp.* and *Colpoda sp.* were isolated from the same soil that was used in the microcosm experiments while *Cercomonas sp.* was isolated from soil from the Danish National Agricultural Research Centre at Askov. By choosing few weeks old isolates laboratory degeneration should be avoided.

Liquid culture experiments were carried out in micro titer plates (Costar, No 3598, 8 x 12 wells). One plate was prepared for each of the three protozoa for each pesticide i.e. 9 micro titer plates were set up. The eight wells in row 1 on the titer plates were added 200 µl 0.3 gl^{-1} Tryptic Soy Broth containing pesticide in a concentration twice the maximum concentration required. The 8 wells in each of the next 11 rows were then added 50 µl 1/100 TSB and successive dilutions were carried out from row 1 to row 11 transferring 150 µl in each step. Finally 50 µl protozoan culture was added to each well. The result of this procedure is that each well contains 100µl liquid with protozoa. The wells in row n+1 will contain pesticide in a concentration 3/4 times that in row n, except row 12 where no pesticide was added.

The protozoa were counted four days after inoculation. For each dilution quantified, at least four wells were counted at 200 times magnification in 12 light fields randomly selected in each well. The results were expressed as number of protozoa for each concentration divided by the number of protozoa in the unamended treatments.

3 Results

3.1 Microcosm experiments

3.1.1 The effect of the three pesticides in soil

The number of protozoa in unamended soil was strongly increasing the first 20 days following inoculation of sterile soil, after which the populations declined (Figure 1). The number of protozoa was significantly reduced on day six in the Dimethoat amended soil (Figure 1a) and for the whole period in the Corbel amended soil (Figure 1c); Pirimor had no significant effect on the protozoan numbers (Figure 1b).

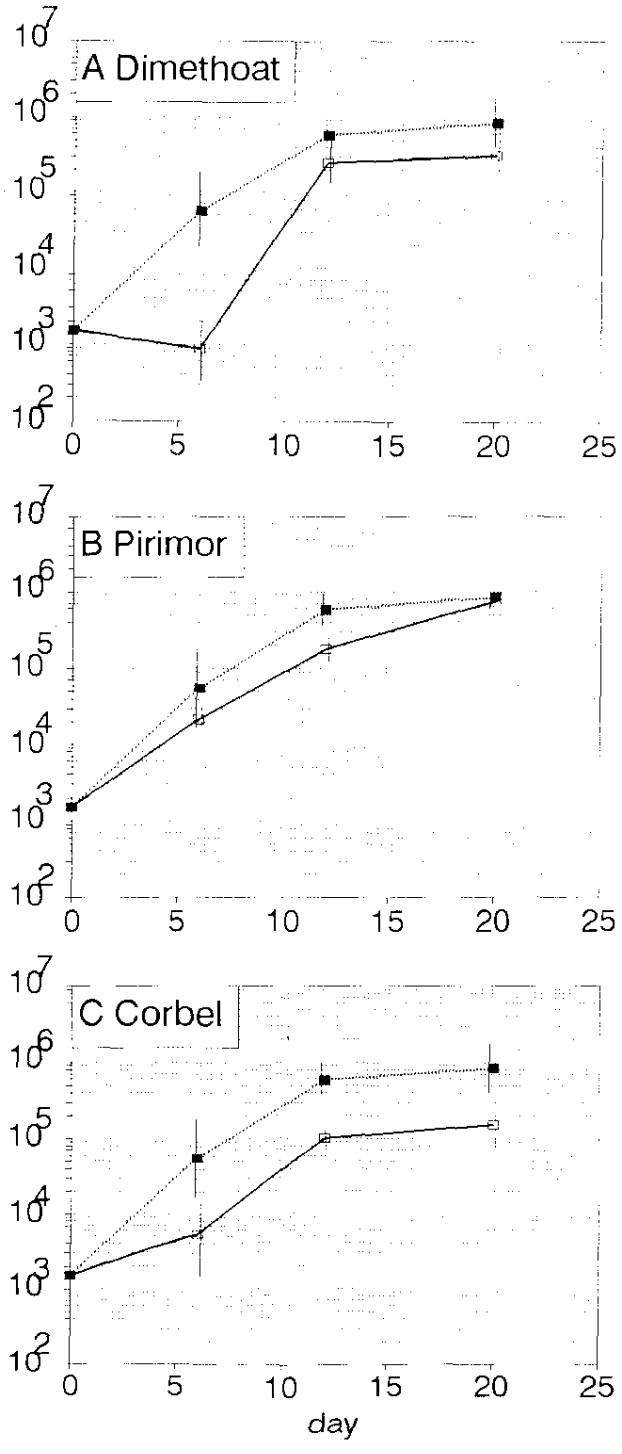


Figure 1
Logarithmic plot showing the number of protozoa in the pesticide amended treatments compared with the unamended in experiment 1. The added pesticides are: Dimethoat 28 100%; Pirimor 100%; Corbel 10%. Open squares represent pesticide treated soil, filled squares represent control soil. The standard deviations are shown as vertical lines. Standard deviation were calculated on logarithmically transformed data and were then re-transformed (N = 3).

Dimethoat 28 and Corbel had a negative effect on soil respiration in the beginning of the experiment compared to the control (Figure 2a and c). After day 12-15 Dimethoat 28 and Corbel stimulated soil respiration. Pirimor had no significant effect on the respiration (Figure 2b). The pesticide effect on the soil respiration can be quantified as the cumulated numeric difference between the three graphs for pesticide amended soils and the control soil in Figure 3, divided by the amount of carbon dioxide evolved in the control soil (Table 2). The cumulated difference in respiration caused by the pesticides was largest in the case of Dimethoat 28, followed by Pirimor and Corbel.

