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OECD GUIDELINES FOR THE TESTING OF CHEMICALS

Predatory mite (*Hypoaspis (Geolaelaps) aculeifer*) reproduction test in soil

INTRODUCTION

1. This test guideline is designed to be used for assessing the effects of chemical substances in soil on the reproductive output of the soil mite species *Hypoaspis (Geolaelaps) aculeifer* Canestrini (Acari: Laelapidae), hence allowing for the estimation of the inhibition of the specific population growth rate (1,2). Reproductive output here means the number of juveniles at the end of the testing period. *H. aculeifer* represents an additional trophic level to the species for which guidelines are already available. A reproduction test without discrimination and quantification of the different stages of the reproductive cycle is considered adequate for the purpose of this Test Guideline. For substances with another exposure scenario than via the soil other approaches might be appropriate (3).

2. *Hypoaspis (Geolaelaps) aculeifer* is considered to be a relevant representative of soil fauna and predatory mites in particular. It is worldwide distributed (5) and can easily be collected and reared in the laboratory. A summary on the biology of *H. aculeifer* is provided in Annex 7. Background information on the ecology of the mite species and the use in ecotoxicological testing is available (4), (5), (6), (7), (8), (9), (10), (11), (12).

PRINCIPLE OF THE TEST

3. Adult females are exposed to a range of concentrations of the test chemical mixed into the soil. The test is started with 10 adult females per replicate vessel. Males are not introduced in the test, because experience has shown that females mate immediately or shortly after hatching from the deutonymph stage, if males are present. In addition, inclusion of males would prolong the test in a way that the demanding discrimination of age stages would become necessary. Thus, mating itself is not part of the test. The females are introduced into the test 28-35 days after the start of the egg laying period in the synchronisation (see Annex 4), as the females can then be considered as already mated and having passed the pre-oviposition stage. At 20 °C the test ends at day 14 after introducing the females (day 0), which allows the first control offspring to reach the deutonymph stage (see Annex 4). For the main measured variable, the number of juveniles per test vessels and additionally the number of surviving females are determined. The reproductive output of the mites exposed to the test chemical is compared to that of the controls in order to determine the ECx (e.g. EC10, EC50) or the no observed effect concentration (NOEC) (see Annex 1 for definitions), depending on the experimental design (see § 29). An overview of the test schedule is given in Annex 8.

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INFORMATION ON THE TEST CHEMICAL

4. The water solubility, the log K_{ow}, the soil water partition coefficient and the vapour pressure of the test chemical should preferably be known. Additional information on the fate of the test chemical in soil, such as the rates of biotic and abiotic degradation, is desirable.

5. This Guideline can be used for water soluble or insoluble substances. However, the mode of application of the test chemical will differ accordingly. The Guideline may not be applicable to substances for which the air/soil partition coefficient is greater than one, or to substances with vapour pressure exceeding 300 Pa at 25°C. Other factors such as high water solubility or high adsorption to soil limiting the volatilisation potential should be taken into account when deciding whether or not the test chemical can be tested. For volatile, unstable or readily degrading substances (e.g. using data generated from a TG 307 study), or where there is otherwise uncertainty in maintaining the nominal soil concentration, analytical measurements of the exposure concentrations at the beginning, during and at the end of the test should be considered.

6. Before use of the Test Guideline for the testing of a mixture intended for a regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

VALIDITY OF THE TEST

6. The following criteria should be satisfied in the untreated controls for a test result to be considered valid:
- Mean adult female mortality should not exceed 20% at the end of the test;
- The mean number of juveniles per replicate (with 10 adult females introduced) should be at least 50 at the end of the test;
- The coefficient of variation calculated for the number of juvenile mites per replicate should not be higher than 30% at the end of the definitive test.

REFERENCE SUBSTANCE

7. The ECx and/or NOEC of a reference substance must be determined to provide assurance that the laboratory test conditions are adequate and to verify that the response of the test organisms did not change over time. Dimethoate (CAS 60-51-5) is a suitable reference substance that has shown to affect population size (4). Boric acid (CAS 10043-35-3) may be used as an alternative reference substance. Less experience has been gained with this substance. Two design options are possible:

- The reference substance can be tested in parallel to the determination of the toxicity of each test chemical at one concentration, which has to be demonstrated beforehand in a dose response study to result in an effect of > 50% reduction of offspring.. In this case, the number of replicates should be the same as that in the controls (see § 29).

- Alternatively, the reference substance is tested 1 – 2 times a year in a dose-response test. Depending on the design chosen, the number of concentrations and replicates and the spacing factor differ (see § 29), but a response of 10 - 90 % effect should be achieved (spacing factor of 1.8). The EC50 for dimethoate based on the number of juveniles should fall in the range between 3.0 and 7.0 mg a.s./kg soil (dw). Based on the results obtained with boric acid so far, the EC50 based on the number of juveniles should fall in the range between 100 and 500 mg/kg dw soil.

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DESCRIPTION OF THE TEST

Test vessels and equipment

8. Test vessels of 3 - 5 cm diameter (height of soil ≥1.5 cm), made of glass or other chemically inert material and having a close fitting cover, should be used. Screw lids are preferred and in that case, the vessels could be aerated twice a week. Alternatively, covers that permit direct gaseous exchange between the substrate and the atmosphere (e.g. gauze) can be used. Since moisture content must be kept high enough during the test, it is essential to control the weight of each experimental vessel during the test and replenish water if necessary. This may be especially important if no screw lids are available. If a non-transparent test vessel is used, the cover should be made of material that allows for access to light (e.g. by means of a perforated transparent cover) whilst preventing the mites from escaping. The size and type of the test vessel depends on the extraction method (see Annex 5 for details). If heat extraction is applied directly to the test vessel, then a bottom mesh of appropriate mesh size could be added (sealed until extraction), and soil depth should be sufficient to allow for a temperature and moisture gradient.

