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PROPOSAL FOR A NEW GUIDELINE

(Written in OECD format)

Determination of Developmental Toxicity of a Test Chemical to Dipteran Dung Flies (*Scathophaga stercoraria* L. (Scathophagidae), *Musca autumnalis* De Geer (Muscidae))

INTRODUCTION

1. This study is designed to estimate the developmental toxicity of a test chemical to the dung dwelling life stages of dung-dependent dipteran species. In this test, insects are exposed under controlled conditions [1].

2. *Scathophaga stercoraria* L. (Scathophagidae) and *Musca autumnalis* De Geer (Muscidae)) are considered to be suitable indicator species for estimating the developmental toxicity of parasiticides on dung dependant diptera for the following main reasons: Collectively, the species cover a wide geographic range. *S. stercoraria* and *M. autumnalis* are widespread in Europe, Asia, Africa and North America [2, 3, 4, 5, 6, 7].

3. Both species are dung-dependent, are multi-voltine, do not undergo obligate diapause and are easy to culture and have a short life-cycle which makes it possible to determine effects on development and survival in the laboratory. Background information on the ecology of the dung fly species and their use in ecotoxicological testing is available [8, 9, 10, 11, 12, 13, 14].

PRINCIPLE OF THE TEST

4. This study is designed to estimate the developmental toxicity of a test chemical to the dung dwelling life stages of dung-dependent dipteran species. Negative control(s) are included as comparisons with contaminated dung. A positive control should be tested periodically. In this test, insects are exposed under controlled conditions. The test chemical is mixed with bovine faeces, to which eggs or larvae are added. The impact of the test chemical on maturation of the flies to adults is assessed. The number of emerged flies after exposure of the eggs/larvae to the test substance is compared to that of the control(s) in order to determine the No Observed Effect Concentration (NOEC) or the ECx (Effect concentration for x% effect e.g. EC_{50}), depending on the experimental design.

INFORMATION ON THE TEST SUBSTANCE

5. The water solubility, the log K_{ow}, and the vapor pressure of the test substance should preferably be known. Additional information on the fate of the test substance in dung, such as degradation times, is desirable. Details of the source, batch or lot number and purity of the test and reference chemicals also need

to be provided.

6. This Guideline can be used for water soluble or insoluble substances. However, the mode of application of the test substance will differ accordingly. The Guideline is not applicable to volatile substances, i.e. substances for which the Henry's constant or the air/water partition coefficient is greater than one, or substances for which the vapour pressure exceeds 0.0133 Pa at 25 °C.

REFERENCE SUBSTANCE

7. Ivermectin (tech.) is a suitable reference substance that has been shown to affect fly emergence [9, 10, 11, 15]. Two options are possible:
- The NOEC (or the ECx) of a reference substance can be determined 1 - 2 per year to provide assurance that the laboratory test conditions are adequate and to verify that the response of the test organisms does not change significantly statistically over time. The EC50 for the endpoint emergence should be between 50 and 150 µg active ingredient (a.i.)/kg d.w. (*S. stercoraria*) and 1 and 15 µg active ingredient (a.i.)/kg d.w. (*M. autumnalis*) respectively.
- However, it is more advisable to test a reference substance in parallel to the determination of the toxicity of a test substance. In this case, one concentration is used and the number of replicates should be the same as that in the water controls. Significant effects should be observed at concentrations >100 µg active ingredient (a.i.)/kg d.w. (*S. stercoraria*) and 12.5 µg active ingredient (a.i.)/kg d.w. (*M. autumnalis*) respectively. ..

After completion of the evaluation of all ring test data, the number in § 7 may be modified.

VALIDITY OF THE TEST

8. The definitive/limit test is valid if in the controls
- Hatching of larvae is ≥ 70% of the number of introduced eggs (*S. stercoraria*);
- Emergence of adults is ≥ 70% of the hatched larvae (*S. stercoraria*);
- Emergence of adults is ≥ 50% of the introduced larvae (*M. autumnalis*).

When a test fails to meet the above validity criteria the test should be terminated unless a justification for proceeding with the test can be provided. The justification should be included in the report.

DESCRIPTION OF THE TEST

Equipment

9. Test vessels must be of an appropriate size (e.g. plastic or glass beakers 250 – 500 mL). Ventilation will be provided through a piece of cotton or muslin cloth secured over the top of the beaker with a rubber band.

10. Standard laboratory equipment is required, specifically the following:
- Vermiculite;

- Drying cabinet;
- Stereomicroscope;
- Brushes for transferring eggs/larvae
- pH-meter and lux meter;
- Suitable accurate balances;
- Adequate equipment for temperature control;
- Adequate equipment for humidity control (not essential if exposure vessels are covered by lids).