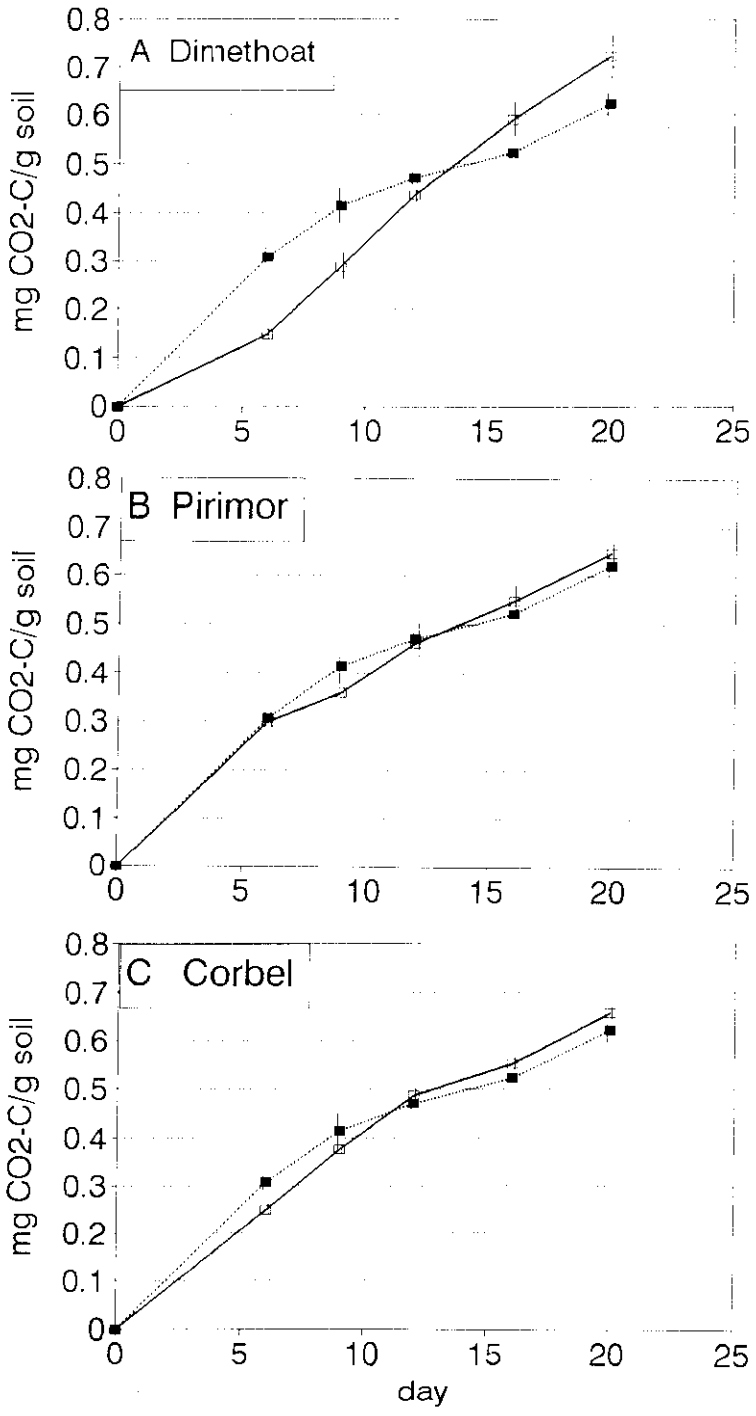


Figure 2
The cumulated carbon dioxide evolution in pesticide amended soil compared to unamended (experiment 1). Each point represents the arithmetical of measurements of carbon dioxide in three different serum bottles. The vertical bars represents the standard deviation (N = 3). Open squares represent pesticide treated soil, filled squares represent control soil.

Figure 3

Logarithmic plot showing the number of protozoa in the soil amended with four concentrations of Corbel compared with the unamended soil. The standard deviations are shown as vertical lines. Standard deviations were calculated on logarithmically transformed data and were then re-transformed (N = 3). Filled squares represent control soil, all other symbols represent Corbel added soil.

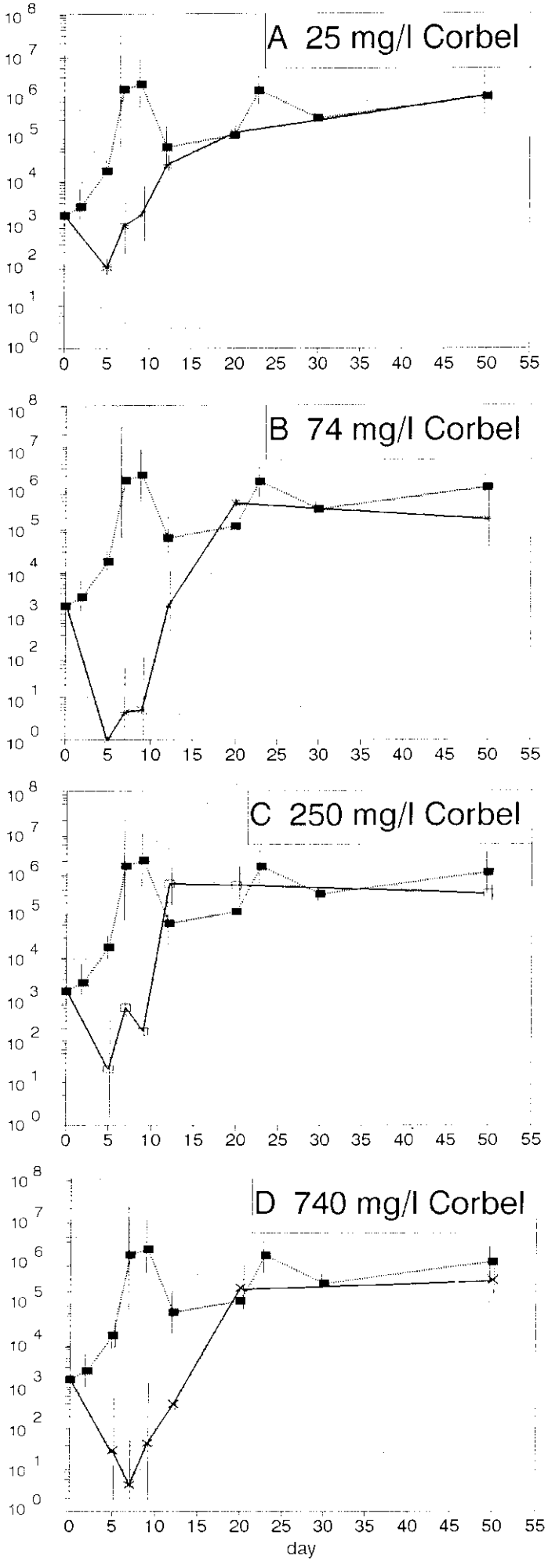


Table 2

Column one shows the ratio between the respiration rate in pesticide amended soil and unamended soil from day 0 to day 6 (relative rate). Second column shows the area between the curves for respiration rates in amended soil and the corresponding curve for unamended soil (Figures 7 a-c) divided by the cumulated carbon dioxide evolution in the control soil. This area measures how different the two curves are.

	Relative area
Dimethoat 28, 930 mg/l	0.48
Pirimor, 500 mg/l	0.23
Corbel, 250 mg/l	0.19

3.1.2. **The effect of four concentrations of Corbel (experiment 2)**

The number of protozoa varied greatly in the unamended soil during the experiment (Figure 3). Corbel had a significantly negative effect on the number of protozoa, for the first 9-12 days, even at a concentration of 25 mg/l. There was no significant difference between the number of protozoa at the four different concentrations of Corbel (Figure 3).

The initial burst of respiration activity lasting three weeks in the unamended soil was increasingly affected by increasing amounts of Corbel (Figure 4). All concentrations of Corbel decreased the respi-ration rate during the first week. Respiration rate in the Corbel amended soil approached the rate in unamended soil at week seven. A calculation of the relative area shows that 25 mg/l of Corbel had a smaller effect than the three higher concentrations (Table 3). The ratio between the respiration rate in the amended compared to the unamended soil in the beginning of the experiment is virtually the same for all four concentrations (Figure 4).