9. Standard laboratory equipment is required, specifically the following:
- preferably glass vessels with screw lids;
- drying cabinet;
- stereomicroscope;
- brushes for transferring mites
- pH-meter and luxmeter;
- suitable accurate balances;
- adequate equipment for temperature control;
- adequate equipment for air humidity control (not essential if exposure vessels are covered by lids);
- temperature-controlled incubator or small room;
- equipment for extraction (see Annex 5) (13)
- overhead light panel with light control
- collection jars for extracted mites.

Preparation of the artificial soil

10. For this test, an artificial soil is used. The artificial soil consists of the following components (all values based on dry mass):
- 5% sphagnum peat, air-dried and finely ground (a particle size of 2 +/- 1 mm is acceptable);
- 20% kaolin clay (kaolinite content preferably above 30%);
- approximately 74% air-dried industrial sand (depending on the amount of $CaCO_3$ needed), predominantly fine sand with more than 50% of the particles between 50 and 200 microns. The exact amount of sand depends on the amount of $CaCO_3$ (see below), together they should add up to 75 %.
- < 1.0% calcium carbonate ($CaCO_3$, pulverised, analytical grade) to obtain a pH of 6.0 ± 0.5; the amount of calcium carbonate to be added may depend principally on the quality/nature of the peat (see Note 1).

Note 1: The amount of $CaCO_3$ required will depend on the components of the soil substrate and should be determined by measuring the pH of soil sub-samples immediately before the test (14).

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Note 2: The peat content of the artificial soil deviates from other OECD guidelines on soil organisms, where in most cases 10% peat is used (e.g. 15). However, according to EPPO (16) a typical agricultural soil has not more than 5% organic matter, and the reduction in peat content thus reflects the decreased possibilities of a natural soil for sorption of the test chemical to organic carbon.

Note 3: If required, e.g. for specific testing purposes, natural soils from unpolluted sites may also serve as test and/or culture substrate. However, if natural soil is used, it should be characterised at least by origin (collection site), pH, texture (particle size distribution) and organic matter content. If available, the type and name of the soil according to soil classification should be included, and the soil should be free from any contamination. In case the test chemical is a metal or organo-metal, the cation exchange capacity (CEC) of the natural soil should also be determined. Special attention should be paid to meet the validity criteria as background information on natural soils typically is rare.

11. The dry constituents of the soil are mixed thoroughly (e.g. in a large-scale laboratory mixer). For the determination of pH a mixture of soil and 1 M potassium chloride (KCl) or 0.01 M calcium chloride ($CaCl_2$) solution in a 1:5 ratio is used (see (14) and Annex 3). If the soil is more acidic than the required range (see § 10), it can be adjusted by addition of an appropriate amount of $CaCO_3$. If the soil is too alkaline it can be adjusted by the addition of more of the mixture comprising the first three components described in § 10, but excluding the $CaCO_3$.

12. The maximum water holding capacity (WHC) of the artificial soil is determined in accordance with procedures described in Annex 2. Two to seven days before starting the test, the dry artificial soil is pre-moistened by adding enough distilled or de-ionised water to obtain approximately half of the final water content, that being 40 to 60% of the maximum WHC. The moisture content is adjusted to 40-60 % of the maximum WHC by the addition of the test chemical solution and/or by adding distilled or de-ionised water (see § 16-18). An additional rough check of the soil moisture content should be obtained by gently squeezing the soil in the hand, if the moisture content is correct small drops of water should appear between the fingers.

13. Soil moisture content is determined at the beginning and at the end of the test by drying to constant weight at 105 °C in accordance with ISO 11465 (17) and soil pH in accordance with Annex 3 or ISO 10390 (14) . These measurements should be carried out in additional samples without mites, both from the control soil and from each test concentration soil. The soil pH should not be adjusted when acidic or basic substances are tested. The moisture content should be monitored throughout the test by weighing the vessels periodically (see § 20 and 24).

Selection and preparation of test animals

14. The species used in the test is *Hypoaspis (Geolaelaps) aculeifer* (Canestrini, 1883). Adult female mites, obtained from a synchronised cohort are required to start the test. Mites should be introduced ca. 7-14 days after becoming adult, 28 – 35 days after the start of the egg laying in the synchronisation (see § 3 and Annex 4). The source of the mites or the supplier and maintenance of the laboratory culture should be recorded. If a laboratory culture is kept, it is recommended that the identity of the species is confirmed at least once a year. An identification sheet is included as Annex 6.

Preparation of test concentrations

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15. The test chemical is mixed into the soil. Organic solvents used to aid treatment of the soil with the test chemical should be selected on the basis of their low toxicity to mites and appropriate solvent control must be included in the test design (see § 29).

Test chemical soluble in water

16. A solution of the test chemical is prepared in deionised water in a quantity sufficient for all replicates of one test concentration. It is recommended to use an appropriate quantity of water to reach the required moisture content, i.e. 40 to 60% of the maximum WHC (see § 12). Each solution of test chemical is mixed thoroughly with one batch of pre-moistened soil before being introduced into the test vessel.

