OECD/OCDE **237**

Adopted:
26 July 2013

OECD GUIDELINES FOR THE TESTING OF CHEMICALS

Honey bee (*Apis mellifera*) larval toxicity test, single exposure

INTRODUCTION

1. This Test Guideline (TG) 237 describes a honey bee brood acute toxicity test under laboratory conditions. It is based on a method developed in France (1)(2)(3) that has been ring-tested from 2005 to 2008 in seven European laboratories (4).

2. This Test Guideline addresses the requirements formulated by the United States, Canada, and Europe (5)(6)(7) to test the toxicity of chemicals on larvae fed with spiked food under laboratory conditions in a tier 1 strategy.

3. The method aims at the determination of the lethal dose seventy-two hours (72-h LD_{50}) following single exposure of larvae to a chemical (particularly pesticide active ingredient or formulation). The data should be used in an appropriate honeybee brood risk assessment scheme. This Test Guideline on honey bee larvae complements OECD TG 213 (8) and TG 214 (9) on young adult honey bees and should be seen as a lower tier test in the context of an overall risk assessment scheme for bees (4).

PRINCIPLE OF THE TEST

4. On day 1 (D1) of the study, first instar (L1) synchronised larvae (i.e. larvae of the same age) are taken from the comb of three colonies and individually placed into 48 well-plates where they are fed a standardized amount of artificial diet. On day 4 (D4) of the test, a single dose of the test chemical is administered to the larvae with the diet in a range of five increasing concentrations. Mortalities are recorded on D5, D6, and D7 of the test. The 72-hr LD_{50} is calculated for larvae (cumulative mortality at D7).

INFORMATION ON THE TEST CHEMICAL

5. The water solubility, solubility in solvent, and the vapour pressure of the test chemical should be known. Useful information on the test chemical including structural formula, purity, stability in water and light, octanol-water partition coefficient (K_{ow}) should be reported. The physical appearance and source (batch, lot number) of the test chemical should be described. Guidance for testing substances with physical-chemical properties that make them difficult to test is provided in OECD Guidance Document No. 23 (10).

REFERENCE CHEMICAL

6. The toxic reference chemical is technical grade dimethoate (CAS RN. 60-51-5). The reference chemical is tested as a means to ensure that the test system and conditions are responsive and reliable. A dose of 8.8±0.5 µg active ingredient (a.i.)/larva, dissolved in maximum 3 µL of water, is mixed with the diet just prior to administration to the larvae and provided on D4 (2)(3).

VALIDITY OF THE TEST

7. For the test to be valid, the following criteria apply:

- In the control plate(s), cumulative larval mortality from D4 to D7 should be $\leqq$ 15% across replicates;
- In the reference chemical treatment, larval mortality (after adjustment, see paragraph 33) should be $\geqq$ 50% at D7.

DESCRIPTION OF THE TEST

Apparatus

8. Larvae are reared in crystal polystyrene grafting cells (*e.g.*, ref CNE/3, NICOTPLAST Society) having an internal diameter of 9 mm and a depth of 8 mm. The cells are initially sterilised *e.g.* by immerging for 30 min in ethanol or other sterilising solution, and then dried in a laminar-flow hood. Each cell is placed into a well of a 48-well plate. The top of the grafting cell may be maintained at the level of the plate, *e.g.* by placing a piece of dental roll wetted with 500 µL of the sterilising solution enhanced with 15% weight/volume glycerol at the bottom of the wells (Figure 1).

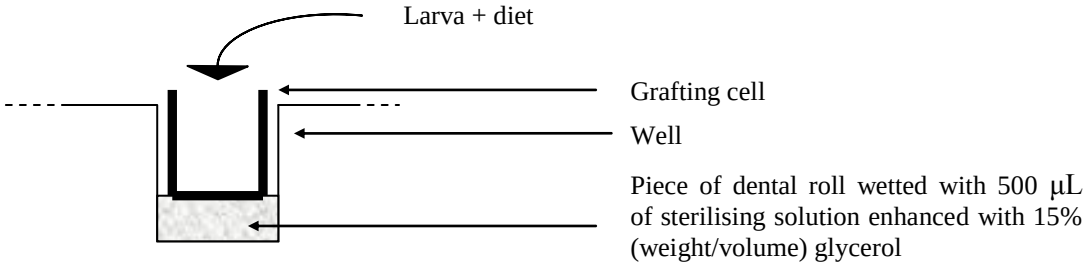

Figure 1: Larval cell in a tissue culture well.