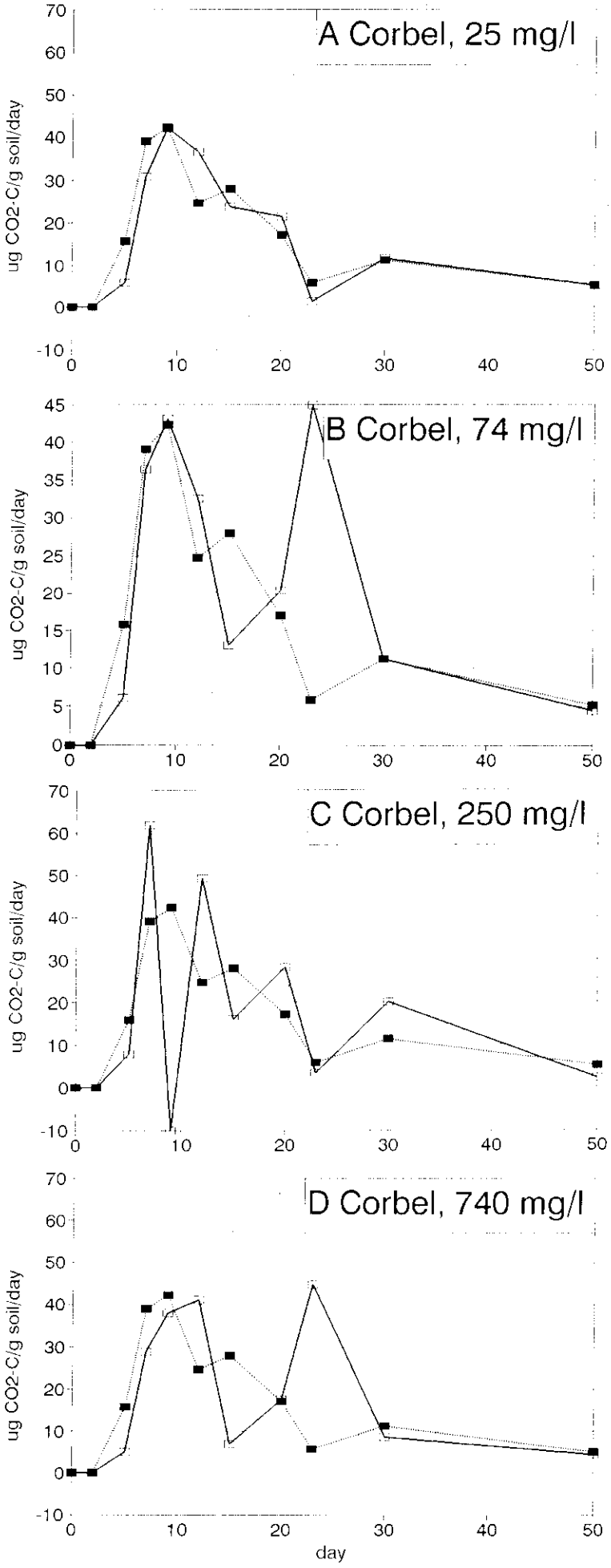
Table 3

Column one shows the ratio between respiration rate in pesticide amended soil and unamended from day 0 to day 5 (relative rate). Second column shows the area between the curves for respiration rates in amended soil and the corresponding curve for unamended soil (Figures 11a-d) divided by the cumulated carbon dioxide evolution in the control soil. This area measures how different the two curves are.

Corbel conc.	Relative area (day 0-50)
25 mg/l	0.19
74 mg/l	0.47
250 mg/l	0.74
740 mg/l	0.60

Figure 4

The respiration rate in soil treated with different concentrations of Corbel. Standard deviation bars (N = 3) are below the size of the symbols.



3.2 **Liquid culture experiments**

The number of protozoa in liquid culture generally decreased with increasing amounts of pesticides (Figure 5-7). The horizontal dotted line on the figures shows the number of protozoa at day zero expressed as fraction of the number after four days in unamended cultures. The intersection between this line and the curves therefore indicates the concentration of pesticide at which there has been no growth of protozoa during the four day incubation. At pesticide concentrations to the left of the intersection net growth of protozoa occurred, either at a rate lower than in unamended cultures (inhibition, ordinate < 1) or in some cases at a higher rate (stimulation, ordinate > 1). At pesticide concentrations to the right of the intersection, the protozoan inoculum was partly or totally killed.

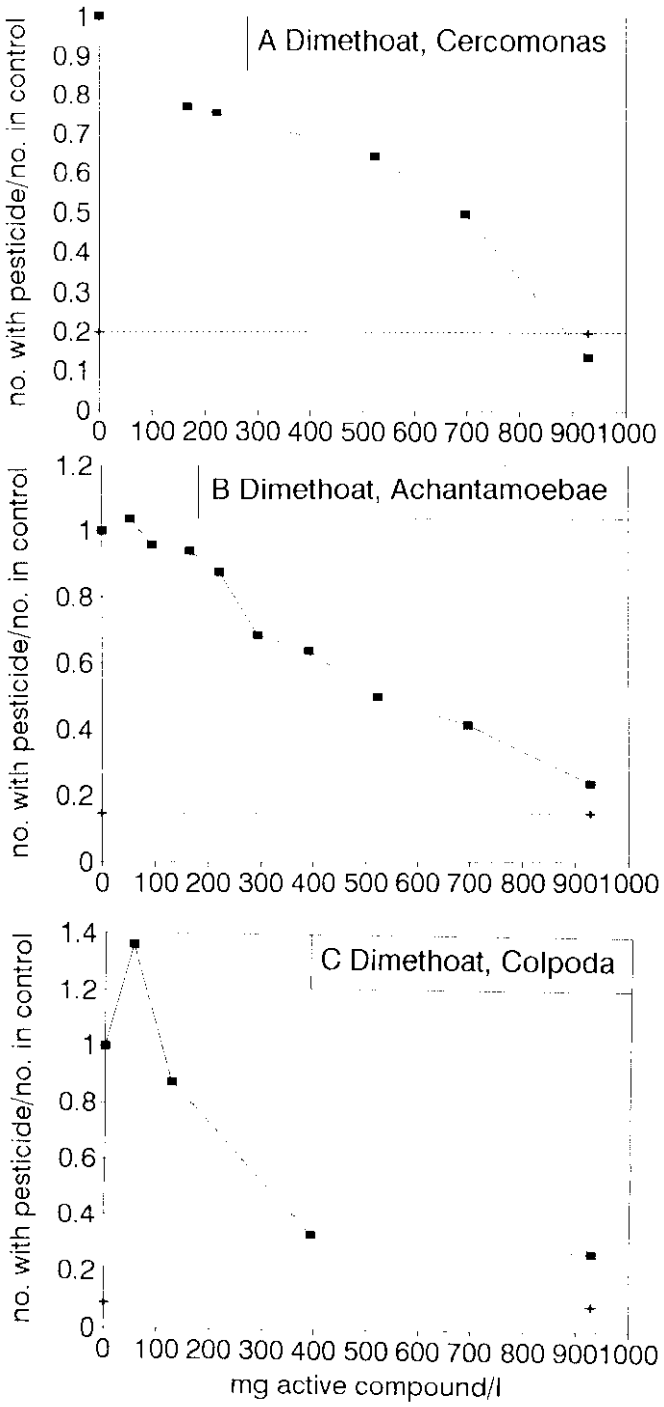
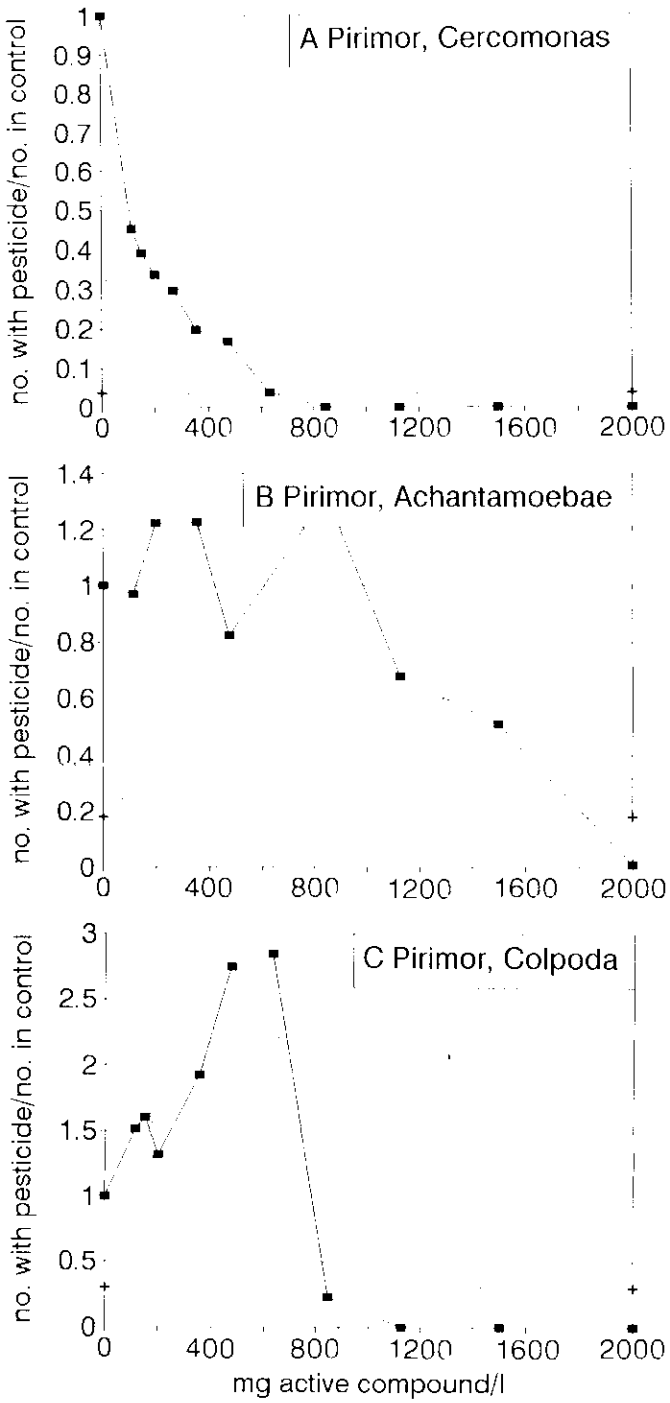