Selection and collection of the dung

11. Non-contaminated bovine dung will be obtained from cattle of documented veterinary history. At the time of collection the animals will not have been treated with any veterinary pharmaceuticals for at least 12 weeks or for at least 5 months if the treatment product is long-acting. No contaminants should be expected in the dung that might interfere with the conduct of the study.

12. The dung may be collected directly from cattle (internal or bag collection) or ground collected. If dung is ground collected, care should be taken to avoid urine contamination. Ground collected dung should be less than 2 hours old at the time of collection to minimise dung fauna colonisation and should be frozen at ca –20^{o}C for at least 1 week before use (preferably longer (e.g. four weeks), in order to avoid mite contamination). The husbandry, in particular the diet, of the cattle providing the dung should be recorded. Samples of the dung should be taken to determine moisture and pH (see ANNEX 2). When testing dung from treated cattle nitrogen and carbon content (incl. C/N ratio) should be determined.

Selection and preparation of test animals

13. The species to be used in this bioassay are *Scathophaga stercoraria* (Fig. 1) or *Musca autumnalis* (Fig. 2). Flies will be obtained from an established laboratory culture (see ANNEX 3). Where field-collection of adult flies to initiate a culture is conducted, the species should be verified using an appropriate key [16]. Colonies initiated from field-collected organisms should be cultured for a minimum of one generation prior to test initiation. The source and history of the organisms will be documented. Newly oviposited eggs or eclosed larvae (less than 12 hours old) will be used in the test.

Fig. 1: *Scathophaga stercoraria* Fig. 2: *Musca autumnalis*

Test conditions

14. Holding and test vessels will be maintained within the laboratory at a temperature of 20°C ± 2°C for *S. stercoraria* and 26°C ± 2°C for *M. autumnalis*. In tests with *S. stercoraria*, humidity (RH) is recommended to be > 60% in the first phase of the test in order to avoid desiccation of the eggs. A light cycle of 16 h light and 8 h dark will be maintained, illumination being provided by fluorescent tubes. Light intensity in the area of the test vessels approximately at the level of the dung surface should be recorded at the start of the test.

PROCEDURE

Dung Preparation

15. Dung should be removed from the freezer in time to ensure that it is completely thawed before use. The dung should be homogenised for ca 10 minutes in a large-scale laboratory mixer or electric cement mixer prior to preparation of the separate treatment groups.

16. Moisture content and pH of a sample of untreated dung will be determined at the start of each test. When testing dung from treated cattle nitrogen and carbon content (incl. C/N ratio) should be determined. The methods used for measuring these parameters will be recorded. Possible methods for parameter determination are included in ANNEX 2. In principle, modified methods already standardised by ISO for soil properties should be used.

17. Moisture content can be determined by weighing three replicate dung samples (ca 20 g) into vessels and drying overnight in an oven at ca 105°C [17]. The samples are then removed, cooled at room temperature in a desiccator and reweighed, the moisture content calculated and expressed on an oven dry basis. Dung pH can be determined by adding a weighed amount of dung to a 1.0 M potassium chloride solution or 0.01 M calcium chloride in a vial and measuring with a calibrated pH meter [18]. The ration between dung and aqueous phase should be 1 : 5 v/v. Nitrogen content can be determined using the method of Tilman and Wedin [19] or the micro-Kjeldahl procedure as described by Hesse [20]. Again, ISO methods should be preferred [21, 22, 23].

Application of Test Chemicals

18. All test concentrations have to be given on a dry weight basis in order to ensure comparability of the results from different studies.

19. A known amount of dung will be placed into a large-scale laboratory mixer or electric cement mixer. Test and reference chemicals will be introduced in a known amount of water. If chemicals are not

water soluble, they will be introduced in a known amount of an organic volatile solvent (e.g. acetone or ethanol) and mixed thoroughly for ca 10 minutes. Control dung will be inoculated either with a known amount of solvent (solvent only control) or with an appropriate amount of water only (untreated control). Afterwards, the dung and the respective addition will be mixed thoroughly. Where a solvent carrier is used, the solvent must be allowed to fully evaporate for at least 4 hours before the test organisms are added.

Preparation of Test Vessels and Addition of Organisms

20. 100 g (fresh weight) of dung will be added to each test vessel. The egg or larval phase may be used as the starting point of the bioassay and should be obtained as documented in the species-specific culturing methods.

