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OECD GUIDELINEFOR THE TESTING OF CHEMICALS

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

INTRODUCTION

1. This Test Guideline is designed to assess the effects of the test chemical on the development of dung dwelling life stages of dung-dependent dipteran species. In this test, insects are exposed under controlled conditions [1] to the test chemical spiked into the dung. An extended test, in which the flies are exposed to dung originating from livestock treated with the test substance, is described in ANNEX 4.

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]. By including a Musca-species the area which is covered by this guideline is extended: For example, the Australian species *M. vetustissima* could be tested in the same way as *M. autumnalis*, while *S. stercoraria* is not occurring there. Despite some overlap, the two species differ in their temperature preferences: *S. stercoraria* avoids temperatures >25° and has been found in Iceland and at high altitudes in the Alps while *M. autumnalis* prefers just the opposite.

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].

4. Due to the ecological role of the adults of the proposed test species (a predatory and a saprophagous fly from different families; larvae from both species are saprophagous) the relevance of the test results for the protection of the dung fauna and their function is secured. In addition, it is known that these flies are very sensitive indicators against chemical dung contamination, especially endo/ectoparasiticides [14]. First results from the ring test indicate that ivermectin is more toxic to *M. autumnalis* than to *S. stercoraria* [15] but it is not yet possible to decide whether there is a general difference in sensitivity. However, flies are not representative for the whole dung community. For

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example, due to their larger size and different feeding habits dung beetles are living in a different ecological niche than dung flies.

PRINCIPLE OF THE TEST

5. This study is designed to estimate the developmental toxicity of a test chemical to the dung dwelling life stages of dung-dependent dipteran species. The possible impact of the test chemical spiked in to the dung on the maturation of the flies to adults is compared to the negative control(s) (an extended test using dung from drug-treated livestock as test substrate is described in ANNEX 4). A positive control should be tested periodically (see §8). The test chemical is mixed with bovine faeces, to which eggs (*S. stercoraria*) or larvae (*M. autumnalis*) are added. Then the effects of the test chemical on the following measurement endpoints are assessed under controlled conditions after exposure of the eggs/larvae to the test substance (always in comparison to the control):

- Emergence, i.e. the sex and total number of emerged adult flies;
- Retardation of emergence, indicated by the developmental rate, i.e. the number of emerged flies per day after introduction into the dung
- Morphological change, i.e. any visual morphological abnormalities, including body size, failures to emerge properly etc.

Depending on the experimental design, the No Observed Effect Concentration (NOEC) or the ECx (Effect concentration for x% effect e.g. EC_{50}) can be determined.

INFORMATION ON THE TEST SUBSTANCE

6. The water solubility, the log K_{ow}, and the vapor pressure of the test substance should preferably be known to assist the test design. 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.

7. 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.

REFERENCE SUBSTANCE

8. Ivermectin (tech.) is a suitable reference substance that has been shown to affect fly emergence [9, 10, 11, 15]. The reference substance has to be tested regularly, but two options are possible:

- The ECx of a reference substance can be determined 1 - 2 times 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 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 20 and 60 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 solvent control (eight). Significant effects on adult emergence should be observed at concentrations 100 µg active ingredient (a.i.)/kg d.w. (*S. stercoraria*) and 40 µg active ingredient (a.i.)/kg d.w. (*M. autumnalis*) respectively.

The performance of a reference test is always required when a new batch of flies is tested for the first time, independently whether they were bought from an existing culture or whether they were collected in the field.

VALIDITY OF THE TEST

9. 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 ≥ 60% of the introduced larvae (*M. autumnalis*);
- Emergence of adult flies starts after 18 ± 2 days (*S. stercoraria*) or after 13 ± 2 days (*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 (e.g. the test results are usable for the selection of concentrations in a new test). The justification should be included in the report.

DESCRIPTION OF THE TEST

Equipment

10. 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.

11. Standard laboratory equipment is required, specifically the following:

- 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

12. Non-contaminated bovine dung will be obtained from cattle of documented veterinary history. The cattle should not be treated with any pharmaceutical products for as least 8 weeks, or with an anthelminthic bolus for at least 5 months, prior to collection. No contaminants should be expected in the dung that might interfere with the conduct of the study.

13. 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 –20oC for at least 1 week before use (preferably longer (e.g. four weeks), in order to avoid mite contamination). Since mite infection is very unlikely for dung collected directly from the intestinal tract freezing to get rid of mites is not necessary. Independently from the way the dung is collected it could be frozen if not needed immediately. 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).