Figure 5
Dose response Curves for three protozoa in liquid culture added different concentrations of Dimethoat (N = 4).

Figure 6
Dose response Curves for three protozoa in liquid culture added different concentrations of Pirimor (N = 4).



There are marked differences in the effect of the pesticides on the protozoa. Cercomonas was the most sensitive organism to Corbel and Pirimor (Figures 6a and 7a); while Colpoda was the most sensitive to Dimethoat (Figure 5c). In all cases Acanthamoeba was less sensitive than the two other organisms (Figures 5b, 6b, and 7b). Apparently the growth of Colpoda is stimulated by low concentrations of Corbel and Pirimor (Figure 6c and 7c).

Corbel had the most pronounced effect on the growth of the protozoa, even at a concentration of 2 mg/l (Figure 7) as opposed to Dimethoat and

Pirimor that apparently only affected protozoan growth markedly at concentrations above 100 mg/l (Figure 5 and 6).

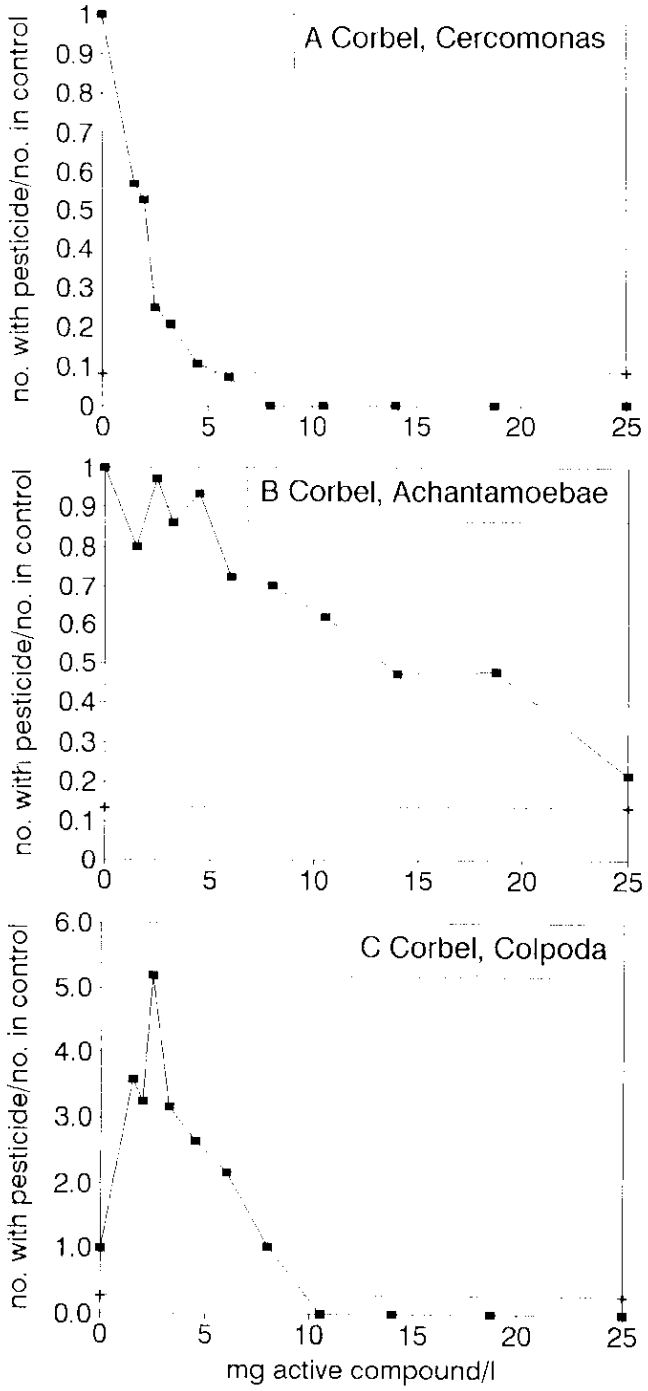


Figure 7
Dose response Curves for three protozoa in liquid culture added different concentrations of Corbel (N = 4).

4 **Discussion**

4.1 **Respiration during recolonisation of sterilised soil**

Large amounts of labile organic matter was liberated when the soil was sterilised by dry heat enabling vigorous growth of the inoculated microor-

ganisms. Probably, the respiration peak during the first week following inoculation covers successive patterns of microbial growth. The fluctuations in carbon dioxide evolution in the pesticide amended soil starting with a marked reduction in respiration in the beginning of the experiment, suggest that the balance between the different microbial populations has been altered. These effects are most clearly demonstrated in the experiments with different concentrations of Corbel. The pesticide treatments always resulted in an increased respiration later during the incubation probably because micro-organisms killed by the pesticides were broken down by the surviving organisms. In the experiment with different concentrations of Corbel, the lower temperature allowed us to observe the increase in activity in the early phase in contrast to the experiment with different pesticides conducted at 15°C, where the respiration rate was decreasing already from the first measurement on day 6.