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Test chemical insoluble in water

17. For substances insoluble in water but soluble in organic solvents, the test chemical can be dissolved in the smallest possible volume of a suitable vehicle (e.g. acetone). Only volatile solvents should be used. When such vehicles are used, all test concentrations and the control should contain the same minimum amount of the vehicle. The vehicle is sprayed on or mixed with a small amount, for example 10g, of fine quartz sand. The total sand content of the substrate should be corrected for this amount. The vehicle is eliminated by evaporation under a fume hood for at least one hour. This mixture of quartz sand and test chemical is added to the pre-moistened soil and thoroughly mixed by adding an appropriate amount of de-ionised water to obtain the moisture required. The final mixture is introduced into the test vessels. Note that some solvents may be toxic to mites. It is therefore recommended to use an additional water control without vehicle if the toxicity of the solvent to mites is not known. If it is adequately demonstrated that the solvent (in the concentrations to be applied) has no effects, the water control may be excluded.

Test chemical poorly soluble in water and organic solvents

18. For substances that are poorly soluble in water and organic solvents, the equivalent of 2.5 g of finely ground quartz sand per test vessel (for example 10 g of fine quartz sand for four replicates) is mixed with the quantity of test chemical to obtain the desired test concentration. The total sand content of the substrate should be corrected for this amount. This mixture of quartz sand and test chemical is added to the pre-moistened soil and thoroughly mixed after adding an appropriate amount of deionised water to obtain the required moisture content. The final mixture is divided between the test vessels. The procedure is repeated for each test concentration and an appropriate control is also prepared.

PROCEDURE

Test groups and controls

19. Ten adult females in 20 g dry mass of artificial soil are recommended for each control and treatment vessel. Test organisms should be added within two hours after preparation of the final test substrate (i.e. after application of the test item). In specific cases (e.g. when ageing is considered to be a determining factor), the time between preparation of the final test substrate and the addition of the mites can be prolonged (for details of such ageing, see (18)). However, in such cases a scientific justification must be provided.

20. After the addition of the mites to the soil, the mites are provided with food and the initial weight of each test vessel should be measured to be used as reference for monitoring soil moisture content throughout the test as described in § 24. The test vessels are then covered as described in § 8 and placed in the test chamber.

21. Appropriate controls are prepared for each of the methods of test chemical application described in § 15 to 18. The relevant procedures described are followed for preparing the controls except that the test chemical is not added. Thus, where appropriate, organic solvents, quartz sand or other vehicles are applied to the controls in concentrations/amounts like in the treatments. Where a solvent or other vehicle is used to add the test chemical, an additional control without the vehicle or test chemical should also be prepared and tested in case the toxicity of the solvent is not known (see § 17).

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Test conditions

22. The test temperature should be 20 ± 2 °C. Temperature should be recorded at least daily and adjusted, if necessary. The test is carried out under controlled light-dark cycles (preferably 16 hours light and 8 hours dark) with illumination of 400 to 800 lux in the vicinity of the test vessels. For reasons of comparability, these conditions are the same as in other soil ecotoxicological tests (e.g. 15).

23. Gaseous exchange should be guaranteed by aerating the test vessels at least twice a week in case screw lids are used. If gauze covers are used, special attention should be paid to the maintenance of the soil moisture content (see §§ 8 and 24).

24. The water content of the soil substrate in the test vessels is maintained throughout the test by weighing and if needed re-watering the test vessels periodically (e.g. once per week). Losses are replenished as necessary with de-ionised water. The moisture content during the test should not differ by more than 10% from the start value.

Feeding

25. Cheese mites (*Tyrophagus putrescentiae* (Schrank, 1781)) have been shown to be a suitable food source. Small collembolans (e.g. juvenile *Folsomia candida* Willem, 1902 or Onychiurus *fimatus* (19, 20), enchytraeids (e.g. *Enchytraeus crypticus* Westheide & Graefe, 1992) or nematodes (e.g. *Turbatrix silusiae* de Man, 1913)) may be also suitable (21). It is recommended to check the food before using it in a test. The type and amount of food should secure an adequate number of juveniles in order to fulfil the validity criteria (§ 6). For the prey selection, the mode of action of the test item should be considered (e.g. an acaricide may be toxic to the food mites too, see § 26).

26. Food should be provided *ad libitum* (i.e. each time a small amount (tip of a spatula)). For this purpose, also low suction exhaustor as proposed in the collembolan test or a fine paint brush can also be used. Supplying food at the beginning of the test and two to three times a week will usually be sufficient. When the test chemical appears to be toxic to the prey, an increased feeding rate and/or an alternative food source should be considered.

Range-finding test

27. Prior knowledge of the toxicity of the test chemical should help in selecting appropriate test concentrations, e.g. from range-finding studies. When necessary, a range-finding test is conducted with five concentrations of the test chemical in the range of 0.1 – 1000 mg/kg dry soil, with at least one replicate for treatments and control. The duration of the range finding test is 14 days, after which mortality of the adult mites and the number of juveniles is determined. The concentration range in the final test should preferably be chosen so that it includes concentrations at which juvenile numbers are affected while survival of the maternal generation is not. This, however, may not be possible for test chemicals that cause lethal and sub-lethal effects at almost similar concentrations. The effect concentration (e.g. EC50 EC25, EC10) and the concentration range, over which the effect of the test chemical is of interest, should be bracketed by the concentrations included in the test. Extrapolating much below the lowest concentration affecting the test organisms or above the highest tested concentration should be done only in exceptional cases, and a full explanation should be given in the report.

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Experimental design

Dose response tests

28. Three test designs are proposed, based on the recommendations arising from another ring test (Enchytraeid reproduction test (22)). The general suitability of all these designs was confirmed by the outcome of *H. aculeifer* validation.