9. These plates are placed into a hermetic Plexiglas desiccator (e.g. NALGENE 5314-0120 or 5317-0180 according to the volume required) with a dish filled with potassium sulphate (K_2SO_4) saturated solution in order to keep a water saturated atmosphere. The desiccator is placed into an incubator equipped with a forced air circulation system at 34 to 35°C to homogenise temperature around the desiccator, and as close as possible within that range for the duration of the test.

Test organisms

Origin of the larvae

10. Larvae are collected from three different colonies, each representing a replicate (see paragraph 19). Colonies should be adequately fed, healthy (*i.e.*, as far as possible disease- and parasite-free), with known history and physiological status.

11. Tests are conducted during the egg laying period of the queen. In case of sanitary treatment (*e.g.* mite or disease treatment measures), the date of treatment application to the colony and the product identity are reported. No treatment is allowed within the four weeks preceding the start of the test.

12. At D-3 (Figure 3), in order to ensure the production of larvae from three colonies, the queens of a minimum of three colonies are confined in their own colony in an exclusion cage containing an empty comb or a comb with emerging worker brood and empty cells (Figure 2). The exclusion cage is placed close to combs containing brood. At D-2, maximum 30 hours after encaging, the queen is released from the cage, after checking the presence of fresh laid eggs. Depending on the fertility of the queen, it is recommended reducing the isolation time in order to minimise the variability in size and age between larvae. The comb containing the eggs is left in the cage, near the brood, during the incubation stage and until hatching (D1).

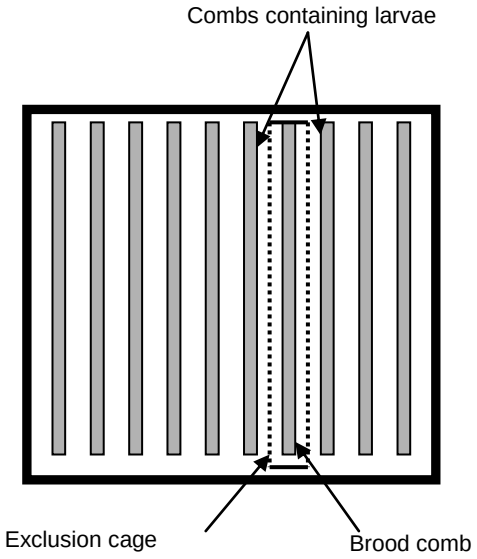

Figure 2: Cross section of a hive with the exclusion cage

Preparation of rearing material

Larval food

13. The food is composed of the three following diets, adapted to the needs of the larvae at different stages of development:

- Diet A (D1): 50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 2% weight of yeast extract, 12% weight of glucose and 12% weight of fructose.

- Diet B (D3): 50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 3% weight of yeast extract, 15% weight of glucose and 15% weight of fructose.

- Diet C (from D4 to D6): 50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 4% weight of yeast extract, 18% weight of glucose and 18% weight of fructose.

14. If some aggregates remain in sugar solutions they are completely dissolved before mixing with the royal jelly. A "fresh royal jelly" is a royal jelly collected during the preceding 12 months; it may be divided into 5 g aliquots in order to avoid unfreezing the whole batch at each test, and stored in a freezer at ≦-10°C. Commercial sources of royal jelly might be acceptable if it can be shown that their performance compares to historical data within the testing facility, e.g. mortality does not exceed 15% during the larval period. It is recommended that a multi-residues analysis of each royal jelly batch be conducted in order to verify the absence of contaminants (mainly antibiotics and insecticides).

15. The diets, freshly prepared prior to each test, are stored in a fridge at ≦ +5°C (but not frozen) during the whole duration of the test. They may be prepared in advance and subsequently stored deep-frozen until use.

Test solutions

16. The test chemical is normally dissolved in osmosed water. For poorly soluble chemicals, a solvent may be used, preferably acetone, to prepare the stock solution. In such case, a solvent-control with diet added with the same volume of solvent is tested in addition to the regular diet-control. The volume of organic solvent, if used, should be kept as low as possible, and in the case of acetone, not exceed 5% of the final diet volume on D4 (exposure day).

17. Dilutions of the stock solutions into the series of five test solutions are made preferably with osmosed water or solvent for poorly soluble substances, preferably just before administration to the larvae, using disposable pipette tips equipped with a filter. The volume of the test solution in the diet should not exceed 10% of the final diet volume if water is used to dissolve the test chemical (*e.g.* 3 µL of test solution for a diet volume of 30 µL on D4) or 5% if acetone is required (*e.g.* 1.5 µL of test solution for a diet volume of 30 µL on D4).

18. A sample of the stock solution will be stored in a freezer at ≦-10°C in order to be checked for analytical determination of the concentration of the test chemical.