4.2 **Colonisation of sterilised soil**

The initial colonisation of sterilised soil was fast with growth rates corresponding to 0.5 and 1.0 day^{-1} during the first week. Populations of $1\text{-}2 \bullet 10^6$ were formed based upon the organic matter liberated during the dry heat sterilisation. The pesticide amendments had a pronounced negative effect on the colonisation of sterilised soil by a mixed population of soil protozoa especially during the first two weeks (Figure 1 and 3). 250 mg/l of Corbel had a greater effect in experiment 2 than in experiment 1, probably because the pesticide was broken down faster in experiment 1 at 15°C than at 10°C in experiment 2. These experiments were not conducted at sufficiently low pesticide concentrations to draw any conclusions about the sensitivity of the method with respect to Corbel, the lowest concentration tested in soil microcosms (25 mg/l) had a dramatic effect.

Cairns Jr. et al. (1986) argued that the colonisation process is very sensitive to low concentrations of xenobiotes: Side effects of such compounds may either be lethal, affect the reproduction, or the general behaviour of the organisms and any of these effects will influence the colonisation. Therefore pesticide effects on many different physiological processes are integrated in the colonisation behaviour; moreover the protozoa are active and therefore more sensitive to xenobiotes during growth than when in the encysted resting stage (Corliss & Esser, 1974).

The colonisation of an artificial substrate (polyurethane) by a natural population of protozoa in freshwater, was strongly affected also by sublethal doses of chemicals Cairns Jr. et al. (1986). It has been shown that the movement of soil protozoa from one aggregate to another is a very critical and slow process (Hattori, 1988), and we observed that very

low sublethal concentrations of pesticides sometimes had an effect on the movements of the organisms (data not shown). Therefore, we suggest that extremely small doses of pesticides may have an effect on the colonisation of soil aggregates by protozoa.

We suggest that the maximum sensitivity of soil assays to pesticide effects are obtained by the proportion between the number of protozoa in the amended soil compared to the unamended during early growth.

Much more detailed information could probably be obtained by comparing the species composition in amended soil compared to unamended. Unfortunately the diversity of soil protozoa has not been studied intensively which make such a comparison impossible at present.

4.2.1 **The relation between growth of protozoa and respiration**

If it is assumed: - that the total protozoan production during the experiment is reflected in the changes in protozoan numbers that we observe (Figure 1, Figure 3)- that the protozoa have a yield of about 0.5, - and that half of the protozoa are amoebae and half of them flagellates with an average C-content of 5 pg and 40 pg respectively (Stout & Heal 1967), then the total cumulated protozoan respiration in the unamended soil will be 0,02 mg per gram of soil in experiment 1 and 0,1 mg per gram of soil in experiment 2. These figures make up 3% and 14% of the total respiration in the two experiments, respectively. Therefore the pesticide effect on the soil respiration was only to a minor extent due to the direct effect on soil protozoa.

4.3 **Liquid culture experiments**

The use of micro titer plates makes it possible to study the effect of a wide spectrum of pesticide concentrations in many replica using very little equipment and space. Furthermore it is possible to use very small amounts of pesticide thereby minimising the health risks.

In general the shape of the dose response curve was the same for the three organisms regardless of the pesticide tested (Figures 5 - 7). In general *Acanthamoeba* was the least sensitive and *Cercomonas* the most sensitive. *Cercomonas* showed a simple relation between pesticide dose and response (Figures 5a, 6a, and 7a) whereas this correlation was less pronounced for *Acanthamoeba* (Figures 5b, 6b, and 7b). *Colpoda* tended to be stimulated by low concentrations of pesticide and this phenomenon was also shown by Schreiber & Brink (1989) and Lord & Wright (1984). For all three

pesticides tested, *Colpoda* cells had a much larger volume at concentrations just below lethal dose.

Of the three animals that we tested *Cercomonas* seemed to be the most suitable organism for pesticide tests. It is the most sensitive to two of the three pesticides tested, and it produces the dose response curves that is most easy to interpret. Furthermore it is easy to count, also when alive, because it moves rather slowly as opposed to *Colpoda* that swims rather fast. Furthermore the shape of *Cercomonas* cells is very characteristic, whereas *Acanthamoeba* can
sometimes be difficult to distinguish from particles of organic matter. Moreover, *Cercomonas* is easy to culture and will grow as soon as a suitable food source is offered.

Flagellates are much more important in agricultural soils than ciliates, which is another argument for using them as test organisms. *Amoebae* are also important in agricultural soils. Our strain of *Acanthamoeba* turned out to be a less suitable test organism due to its low sensitivity to pesticides. However, Dive et al. (1984), found that some strains of *Acanthamoeba* were among the most sensitive for the fungicide Thiram. Possibly other strains of Acanthamoeba than ours would be more suitable as test organisms.

It is not possible to judge from our work whether liquid culture experiments with single species or community experiments carried out in soil should be preferred. Single species based tests are much easier to interpret than community tests, but much more remote from the natural system they are supposed to reflect. At present the best way to go is probably to combine the two types of approach in order to get a broader understanding of the effect of pesticides on the soil protozoa and thereby on the soil ecosystem as a whole.

Annex II

Acta Zoologica Fennica (in press)

Effects of pesticides on the reproduction of *Hypoaspis aculeifer* (Gamasida: Laelapidae) in the laboratory

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Abstract

A single species reproductive test system with the predaceous mite *Hypoaspis aculeifer* Canestrini (Gamasida: Laelapidae) is presented. This mite breeds successfully on the prey organisms *Folsomia fimetaria* L. or *Folsomia candida* Willem (Collembola). Trials testing fenpropimorph and pirimicarb showed no negative effects on the reproductive output of the mite up to the maximum dosage of approx. 5× the recommended field dosage. Dimethoate showed a stimulatory effect at .4 mg/kg (LOEC) and a reduction at 3× the recommended field dosage (LOAEC), and an EC_{50} in the range of $.8<EC_{50}<1.0$ mg/kg.

The study shows that the following points may be of importance when performing toxicity testing with Gamasida 1) Stimulations of the reproductive output of the mite at low doses of pesticide below and around field rates 2) An uneven distribution of pesticide due to surface application compared to a homogeneous distribution in the test soil.

1 Introduction

In recommendations for the development of test systems in ecotoxicological risk assessment, it is often put forward that single species tests do not take into account the real species interactions. Some of the early explanations for field observations of soil microarthropod responses to pesticides indeed involved predator/prey interactions, and the potential role of predators in the soil ecosystem has been recognized since the childhood of soil ecology/ecotoxicology (e.g. Edwards et al. 1967, Sheals 1956, Usher

1985). Still, however, this scientific background knowledge has not yet led to internationally accepted test guidelines for this important group of soil invertebrates.

This paper presents the results of experiments with the sexually reproducing predaceous mite *Hypoaspis aculeifer* Canestrini (Gamasida: Laelapidae) under the influence of three pesticides. The prey has been eliminated as a factor by ensuring its surplus in the test-system ("satiation" of the predator).

2 **Materials and methods**

2.1 **Pesticides**

Dimethoate, O,O-dimethyl-S-(N-methylcarbomoyl-methyl)-phosphorodithionate, is an inhibitor of cholinesterase. It has a half life in soil of 5-9 days depending on soil texture (Kolbe et al. 1991). It is a broad spectrum insecticide with pronounced effects on the arthropod fauna at the recommended dose level (e.g. Frampton 1988, Goodwin 1984, Vickerman & Sunderland 1977, Powell et al. 1985). Dimethoate has been selected in our laboratory as a reference chemical in toxicity studies.