29. In setting the range of concentrations, the following should be borne in mind:

- For determination of the ECx (e.g. EC10, EC50), twelve concentrations should be tested. At least two replicates for each test concentration and six control replicates are recommended. The spacing factor may vary, i.e. less than or equal to 1.8 in the expected effect range and above 1.8 at the higher and lower concentrations.
- For determination of the NOEC, at least five concentrations in a geometric series should be tested. Four replicates for each test concentration plus eight controls are recommended. The concentrations should be spaced by a factor not exceeding 2.0.
- A combined approach allows for determination of both the NOEC and ECx. Eight treatment concentrations in a geometric series should be used. Four replicates for each treatment plus eight controls are recommended. The concentrations should be spaced by a factor not exceeding 1.8.

Limit test

30. If no effects are observed at the highest concentration in the range-finding test (i.e. 1000 mg/kg dw soil), the definitive reproduction test can be performed as a limit test, using a test concentration of 1000 mg/kg dw soil. A limit test will provide the opportunity to demonstrate that the NOEC or the EC10 for reproduction is greater than the limit concentration, whilst minimising the number of mites used in the test. Eight replicates should be used for both the treated soil and the control.

Test duration and measurements

31. Any observed differences between the behaviour and the morphology of the mites in the control and the treated vessels should be recorded.

32. On day 14 the surviving mites are extracted from the soil via heat/light extraction or by another appropriate method (see Annex 5). The numbers of juveniles (i.e. larvae, protonymphs and deutonymphs) and adults are counted separately. Any adult mites not found at this time are to be recorded as dead, assuming that such mites have died and decomposed prior to the assessment. At the end of the test, adults and juveniles are humanely euthanized, preferably by rapid freezing at -80°C or cryopreservation. Extraction efficiency must be validated once or twice a year in controls with known numbers of adults and juveniles. Efficiency should be above 90% on average combined for all developmental stages (see Annex 5). Adult and juvenile counts are not adjusted for efficiency.

DATA AND REPORTING

Treatment of results

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33. Information on the statistical methods that may be used for analysing the test results is given in §§ 36 to 41. In addition, OECD Document 54 on the "Current Approaches in the Statistical Analysis of Ecotoxicity Data: a Guidance to Application" should be consulted.

34. Test main endpoint is the reproductive output, here the number of juveniles produced per replicate test vessel (with 10 adult females introduced). The statistical analysis requires the arithmetic mean (X) and the variance (s^2) for the reproductive output to be calculated per treatment and per control. X and s^2 are used for ANOVA procedures such as the Student t test, Dunnett test, or Williams' test as well as for the computation of 95% confidence intervals.

Note: This main endpoint is equivalent with fecundity measured as the number of living juveniles produced during the test divided by the number of parental females introduced at the start of the test.

35. The number of surviving females in the untreated controls is a major validity criterion and has to be documented. As in the range-finding test, all other harmful signs should be recorded in the final report as well.

ECx

36. ECx-values including their associated lower and upper 95% confidence limits for the parameter described in § 34 are calculated using appropriate statistical methods (e.g. probit analysis, logistic or Weibull function, trimmed Spearman-Karber method, or simple interpolation). An ECx is obtained by inserting a value corresponding to x% of the control mean into the equation found. To compute the EC50 or any other ECx, the per treatment means (X) should be subjected to regression analysis.

NOEC/LOEC

37. If a statistical analysis is intended to determine the NOEC/LOEC, per-vessel statistics (individual vessels are considered replicates) are necessary. Appropriate statistical methods should be used (according to OECD Document 54 on the Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application). In general, adverse effects of the test chemical compared to the control are investigated using one-tailed (smaller) hypothesis testing at $p \leq 0.05$. Examples are given in the following paragraphs.

38. Normal distribution of data can be tested e.g. with the Kolmogorov-Smirnov goodness-of-fit test, the Range-to-standard-deviation ratio test (R/s-test) or the Shapiro-Wilk test (two-sided, $p \leq 0.05$). Cochran's test, Levene test or Bartlett's test, (two-sided, $p \leq 0.05$) may be used to test variance homogeneity. If the prerequisites of parametric test procedures (normality, variance homogeneity) are fulfilled, One-way Analysis of Variance (ANOVA) and subsequent multi-comparison tests can be performed. Multiple comparisons (e.g. Dunnett's t-test) or step-down trend tests (e.g. Williams' test in case of a monotonous dose-response relationship) can be used to calculate whether there are significant differences ($p \leq 0.05$) between the controls and the various test item concentrations (selection of the recommended test according to OECD Document 54 on the Current Approaches in the Statistical Analysis of Ecotoxicity Data: a Guidance to Application). Otherwise, non-parametric methods (e.g. Bonferroni-U-test according to Holm or Jonckheere-Terpstra trend test) should be used to determine the NOEC and the LOEC.

Limit test

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39. If a limit test (comparison of control and one treatment only) has been performed and the prerequisites of parametric test procedures (normality, homogeneity) are fulfilled, metric responses can be evaluated by the Student test (t-test). The unequal-variance t-test (Welch t-test) or a non parametric test, such as the Mann-Whitney-U-test may be used, if these requirements are not fulfilled.

40. To determine significant differences between the controls (control and solvent control), the replicates of each control can be tested as described for the limit test. If these tests do not detect significant differences, all control and solvent control replicates may be pooled. Otherwise all treatments should be compared with the solvent control.