Selection and preparation of test animals

14. 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 identity must 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 species confirmation, source and history of the organisms should 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

15. The rearing vessels for laboratory culturing of flies 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*, relative 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

16. Dung should be removed from the freezer in time to ensure that it is completely thawed before use. (directly collected dung could be used immediately; see §13). The dung should be homogenised for ca 10 minutes, for example in a large-scale laboratory mixer, prior to preparation of the separate treatment groups. No change of the moisture is usually required (experience has shown that a moisture content of 80% fw is suitable for the flies).

17. Moisture content and pH of a sample of dung from cattle which has not been treated with any pharmaceutical products for as least 8 weeks, or with an anthelminthic bolus for at least 5 months, prior to collection will be determined at the start of each test. 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. 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.

Application of Test Chemicals

18. All test concentrations must 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. Test and reference chemicals will be introduced in a known amount of water. If chemicals are poorly soluble in water, they will be introduced in a known amount (depending on the solubility of the test substance 1 – 10 mL/120 g dw of dung have been proved to be suitable) 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 at room temperature before the test organisms are added.

20. The concentrations of application must be confirmed by an appropriate analytical verification. For soluble substances, verification of all test concentrations can be confirmed by analysis of the highest test solution used for the test with documentation on subsequent dilution and use of calibrated application equipment (e.g., calibrated analytical glassware, calibration of sprayer application equipment).

Preparation of Test Vessels and Addition of Organisms

21. 100 g (fresh weight) of dung will be added to each test vessel, leading to a depth of dung in the vessels of 5 – 8 cm. The egg (*S. stercoraria*) or larvae (*M. autumnalis*) are used as the starting point of the bioassay and should be obtained as documented in the species-specific culturing methods.

22. 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. Harvested eggs/larvae should be divided into separate groups corresponding to the number of treatments prior to addition. 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.

23. 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 (validity criterion). Immediately after hatching the larvae of S. stercoraria will leave the piece of filter paper, thus being exposed to the dung.

24. 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 not considered to be necessary for tests with *S. stercoraria*.

Observations

25. 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 repeated assessment of the sex. Any visual morphological abnormalities (including body size, failures to emerge properly etc.) will also be recorded. Emergent (including dead) 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. Adults are then humanely euthanized, preferably by rapid freezing at -80°C or cryopreservation.

Test design

26. 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) plus an untreated control and a solvent control (if solvent is not water) should be conducted. If information about the toxicity is available, the test concentrations can be adapted accordingly (see §28). All test concentrations have also to be given on a dry weight basis.

27. Limit or Definitive Test: If the range finding test indicates that the no-observed effect concentration (NOEC) of the 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 eight test chemical vessels and eight untreated vessels. A reference substance and a solvent control (if solvent is not water) will also be included (eight replicates each). This design was selected in accordance with OECD Guidance Document No. 54 [24].

28. 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. It can be performed following either a NOEC or an ECx approach:

- For determination of the NOEC, at least five concentrations in a geometric series should be tested. Four replicates for each test concentration treatment plus eight controls are recommended. The concentrations should be spaced by a factor not exceeding 1.8.
- 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 two in the expected effect range at low concentrations and

more than two at higher and lower concentrations.

Besides an untreated control and a solvent control (if solvent is not water) a reference substance (not always, see §8) are tested.

29. 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

30. No definitive statistical guidance for analysing test results is given in this guideline. However, based on recent recommendations in other OECD guidelines (mainly the Guidance Document on statistics [24] but also other recently published guidelines [26], in particular [27]) some proposals can be made. This Guideline primarily focuses on the determination of the ECx. According to the recent VICH guideline [28] the EC50 is required by many regulatory authorities (e.g. in the European Union), mainly resulting from statistical and ecological considerations. However, for reasons of flexibility guidance is also given for the determination of the NOEC [26, 27].

31. 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 morphological changes, always compared to the control, will be used. The developmental rate is determined using the method already described in the Sediment-Water Chironomid Toxicity Test Using Spiked Sediment [27].

TEST REPORT

32. On completion of the study a final report will be prepared. The report must include the following information (but not be limited to):

Test substance:
- Test chemical (name, common name, chemical name, Batch no., purity etc)
- Reference chemical (name, common name, Batch no., purity etc)
- properties of the test substance (e.g. log Kow, water solubility, vapour pressure and information on fate and behaviour), if possible

Test species:
- Test species used (confirmation of species, source of organism, breeding conditions)
- Handling of organisms
- Age of organisms when added to test vessels

Test conditions:
- Source of dung and recent veterinary history of livestock used
- pH and moisture content of the dung
- Depth of dung in the test vessels
- Depth of vermiculite in the test vessels (only for *M. autumnalis)*
- 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, humidity)

Test results:

- 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) per replicate
- Developmental rate per replicate
- Hatching rate (in the tests started with eggs) per replicate
- Results of the tests with the reference substance
- Results presented in tabular and/or graphical form
- Estimates of toxic endpoints (e.g. ECx, NOEC), and the statistical methods used for their determination

Evaluation of the test results:

- Fulfilment of validity criteria
- 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 highest 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 of 50% on a test endpoint in 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 dung.