Pirimicarb, 2-dimethylamino-5,6-dimethylpyrimidin-4-yl dimethylcarbamate, is a selective aphicide, but direct mortality has been observed for *Phytoseiulus persimilis* (Acarina: Gamasida) at recommended field concentrations for both adults and nymphs (Goodwin 1984).

Fenpropimorph, (±)-*cis*-4-[3-(4-*tert*-butylphenyl)-2-methylpropyl]-2,6-]-2,6-dimethylmorpholine, a systemic fungicide, was termed harmless (mortality < 50%) to predaceous mites (Hassan et al. 1988).

Recommended dosage and calculated concentrations assuming even distribution in the upper 5 cm of soil is presented in Table 1.

Table 1

Field dosage levels compared with laboratory dosage level of the pesticides.

Active ingredient	Commercial name	Recommended dosage pr. ha (formulated product)	Active ingredient pr. ha	Calculated A.I. in soil (upper 5 cm)
Dimethoate	Dimethoate 28	1 *l*	280 g	0.389 mg/kg
Pirimicarb	Pirimor	0.3 kg	150 g	0.209 mg/kg
Fenpropimorph	Corbel	1 *l*	750 g	1.042 mg/kg

2.2 **Characterization of *H. aculeifer***

Eggs produced overnight (0-24 hours old) were transferred individually to isolated chambers of 2 cm^2 in a multidish with the bottom covered by gypsum/charcoal substrate. They were observed daily and any changes registered. Mite growth (length, width and area of dorsal shield) was measured using DIP (Digital Image Processing) and registered until cease of growth (ca. 30 days). Rate of oviposition was determined by counting and removing eggs daily. The immature stages were fed 0-7 days old juveniles (average length: .4 mm) of sexually reproducing *Folsomia fimetaria* L. (Collembola: Isotomida)). The number of *F. fimetaria* was kept continuously at the level of about 30 individuals by daily compensating for eaten collembolas. Consumption was estimated from counts of the remaining prey the following day. After sexual maturation (first day of oviposition) the mites were fed with 13-20 days old adult *F. fimetaria* (average length: .7 mm).

To study the relationship between available food and reproduction, 10 female and 5 male *H. aculeifer* in soil were allowed to reproduce in the presence of 0, 100, 800 and 2000 individuals of 9-12 days old parthenogenetically reproducing *Folsomia candida* Willem (Collembola: Isotomida) added weekly. The procedure was performed as described in Table 2 and paragraph 2.3 exept for the variation in prey numbers and no addition of pesticides.

2.3 **Laboratory cultures, basic test protocol and toxicity testing**

In a screening for suitable representatives of predaceous gamasid mites *H. aculeifer* was successfully reproducing under the conditions offered: plaster/charcoal substrate and *F. fimetaria* or *F. candida* as prey.

The tests were executed at 20 °C in a sandy loam soil presently used as a standard test soil in our ecotoxicological laboratory. It is a typically Danish agricultural sandy loam with the following composition: O.M. 1.7 %; clay 11%; silt 13 %; sand 48 %; coarse sand 27 %; pH_{H_2O} = 5.8.

In order to eliminate fauna, the soil was alternately dried (60 °C), frozen (-35 °C) and incubated (-35 °C) in wetted condition. The soil was then sieved through a 2 mm mesh. For the purpose of long time storage it was kept at -18 °C.

The soil was moistened and inoculated with a suspension of soil extract. The extract was produced from ½ kg fresh soil which had been stored at 5° C for no longer than 2 months, added ½ l demineralized water, incu-

bated for 24 hours for extraction of microbes and then sieved through a 40 µm mesh.

Experiences with the abovementioned type of defaunation have shown that it cannot completely eliminate the nematodes (Huhta *et al.* 1989). However, observations of starving *H. aculeifer* have shown that under the standard test conditions they neither grow nor reproduce in the inoculated soil. Consequently, none of the inoculated organisms acts as important food sources.

Along the lines followed in toxicity testing with *F. fimetaria* and *F. candida* currently in use at our laboratory, a procedure for *H. aculeifer* was formulated and proved successful. 10 female and 5 male adults, roughly the same proportion as found in our laboratory cultures, were added to microcosms containing 30 g of moist soil. The microcosms were incubated for three weeks and the resulting reproductive output was estimated as numbers of juveniles extracted from the soil with a high gradient extractor (MacFadyen type). The treatments consisted of 6 concentrations including a control and each treatment was replicated 4 times. A brief description of the rearing and toxicity testing of *H. aculeifer* is displayed in Table 2.

Table 2

Overview of the procedure for production of synchroneous cultures for testing of chemicals with H. aculeifer.

Day no.	Day of Week	Operation
1	F	Adult *H. aculeifer* are transferred to a new substrate on a Friday and allowed to oviposit over the weekend
4	M	Adults are removed and eggs left on the substrate
8	F	Hatching starts. A surplus of prey is added
11	M	Immatures are 0-3 days old. Unhatched eggs are removed/destroyed
27	W	The mature individuals being now 16-19 days old are used in tests Pesticides and inoculate are mixed with defaunated soil Transferring of 16-19 days old mites to test soil Addition of 700-900 prey ind. of 16-19 days old *F. fimetaria* Incubation in climate chamber at 20° C
48	W	Termination of test after 3 weeks. Extraction of microarthropods from soil in high gradient extractor at 25° C increasing to 40° C during two days.

A first step towards simulation of the spatially complex distribution of pesticides was taken by putting a 1 cm layer of 30 g contaminated soil on top of another 1 cm of 30 g uncontaminated soil. The test procedure was identical to the one with a single layer of soil (Table 2).

2.4 **Statistical analysis**

Calculation of EC_{50} was done by fitting a model to the data, which allows for a stimulatory effect at low concentrations. The model was adopted from Brain & Housens (1989) using a notation in agreement with Lacey & Mallett (1991):

$$y = \frac{c + s \cdot x}{1 + e^{\alpha} \cdot x^{\beta}}$$

y: reproductive output,
x: concentration of pesticide,
c: control reproduction,
s: rate of increase in reproduction,
α: 'slope' parameter,
β: 'shape' parameter.

At $x = EC_{50}$, e^{α} can be expressed as:

$$e^{\alpha} = \frac{c/2 + s \cdot EC_{50}}{EC_{50}^{\beta} \cdot c/2}$$

This reparameterization introduces EC_{50} (Ann Gould, Shell Research Ltd, pers. comm.) into the equation and allows for the calculation of 95% confidence intervals when parameter estimations is done with a nonlinear regression analysis procedure like the SAS procedure NLIN (SAS Institute Inc. 1988A).

The statistical tests for NOEC (No Observed Effect Concentration) and LOEC (Lowest Observed Effect Concentration) were made with a Williams Test by means of the program Toxstat (Gulley et al. 1988). In the case of stimulatory effects of low concentrations, results from those concentrations were omitted from the statistical analysis to be able to estimate the NOAEC (No Observed Adverse Effect Concentration).