Test report

41. The test report should at least include the following information:

Test chemical

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical and environmental fate properties, measured or estimated (e.g. hydrolysis, vapour pressure, log Kow, log Koc, log Kd (soil), log Koa, air/soil partitioning coefficient, biodegradability in soil or other biodegradability information);
- chemical identification, such as IUPAC or Chemical Abstract (CA) Index name, CAS Registry Number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc. (including the organic carbon content, if appropriate).

Multi-constituent substance, UVCBs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant

Test organisms

- identification and supplier of the test organisms, description of the culturing conditions;
- age range of test organisms.

Test conditions

- description of the experimental design and procedure;
- preparation details for the test soil; detailed specification if natural soil is used (origin, history, particle size distribution, pH, organic matter content and if available the soil classification)
- the maximum water holding capacity of the soil;
- a description of the technique used to apply the test chemical to the soil;
- details of auxiliary substances used for administering the test chemical;
- size of test vessels and dry mass of test soil per vessel;
- test conditions: light intensity, duration of light-dark cycles, temperature;
- a description of the feeding regime, the type and amount of food used in the test, feeding dates;
- pH and water content of the soil at the start and during the test (control and each treatment)
- detailed description of the extraction method and extraction efficiency.

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Test results

- the number of juveniles determined in each test vessel at the end of the test;
- number of adult females and adult mortality (%) in each test vessel at the end of the test
- a description of obvious symptoms or distinct changes in behaviour;
- the results obtained with the reference test chemical;
- summary statistics (ECx and/or NOEC) including 95%-confidence limits and a description of the method of calculation;
- a plot of the concentration-response-relationship;
- deviations from procedures described in this guideline and any unusual occurrences during the test.

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ANNEX 1

DEFINITIONS

The following definitions are applicable to this Guideline (in this test all effect concentrations are expressed as a mass of test chemical per dry mass of the test soil):

NOEC (no observed effect concentration) is the test chemical concentration at which no effect is observed. In this test, the concentration corresponding to the NOEC, has no statistically significant effect ($p < 0.05$) within a given exposure period when compared with the control.

LOEC (lowest observed effect concentration) is the lowest test chemical concentration that has a statistically significant effect ($p < 0.05$) within a given exposure period when compared with the control.

ECx (effect concentration for x% effect) is the concentration that causes an x% of an effect on test organisms within a given exposure period when compared with a control. For example, an EC_{50} is a concentration estimated to cause an effect on a test end point in 50% of an exposed population over a defined exposure period.

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ANNEX 2

DETERMINATION OF THE MAXIMUM WATER HOLDING CAPACITY OF THE SOIL

The following method for determining the maximum water holding capacity of the soil is considered to be appropriate. It is described in Annex C of the ISO 11268-2 (Soil Quality - Effects of pollutants on earthworms (*Eisenia fetida*). Part 2: Determination of effects on reproduction (23)).

Collect a defined quantity (e.g. 5 g) of the test soil substrate using a suitable sampling device (auger tube etc.). Cover the bottom of the tube with a piece of filter paper filled with water and then places it on a rack in a water bath. The tube should be gradually submerged until the water level is above to the top of the soil. It should then be left in the water for about three hours. Since not all water absorbed by the soil capillaries can be retained, the soil sample should be allowed to drain for a period of two hours by placing the tube onto a bed of very wet finely ground quartz sand contained within a covered vessel (to prevent drying). The sample should then be weighed, dried to constant mass at 105 °C. The water holding capacity (WHC) can then be calculated as follows:

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

Where:

S = water-saturated substrate + mass of tube + mass of filter paper
T = tare (mass of tube + mass of filter paper)
D = dry mass of substrate

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ANNEX 3

DETERMINATION OF SOIL pH

The following method for determining the pH of a soil is based on the description given in ISO 10390: Soil Quality – Determination of pH (16).

A defined quantity of soil is dried at room temperature for at least 12 h. A suspension of the soil (containing at least 5 grams of soil) is then made up in five times its volume of either a 1 M solution of analytical grade potassium chloride (KCl) or a 0.01 M solution of analytical grade calcium chloride ($CaCl_2$). The suspension is then shaken thoroughly for five minutes and then left to settle for at least 2 hours but not for longer than 24 hours. The pH of the liquid phase is then measured using a pH-meter that has been calibrated before each measurement using an appropriate series of buffer solutions (e.g. pH 4.0 and 7.0).

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ANNEX 4

REARING OF *HYPOASPIS (GEOLAELAPS) ACULEIFER* , FOOD MITES AND SYNCHRONISATION OF CULTURE

Rearing of *Hypoaspis (Geolaelaps) aculeifer*:
Cultures can be maintained in plastic vessels or glass jars filled with plaster of Paris / charcoal powder (9:1) mixture. The plaster can be kept moist by adding few drops of distilled or deionised water if required. Rearing temperatures are optimal between 20 ± 2°C, light / dark regime is not relevant for this species. Prey can be *Typrophagus putrescentiae* or *Caloglyphus* sp. mites (food mites should be handled with care since they could cause allergies in humans), but nematodes, enchytraeids and collembolans are also suited as prey items. Their source should be recorded. Population development can start with a single female because males develop in unfertilised eggs. Generations are largely overlapping. A female can live at least 100 days and can deposit approximately 100 eggs during its lifetime. A maximum oviposition rate is reached between 10 and 40 days (after becoming adults) and amounts to 2.2 eggs female^{-1} day^{-1}. Developmental time from egg to adult female is approximately 20 days at 20°C. More than one culture should be maintained and held beforehand.

Rearing of *Typrophagus putrescentiae:*
The mites are kept in a glass vessel filled with fine brewers yeast powder which is put in a plastic bucket filled with KNO_3-solution in order to avoid escaping. The food mites are placed on top of this powder. Afterwards, they are carefully mixed with the powder (which has to be replaced twice a week) using a spatula.