ANNEX 2

DETERMINATION OF DUNG pH

The following method for determining the pH of a dung sample is based on the description given in ISO ~~DIS~~ 10390: Soil Quality – Determination of pH [18]. A defined quantity of dung is dried at room temperature for at least 12 h. A suspension of the dung (containing at least 5 grams of dung) 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).

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^{o}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 ratio 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]. Accordingly, the carbon content in dung should be determined by using modified ISO guidelines [e.g. 31].

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 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 *Musca autumnalis* De Geer (Diptera: Muscidae)

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Laboratory Culture Method for *Scathophaga stercoraria* L. (Diptera: Scathophagidae)

This document describes the procedure used for rearing *S. stercoraria* as provided by the University of Zurich (Prof. Dr. W. Blanckenhorn), partly supplemented by literature information [e.g. 29].

1. Housing and Environmental Conditions

Cultures are housed in mesh cages (e.g. 0.09 m^2), including a water source, a honey pad and a dish of commercially available pollen. This size is suitable for 25 pairs of *S. stercoraria*.

Environmental conditions are 20 $\pm$ 2 ^oC and 60 ± 5 % 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.

2. Feeding

- Water is provided *ad lib* in each cage by inverting a water-filled beaker onto a tray lined with absorbent paper.

- Dishes with 7 g of *Musca domestica* puparia per cage (coming from another laboratory culture), are added twice weekly to ensure an adequate supply of prey.

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^oC 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. The defrosted dung is homogenised using a laboratory mixer for ca 10 minutes before using in the culture.

- Once the flies (*S. stercoraria*) reach 13 days of age, a 15 cm Petri dish containing fresh cow dung is added with the addition of prey.

- This oviposition medium is removed after 24 hours and is added to an additional 1500 – 2000 g of cow dung mounded on a sand substrate in an open plastic tub.

- Pupation is completed within ten days at which time the puparia are removed by flotation from the sand.

Source for *Scathophaga stercoraria* L. (Scathophagidae)

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ANNEX 4

TESTING OF DUNG COLLECTED FROM LIVESTOCK TREATED WITH VETERINARY PHARMACEUTICALS

In contrast to use dung spiked with a test substance the two fly species can also be exposed to dung which was collected from livestock (usually cattle) treated with the test substance. This test design is considered to be more realistically since it includes all metabolism occurring during the passage of the drug through the body of the treated animal. In addition, the exposure situation reflects the real availability of the test substance in the dung which may differ from the one reached after spiking and homogenisation. For these reasons, such an extended laboratory test may be required at higher tiers when assessing the potential risk of veterinary pharmaceuticals for dung organisms. However, it has to be remembered that even when the keeping of the livestock (e.g. the food) is as similar as possible, the results may show a higher variability than in tests with spiked dung due to differences in the individual metabolism of the animals.

Basically the test is performed as described in the main body of this guideline. Therefore, in the following only those issues which need to be modified are listed (for example, no changes are necessary concerning reference testing, validity criteria or the culturing of the two test species). In addition to those changes given below information on the equipment used to treat livestock with the test substance (depending on the formulation used, e.g. a syringe), and a description of the treated animals (e.g. race, age, weight of cattle; husbandry, including feeding; how often and in which frequency the livestock was treated) is needed.

INFORMATION ON THE TEST SUBSTANCE:

Par. 5,6 In addition to the physico-chemical properties of the test substance, the formulation used in the test has to be described.

DESCRIPTION OF THE TEST

Par. 20 Since it is not detail foreseeable how much of the test substance will appear in the dung it is necessary to analyse the dung for the test substance and its main metabolites. Residue analysis has to be performed as long as test substance is appearing in the faeces of the treated livestock.

Par. 21 Dung from treated cattle is collected at different dates after treatment, depending on the excretion profile of the test substance (e.g. for a pour-on formulation containing ivermectin used on cattle, samples were taken up to 12 days after treatment [30]). Dung samples from one animal and from the same day are combined and mixed in order to get a homogenized batch. From each batch, 100 g (f.w.) are taken for each replicate (= vessel).

Par. 26 Depending on the aim of the study, the same test designs could be used as for the tests with spiked dung, since each dung sample from treated livestock contains a different concentration of test substance depending on the excretion profile. Therefore, both limit tests (just one sampling date) or dose-response designs (ECx, NOEC) are possible. For the same reasons, there is also no difference concerning statistical assessment.

Par. 32 In the test report, information referring to the test modifications described in annex 4 should be presented.