21. Harvested eggs/larvae should be divided into separate groups corresponding to the number of treatments prior to addition. For the time being, it is recommended to start the tests with *S. stercoraria* with eggs while the *M. autumnalis* tests should be started with larvae; mainly due to reasons of practicability. This ensures the transfer of organisms to a particular dung type does not result in any chemical cross-contamination. Allocation of eggs/larvae to treatment groups should be done progressively, in small batches, so as to further randomise larval distribution. Each group of eggs should be kept on moist filter paper in a closed container until ready for use in the bioassay.

22. Ten eggs for *S. stercoraria* or 10 larvae of *M. autumnalis* will be placed on the dung surface of each test vessel. If eggs are used they should be placed on a piece of moist filter paper on the dung surface to allow an assessment of egg hatch to be made.

23. After *M. autumnalis* larval addition, when the larvae are no longer visible, the dung surface will be covered with dry vermiculite to a depth of ca 3 cm. The vermiculite provides a suitable substrate in which pupation can occur. This addition is recommended in the case of tests with *M. autumnalis*, while it is not considered to be necessary for tests with *S. stercoraria*.

Observations

24. If eggs are used as the bioassay start point (i.e. in the test with *S. stercoraria*) the number of eggs that hatched successfully should be assessed at 48 hours after egg addition. During the period of adult emergence, the sex and number of emergent adults and subsequent survival will be recorded daily. Where the sex of recently emerged adults is uncertain flies should be left for 2 – 3 hours before re-sexing. Any visual morphological abnormalities (including body size, failures to emerge properly etc.) will also be recorded. Emergent flies should be removed daily. In tests with *S. stercoraria* emergence of adults starts about 18 days after the start of the test. In tests with *M. autumnalis* emergence of adults starts about 13 days after the start of the test. The test will be terminated five days after emergence of the last adult in the control.

Test design

25. Range Finding Test: If the toxicity of the test chemical is unknown, five nominal test concentrations of 0.1, 1.0, 10, 100, and 1000 mg/kg (dry weight of dung) should be conducted. If information about the toxicity is available, the test concentrations can be adapted accordingly. All test concentrations have also to be given on a dry weight basis. Appropriate solvent and or water control treated groups should also be included.

26. Limit or Definitive Test: If the range finding test indicates that the no-observed effect concentration (NOEC) of test chemical is greater than the tested concentrations (e.g. 1000 mg/kg dung d.w.), a limit test at an appropriate concentration (usually 1000 mg/kg dung d.w.) may be carried out instead of a definitive test. The limit test will be conducted with 8 test chemical vessels and 8 untreated vessels. A reference item and solvent only control if necessary will also be included (8 replicates each). This design was selected in accordance with OECD Guideline [24].

27. If effects of the test chemical are observed within the range tested in the range-finding study (corrected for control mortality using Abbott's (1925) formula [25]), a definitive test will be conducted. The definitive test will be conducted with four replicate vessels per test concentration or control group. There will be five evenly spaced test chemical concentrations (spacing factor of 1.8 to 3.2). A positive control and a solvent only control (plus untreated control if solvent is not water).

28. Positional bias will be eliminated by using a randomised complete block design for all studies carried out (range test, limit test or definitive test).

STATISTICAL EVALUATION

29. No definitive statistical guidance for analysing test results is given in this guideline. However, based on recent recommendations in other OECD guidelines (mainly the document on statistics [24] but also other recently published guidelines [26], in particular [27]) some proposals can be made. This Guideline makes provision for the determination of the NOEC and the ECx. A NOEC is likely to be required by regulatory authorities for the foreseeable future. More widespread use of the ECx, resulting from statistical and ecological considerations, may be adopted in the near future.

30. The numbers of emerged adults of each sex will be tabulated along with each concentration of test chemical. In addition, all other observations will be provided in a tabular format. As endpoints the number of emerged adult flies, the developmental rate per treatment and other observations compared to the control will be used.

TEST REPORT

31. On completion of the study a final report will be prepared. The report will incorporate (but not be limited to):

- Test chemical (name, common name, chemical name, Batch no., purity etc)
- Reference chemical (name, common name, Batch no., purity etc)

- Test species used (species, source of organism, breeding conditions)
- Handling of organisms
- Age of organisms when added to test vessels
- Source of dung and recent veterinary history of livestock used
- pH and moisture content of the dung (plus nitrogen and carbon when testing dung from treated cattle)
- Depth of dung in the test vessels
- Depth of vermiculite in the test vessels
- Test vessels (material, dimensions and size)
- Test concentrations and number of replicates
- Description of the preparation of test and reference chemical dosing solutions
- Environmental conditions (temperature, light cycle and intensity)
- Number of emerged male and female flies per vessel per day
- Percent emergence per replicate and treatment rate (male and female flies pooled)
- Morphological abnormalities (e.g. body size)
- Developmental time
- Hatching rate (in the tests started with eggs)
- Results of the tests with the reference substance
- Fulfilment of validity criteria
- Statistical methods used and results of statistical evaluations
- Results presented in tabular and/or graphical form
- Review/discussion of results obtained
- Conclusion reached

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ANNEX 1

DEFINITIONS

The following definitions are applicable to this Guideline:

NOEC (No Observed Effect Concentration) is the test substance 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.