Synchronisation of culture:
Specimens that are used in the test should be of similar age (ca. 7 days after reaching the adult stage). At a rearing temperature of 20 °C this is achieved by

- Transfer females to a clean rearing vessel and add sufficient food
- Allow for two to three days of egg laying, remove females
- Take adult females for testing between the 28th and 35th day after start placing female adults in clean rearing vessels.

Adult females can be easily distinguished from males and other developmental stages by their larger size, bloated shape and their brown dorsal shield (males are slimmer and flat), immatures are white to cream-coloured. The development of the mites follows approximately the pattern described below at 20°C (figure): Egg 5d, Larva 2d, Protonymph 5d, Deutonymph 7d, preoviposition period of female 2d. Afterwards, the mites are adult.

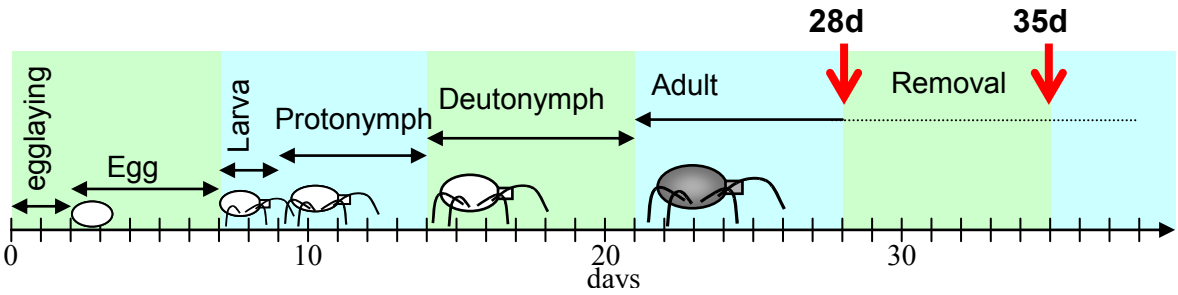

Figure: Development of *Hypoaspis (Geolaelaps) aculeifer* at 20°C. (removal = females used for the test)

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The adult test animals are removed from the synchronised culture and introduced into the test vessels between the 28th and the 35th day after the parental females have started egg laying (i.e. 7 – 14 days after they became adult). This ensures that the test animals have already passed their preoviposition period and have been mated by males that are also present in the culture vessel. Observations in laboratory cultures suggest, that females mate immediately or shortly after becoming adult if males are present (Ruf, Vaninnen, pers. obs.). The period of seven days is chosen to facilitate integration in laboratory routine and to buffer individual developmental variability among mites. The oviposition should be started with at least the same number of females that is eventually needed for the test (If for example 400 females are needed in the test, at least 400 females should be allowed to oviposit for two to three days. At least 1200 eggs should be the starting point for the synchronised population (sex ratio ca. 0.5, mortality ca. 0.2). To avoid cannibalism, it is more feasible to keep not more than 20-30 ovipositing females in one vessel.

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ANNEX 5

EXTRACTION METHODS

For micro-arthropods a heat extraction is an appropriate method to separate specimens from the soil / substrate (see figure below). The method is based on the activity of the organisms, so only mobile specimens will have the chance to be recorded. The principle of the heat extraction is to make conditions for the organisms gradually worse in the sample, so that they will leave the substrate and fall in a fixing liquid (e.g. ethanol). Crucial points are the duration of the extraction and the gradient of good to moderate to bad conditions for the organisms. The duration of extraction for ecotoxicological tests have to be as short as possible, because any population growth during the time of extraction would falsify the results. On the other hand the temperature and moisture conditions in the sample have to be always in a range that allows the mites to move. The heating of a soil sample leads to a desiccation of substrate. If the desiccation is too quick, some mites might also desiccated before they managed to escape.

Therefore the following procedure is proposed (24) (25):
Apparatus: Tullgren funnel or comparable methods like e.g. McFadyen (heating from above, sample is put over a funnel)

Heating regime: 25°C for 12 h, 35°C for 12 h, 45°C for 24 hours (in total 48 h). The temperature should be measured in the substrate.

Fixation liquid: 70% ethanol

Details: Take glass vial that was used for the test. Remove lid and wrap a piece of mesh or fabric around the opening. The fabric should have a mesh size of 1.0 to 1.5 mm. Fix the fabric with an elastic band. Carefully turn the vial upside down and place it in the extraction apparatus. The fabric prevents substrate from trickling in the fixation liquid but allows mites to leave the sample. Start the heating regime after all vials are inserted. End the extraction after 48 hours. Remove fixation vials and count mites by means of a dissecting microscope.

The extraction efficiency of the chosen method must have been proven once or twice a year using vessels containing a known number of juvenile and adult mites kept in untreated test substrate. Efficiency should be ≥ 90% on average combined for all developmental stages.