3 Results

3.1 Characterization of H. aculeifer

The time for eggs to develop is 7 days, and the time for larvae to reach maturation, i.e. time to the first oviposition is about 18 days. The individual mites of the same sex did not differ in growth rate or size, but the males were smaller and grow more slowly than the females (Figure 1A). The same is true for the consumption (Figure 1B). Likewise, the oviposition rate is equal among females (Figure 1C).

The egg production was 3.1 day^{-1} $ind.^{-1}$ of individually isolated mature females (slope of curves in Figure 1C where age is >20 days).

The consumption was 8.8 *F. fimetaria* day^{-1} for mature females and in the order of 1 *F. fimetaria* day^{-1} for mature males (slope of curve in Figure 1C where age is >20 days).

In microcosm replicates where 100 collemboles were added weekly the remaining prey population was dominated by large individuals, possibly because smaller individuals were more easily captured and therefore completely consumed. The point of "satiation" for the mite population is reached at about 1000 prey individuals added to the microcosm (Figure 1D).

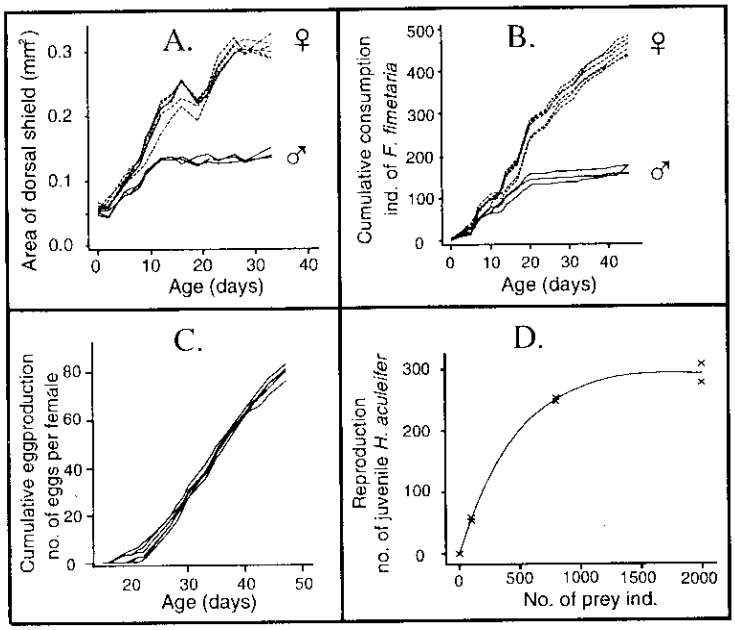

Figure 1
Characterization of individually isolated H. aculeifer through growth dynamics of dorsal shield (A), consumption (B) and oviposition (C) in relation to age. Each line represent an individual mite. Reproduction in soil of 10 females and 5 males as a function of number of added prey individuals per week (D).

3.2 Reproductive tests

In the experiments with single layer of contaminated soil only dimethoate had a clear negative effect on the reproduction of H. aculeifer, and this

drop corresponded directly to the parallel reduction in number of females (Figure 2). The control number of females were quite stable after the three week incubation period with a mortality less than 10%. The production of offspring turned out to be bifasic under the influence of dimethoate with a 25% stimulation at field dosage (.389 mg/kg) and a 90% reduction at 3×-field dosage.

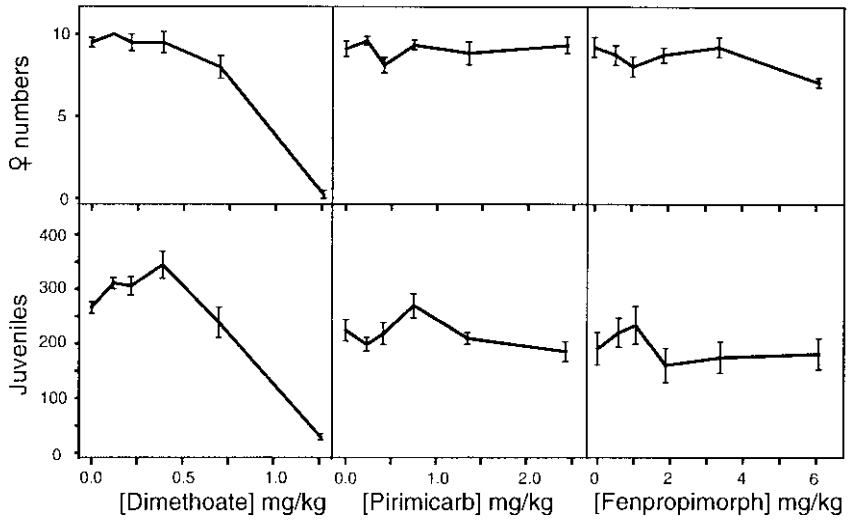

Figure 2
Reproductive output of H. aculeifer under the influence of three pesticides during a test period of 3 weeks. Vertical lines indicates 1 standard error of the mean (n=4). Data for dimethoate: EC_{50} = .87 mg/kg soil ($C.I._{95}$ = [.77;.96]), LOEC=.4 mg/kg, LOAEC=1.3 mg/kg (Lowest Observed Adverse Effect Concentration). Pirimicarb: NOEC > 2.3 mg/kg. Fenpropimorph: NOEC > 6.0 mg/kg.

With the two-layered soil contaminated in the top layer, there were no reductions in reproductive output at the tested dimethoate concentrations (Figure 3). The concentration maximally stimulating the reproduction was now .7 mg/kg, juvenile numbers decreasing to control level at 1.3 mg/kg. All females survived the treatments. The doubling of substrate amount (60 g of soil in contrast with 30 g) while mite numbers were kept stable did not significantly affect the reproduction.

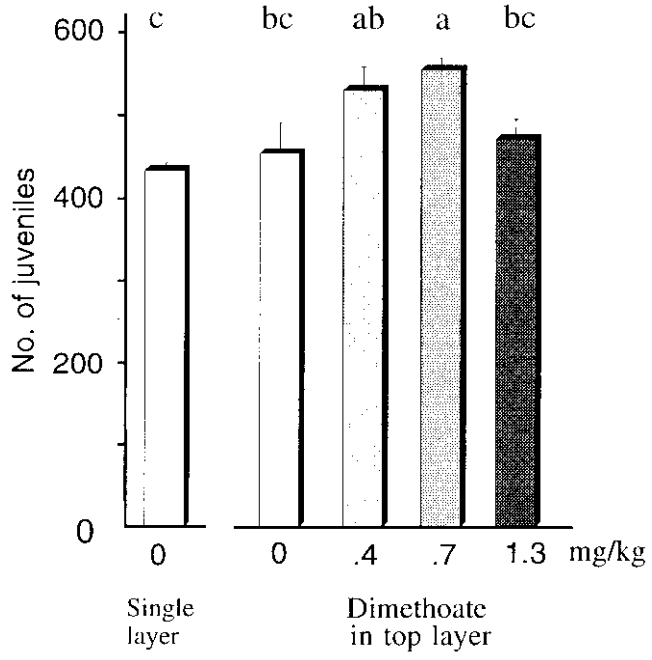

Figure 3
Mean reproduction of H. aculeifer under influence of dimethoate confined to the top layer of soil (n=4). Where letters above the bars are different the means are significantly different at the 95% level (ANOVA/LSD). LOEC = .7 mg/kg in top layer.