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. In this test the effect concentrations are expressed as a mass of test substance per dry mass of the test soil or as a mass of the test substance per unit area of the soil.

ANNEX 2

DETERMINATION OF SOIL pH

The following method for determining the pH of a soil is based on the description given in ISO DIS 10390: Soil Quality – Determination of pH [18].

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 1.0 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).

ANNEX 3

REARING OF THE DUNG FLIES

Laboratory Culture Method for *Musca autumnalis* De Geer (Diptera: Muscidae)

This document describes the procedure used for rearing *M. autumnalis* at Inveresk Research.

1. Housing and Environmental Conditions

Cultures are housed in plastic chambers (*ca* 50 x 50 x 50 cm) with externally mounted heater boxes.

Environmental conditions are 30 $\pm$ 2 ^{o}C and > 60 % RH. All developmental timings reported in this method are based upon rearing at this temperature and must be reassessed if a lower temperature regime is implemented.

The lighting regime is 16:8 h light:dark. In addition to fluorescent lighting, an incandescent light source (such as a tungsten filament spotlight) may be provided to create an area in which the flies can bask.

2. Feeding

- Water is provided *ad lib* in each cage by inverting a water-filled beaker onto a tray lined with absorbent paper.
- Dried egg yolk powder, milk powder and sucrose (1:1:1 ratio) are provided *ad lib*.
- Honey solution soaked cotton wool (25 % honey solution w/v) is provided and replenished *ca* twice a week.
- Fresh pig's liver is provided weekly as an additional protein source for female flies (it is predominately the females that feed upon the protein-rich facial secretions of livestock). Strips of liver are hung from hooks on the walls of the cage.

3. Oviposition

- Dung is collected from cattle with a known veterinary history. The cattle are not treated with any pharmaceutical products for as least 8 weeks, or with an anthelminthic bolus for at least 5 months, prior to collection. Dung is frozen at *ca* –20^{o}C upon collection and stored at this temperature until the day before use. Dung is defrosted at room temperature for *ca* 24 hours before addition to the culture.
- In an unsynchronised culture, bovine dung is provided weekly. In a synchronised culture, bovine dung is provided when adult flies are between 7 and 10 days old, 3 to 4 days after copulation is first observed.

- The defrosted dung is homogenised using a laboratory mixer for ca 5-10 minutes before addition to the culture. The dung should be wet enough to be easily moulded into a *ca* 7 cm diameter ball, but dry enough that the ball will retain its shape. This ball is dropped onto a plastic tray to produce an artificial pat. The pat is then placed into the culture.

- Each batch of egged manure is transferred onto *ca* 1 kg bovine dung in a plastic bucket. If egg densities are high the eggs should be divided up to ensure that no more than *ca* 500 eggs are transferred to each 1 kg batch.

- 48 hours after oviposition, *ca* 3 cm of sawdust is added to the dung surface and a fine mesh or muslin cloth (nappy liner is ideal) secured over the top of the bucket. Larvae will migrate to the dung surface and into the sawdust to pupate.

4. Life Cycle and Developmental Timing

- Eggs are laid both singly and in clumps. Eggs are primarily deposited under the dung surface and only the terminal respiratory horn is apparent. Clumps of eggs can by removed from the dung and gently teased apart for experimental use.

- Eggs hatch occurs after *ca* 24-36 hours. If larvae are required they are removed from the dung at *ca* 48 hours after oviposition.

- There are three larval instars, third instar larvae are cylindrical yellowish white maggots which taper anteriorly, and are *ca* 12 mm long. Larvae usually 'wander' at *ca* 4 days after egg hatch, migrating to the dung surface and sawdust layer to pupate.

- Pupae can be removed from the sawdust at 6 days after oviposition and placed in the culture or be allowed to emerge from the dung naturally. Pupae are white/grey and 5 - 7 mm long.

- Adult eclosion occurs at 4 to 5 days after pupal formation. Therefore, egg to adult development takes 10 to 11 days at 30^{o}C. At 25^{o}C development takes approximately 17 days.

- Adults are 7 to 8 mm long. The females are easily distinguished from the males by the proximity of the eyes, the eyes of the males almost touch, whilst those of the females are distinct.

Source for *Scathophaga stercoraria* L. (Scathophagidae)

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