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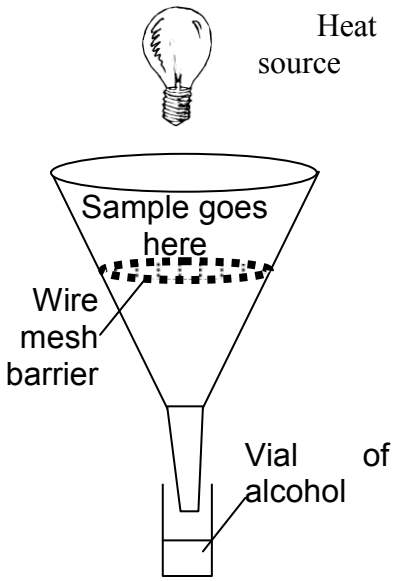
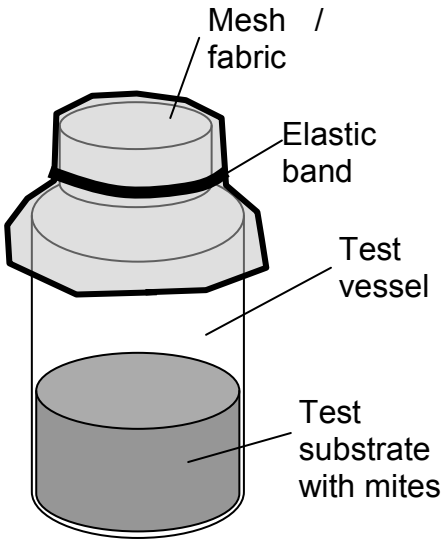
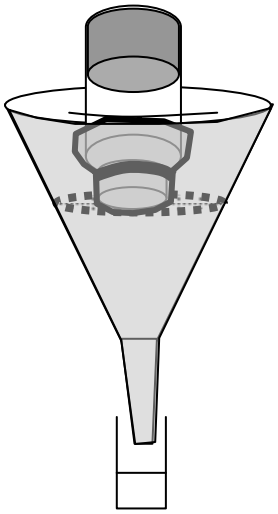
TULLGREN-TYPE EXTRACTING DEVICE



How to prepare the test vial after the test is finished, before extraction





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ANNEX 6

IDENTIFICATION OF *HYPOASPIS (GEOLAELAPS) ACULEIFER*

Subclass/order/suborder:	Family:	Genus/subgenus/species:
Acari/Parasitiformes/ Gamasida	Laelapidae	*Hypoaspis* (*Geolaelaps*) *aculeifer*

Author and Date:	F. Faraji, Ph.D. (MITOX), 23 January 2007

Literature used:	**Karg, W. (1993).** Die freilebenden Gamasina (Gamasides), Raubmilben. Tierwelt Deutschlands 59, 2nd revised edition: 1-523. **Hughes, A.M. (1976).** The mites of stored food and houses. Ministry of Agriculture, Fisheries and Food, Technical Bulletin 9: 400pp. **Krantz, G.W. (1978).** A manual of Acarology. Oregon State University Book Stores, Inc., 509 pp.

Deterministic characteristics:	Tectum with rounded denticulate edge; hypostomal grooves with more than 6 denticles; caudal dorsal setae of **Z4** not very long; dorsal setae setiform; genital shield normal, not very enlarged and not reaching the anal shield; posterior half of dorsal shield without unpaired setae; legs II and IV with some thick macrosetae; dorsal seta **Z5** about two times longer than **J5**; fixed digit of chelicera with 12-14 teeth and movable digit with 2 teeth; Idiosoma 520-685 long. *Hypoaspis miles* is also used in biological control and might get confused with *H. aculeifer*. The main difference is: *H. miles* belongs to subgenus *Cosmolaelaps* and has knife-like dorsal setae while *H. aculeifer* belongs to subgenus *Geolaelaps* and has setiform dorsal setae.

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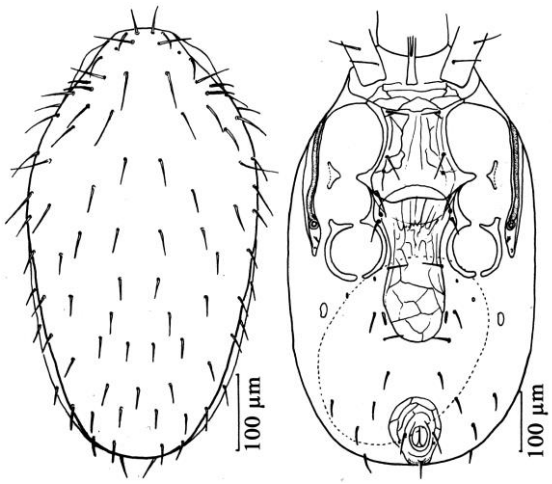
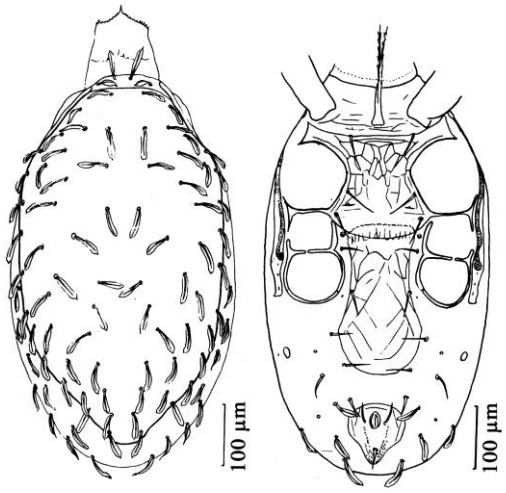
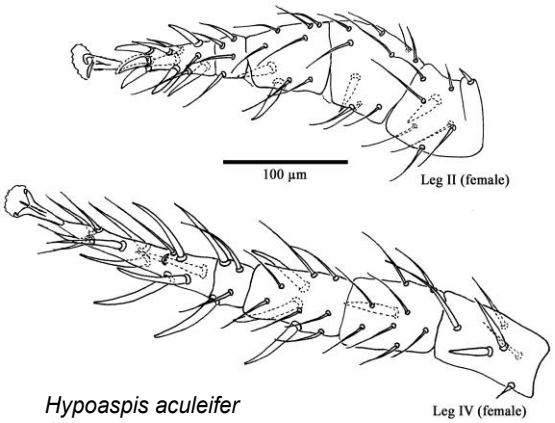