4 **Discussion**

The ecology of *H. aculeifer* has been described by various authors (e.g. Chi 1980, Sharma 1987, Sardar & Murphy 1987, Usher & Davis 1983). Its importance in agriculture is demonstrated by its potential for regulating the populations of plant parasitic nematodes (Sharma 1987).

H. aculeifer could be kept in permanent cultures fed either *F. candida* or *F. fimetaria*. Food supply in the basic test system seems to be at least about the satiation level. According to the rates of consumption by isolated females cultured on the gypsum/charcoal substrate, 10 females and 5 males consumed 650 prey individulas per week. It was expected that the prey would be less accessible in the test soil and therefore we added about 700-900 springtails per replicate.

The level of reproduction in control microcosms with soil substrate was 250 juveniles/3 weeks. A daily egg production rate of 3.1 per isolated female on gypsum/charcoal substrate would result in roughly 450 eggs during 14 days, which would be observed after three weeks as juveniles (the eggs produced in the last week had not hatched by the time of test termination). This larger value could stem from various factors present in the soil habitat, leading to lower consumption rate, decreased egg hatching rate, higher mortality rate, density dependence etc.

Doubtlessly, the mechanism behind the drop in reproduction in the treatment with dimethoate is a direct toxic effect reducing the number of females presumably allready at the beginning of the incubation period. This is supported from the fact that dimethoate is an efficient broad spectrum pesticide. However, it would be desirable to have data on the population size/age structure because this might reveal when dimethoate exerts its effect.

Different mechanisms can be suggested to explain the observed stimulation of the reproduction at low concentrations of dimethoate. When this effect is obviously a result of a direct chemical impact on the organism, then the phenomenon has been unspeci-fically termed hormesis (Stebbing 1982). Thus, the effect is considered to have a physiological/biochemical basis, which results in a stressed condition of affected organism leading to elevation of the level of reproduction. However, only investigations at this biological level will be able to reveal this type of underlying mechanisms. Alternative explanations could be searched for within the many possible indirect effects. The EC_{50} of the prey species *F. candida* is .5 mg/kg (unpublished data). This indicates that the prey animals could have been behaviourally affected by the chemical making them easier to be captured,

which would have resulted in the higher reproductive output of *H. aculeifer* at sublethal doses. This relies on the assumption that the mites are not completely satiated at the level of available prey present in the microcosms containing dimethoate treated soil. An attempt should be made to test the hypothesis that the mites are completely satiated with prey in this situation.

The well known structural complexity of the biotic and abiotic compartments in the soil environment is often in sharp contrast with the simplicity of the design of laboratory test systems. This has stimulated the investigation of a simple simulation of a vertical dimethoate distribution in soil. The manipulation of the distribution of the pesticide results in significant changes of the toxicity in comparison with the simplified homogeneous distribution in the soil.

The pattern in the response of mites to dimethoate in the topsoil is retained in comparison with the homogeneous distribution of the chemical. Thereby, the hormesis explanation is supported, because we know that *F. candida* is considerably less sensitive to dimethoate (unpublished data) in the same situation and will be equally available both in control and treatments.

In conclusion, it can be stated that dimethoate has two ways of affecting *H. aculeifer*: an initial stimulation at low concentrations around field dosage and an adverse effect at doses from about 2× field dosage and upwards. It remains to be investigated if these results have any relevance to the field situation and are valid for other gamasid mites.

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DATA SHEET

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Løkke, Hans (ed)

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Danish National Environmental Research Institute. Department of Terrestrial Ecology. Vejlsøvej 25, DK-8600 Silkeborg

Abstract:
Laboratory tests concerning sublethal effects of pesticides on Protozoa, Enchytraeidae, Collembola, predaceous mites, and on microbial respiration were developed. A semi-field test system concerning Collembola and predaceous mites was developed. Dimethoat caused significant effects on the reproduction of Collembola in the laboratory and in semi-field. Pirimicarb and fenpropimorph showed effects at single sampling dates in the semi-field but not in laboratory tests of 28 days duration.

Terms:
laboratory tests; pesticides; soil; ecotoxicology; methods

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Resumé:
Laboratorietest blev udviklet for at måle effekter af pesticider på encellede dyr, enchytræorme, springhaler, rovmider og på mikrobiel respiration. Semifelttest blev udviklet for at måle effekter på springhaler og rovmider. Dimethoat udviste statistisk klare effekter på springhaler i laboratoriet og under semifeltforhold. Pirimicarb og fenpropimorph udviste effekter i semifelttesten på enkelte prøvetagningsdage, som ikke blev observeret i laboratoriet efter 28 dages forløb.

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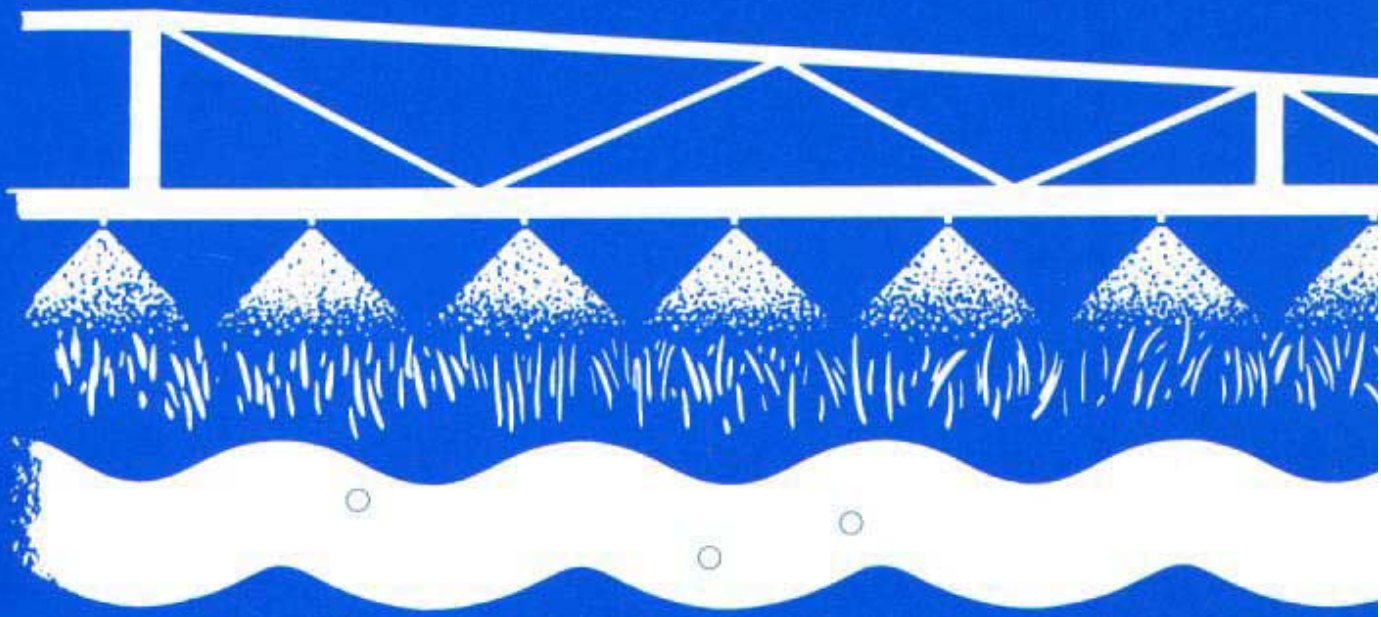

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