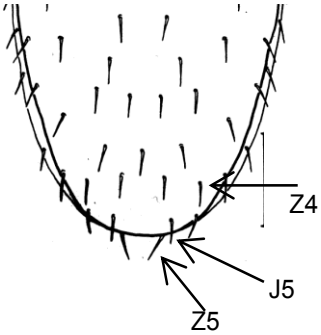
Hypoaspis aculeifer After Hughes, 1976



Hypoaspis miles After Hughes, 1976



Hypoaspis aculeifer
Original drawings by F. Faraji



Hypoaspis aculeifer,
dorsal shield with characteristic setae

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ANNEX 7

Basic information on the biology of *Hypoaspis (Geolaelaps) aculeifer*

Hypoaspis aculeifer belongs to the family Lealapidae, order Acari (mites), class Arachnida, tribe Arthropoda. They are living in all kinds of soil and feed on other mites, nematodes, enchytraeids and collembolans (26). In case of food shortage they switch to cannibalism (27). Predatory mites are segmented in idiosoma and gnathosoma. A clear differentiation of the idiosoma in prosoma (head) and opisthosoma (abdomen) is missing. The gnathosoma (head shield) contains the instruments for feeding such as palps and chelicera. The chelicers are trifurcated and tusked with teeth of different shape. Beside ingestion the males are using their chelicers mainly to transfer the spermatophores to the females. A dorsal shield covers nearly completely the idiosoma. A big part of the female idiosoma is occupied by the reproductive organs, which are in particular distinct shortly before egg deposition. Ventrally, two shields can be found, the sternal and the genital shield. All legs are provided with bristles and thorns. The bristles are used to anchor when moving in or on top of the soil. The first pair of legs is used mainly as antenna. The second pair of legs is used not only for moving but also to clinch the prey. The thorns of the fourth pair of legs can serve as protection as well as 'moving motor' (28). Males are 0.55 – 0.65 mm long and have a weight of 10 – 15 µg. Females are 0.8 – 0.9 mm long and are weighing 50 – 60 µg (8) (28) (Fig 1).

Fig 1: **Female, male, protonymph and larvae of *H. aculeifer*.**

At 23°C, the mites become sexually mature after 16 days (females) and 18 days (males), respectively (6). The females carry over the sperms by the solenostom where they will be then transferred to the ovar. In the ovar the sperms mature and will be stored. Fertilisation takes place only after maturation of the sperms in the ovar. The fertilised or unfertilised eggs will be deposited by the females in clumps or separately, preferably in crevices or holes. Copulated females can bear juveniles of both sexes whereas from eggs of uncopulated females only male juveniles are hatching. During development to the adult four phases of development (egg – larvae, larvae – protonymph, protonymph – deutonymph, deutonymph – adult) are passed through.

The egg is milky white, hyaline, elliptical and approximately 0.37 mm long with a solid mantle. According to (8), the larvae are between 0.42 – 0.45 mm in size. They have only three pairs of legs. In the head region palps and chelicers are developed. The chelicers, having some few small denticles, are used to hatch from the egg. After the first moult, 1 – 2 days after hatching, the protonymphs are developed. They are also white, the size is 0.45 – 0.62 mm (8) and they have four pairs of legs. On the chelicers the teeth are completely present. Beginning with that stadium the mites start to forage. For that reason the cuticula of

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the prey is pierced with the chelicers and a secretion for the extra intestinal digestion is emitted into the prey. The food mash can then be sucked by the mite. The chelicers can also be used to rip bigger particles out of food nuggets (28). After one further moult the deutonymphs are developed. They are 0.60 – 0.80 mm (8) in size and yellow to light brown in colour. Beginning with that phase they can be separated into females and males. After further ecdysis, during which time the animals are inactive and the brown shield is developing (approx. after 14 days) the mites are adult (28) (29) (30).Their life span is between 48 and 100 days at 25°C (27).

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ANNEX 8

SUMMARY AND TIME SCHEDULE OF THE MAIN ACTIONS TO BE TAKEN IN ORDER TO PERFORM THE HYPOASPIS TEST

Time (days) test start = day 0	Activity / task
Day -35 to -28	- Transfer females from stock culture to clean vessels to start synchronisation - 2 days later: removal of females - twice or three times a week: supply with sufficient food
Day -5 (+/- 2)	Prepare artificial soil
Day -4 (+/- 2)	- Determine WHC of artificial soil - Dry over night - Next day: weigh samples and calculate WHC
Day -4 (+/- 2)	Pre moisture artificial soil to achieve 20 - 30 % of WHC
Day 0	- Start test: add test chemical to artificial soil - Introduce 10 females to each replicate - Weigh each replicate - Set up abiotic controls for moisture content and pH, 2 replicates for each treatment - Dry moisture controls over night - Next day: weigh moisture controls - Next day: measure pH of dried abiotic controls
Day 3, 6, 9, 12 (approx.)	- Supply each replicate with sufficient amount of prey organisms - Weigh each replicate and eventually add evaporated water
Day 14	- Terminate test, set up extraction with all replicates plus extraction efficiency controls - Dry water content controls over night - Next day: weigh water content controls - Next day: measure pH of dried controls
Day 16	Terminate extraction
Day 16+	- Record number of adults and juveniles in extracted material - Report results on template tables - Report testing procedure in test protocol sheets

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