PROCEDURE

Conditions of exposure

19. The experimental unit is the individual cell containing a larva. A minimum of twelve larvae from each of three colonies are allocated on the same plate to each treatment level and to the control(s) and reference chemical. For each test, the following treatments and control(s) are used:

- control without solvent (minimum 12 larvae × 3 colonies=36 larvae minimum)

- control with solvent if necessary (minimum 12 larvae × 3 colonies=36 larvae minimum)

- five treatments, i.e. 5 increasing test concentrations (each containing a minimum of 12 larvae × 3 colonies = 36 larvae minimum per treatment) in a geometric series, spaced by a factor not exceeding 3, and covering the LD_{50}; alternatively, when a limit test is performed (see

paragraph 23), a single dose of 100 µg a.i. (or test chemical)/larva or the maximum achievable solubility, whichever is lower, may be tested;

– Reference chemical, dimethoate 8.8 µg/larva (minimum 12 larvae × 3 colonies = 36 larvae minimum)

20. A total of seven to eight (if solvent is used) well-plates are used per test. Each group of a minimum of 12 larvae from each of the three colonies is considered a replicate for a given treatment level and identified as such on the microplate.

21. The plates are kept under dark conditions for the duration of the test. During the test, the temperature in the incubator is kept between 34 and 35°C. However, deviations are allowed, but temperature should not be lower than 23°C or higher than 40°C, and these deviations should not last more than 15 minutes once every 24 hours.

Range-finding test

22. In order to assess the adequate LD_{50} range, it is recommended that a preliminary experiment be run with doses of the test chemical varying according to a geometrical ratio from 5 to 10.

Limit test

23. In some cases (*e.g.* when a test chemical is expected to be of low toxicity or when a chemical is poorly soluble) a limit test may be performed, using 100 µg a.i. (or test chemical)/larva or the maximum achievable solubility for poorly soluble chemicals, whichever is lower, in order to demonstrate that the LD_{50} is greater than this value. Three replicates of a minimum of twelve larvae from three different colonies are used for the limit dose tested, as well as the relevant control(s), and the use of the reference chemical. If statistically significant mortalities occur compared to the control mortality, a full study should be conducted.

Collection of larvae

24. At D1, the comb containing first instars larvae (Figure 3) is carried from the hive to the laboratory in an insulated container in order to avoid temperature variation and then maintained at ambient temperature (not below 20°C). It is then introduced into a laminar-flow hood or under other clean conditions for grafting. In order to avoid bias due to possible heterogeneity of the larvae, it is highly recommended selecting newly hatched larvae that have not yet formed a "C" shape, and randomizing the allocation of larvae into the plates for each colony. A minimum of twelve larvae from each of the three replicate colonies is needed at D4 on the day of administration of the chemical treatment; therefore the test may be initiated on D1 with larvae in excess of that number from each colony.

25. Alternatively this randomization of larvae may be done at D4, just before administration of the chemical treatment.

Grafting and feeding of larvae

26. The diet is warmed in the incubator before use. The grafting is performed preferably on a warming plate maintained at 34 to 35°C, and in any case not beyond 35°C. The micropipettes used to provide the diet into the cells are equipped with disposable tips. On day 1 (D1), 20 µl of diet A is placed into each cell, and one larva is delicately collected from the comb and transferred to each cell, on the surface of the diet, using a grafting tool or a wetted paintbrush (*e.g.* No 3/0). When a plate is filled with a

minimum of 12 larvae from each colony, it is placed in a single layer into the hermetic container, which has previously been placed into a ventilated incubator at 34 to 35°C (see picture 1), and as close as possible within that range for the duration of the test.

Picture 1 : larvae incubation device

27. All larvae are fed once a day (except at D2), preferably on a warming plate that should not be warmed above 35°C, with a transparent sterilised pipette tip following the schedule of Figure 3, in particular the volume of diet provided to individual larva is adjusted on a daily basis. Care should be taken to avoid touching and drowning the larvae when feeding them. Food is placed next to the larva, along the wall of the grafting cell. Additional food should be added to the cell even if the previous allocation has not been totally consumed. The presence of uneaten food at termination of the test should be reported.

Single administration of the chemical in the test solution

28. On D4, a minimum of twelve well-fed larvae from each of the three colonies are selected and treated with 30 µL of the diet C containing the test solution at the suitable concentration. The mixing of the test solution with the diet is performed just before administration on D4, unless the stability of the test chemical in the diet has been demonstrated and is reported. On D4, each treatment (containing the diet) is administered with a different micropipette tip to avoid contamination.

Termination of the test

29. At D7, mortalities are counted and the test is terminated by freezing the plates at $\leqq$ -10°C.

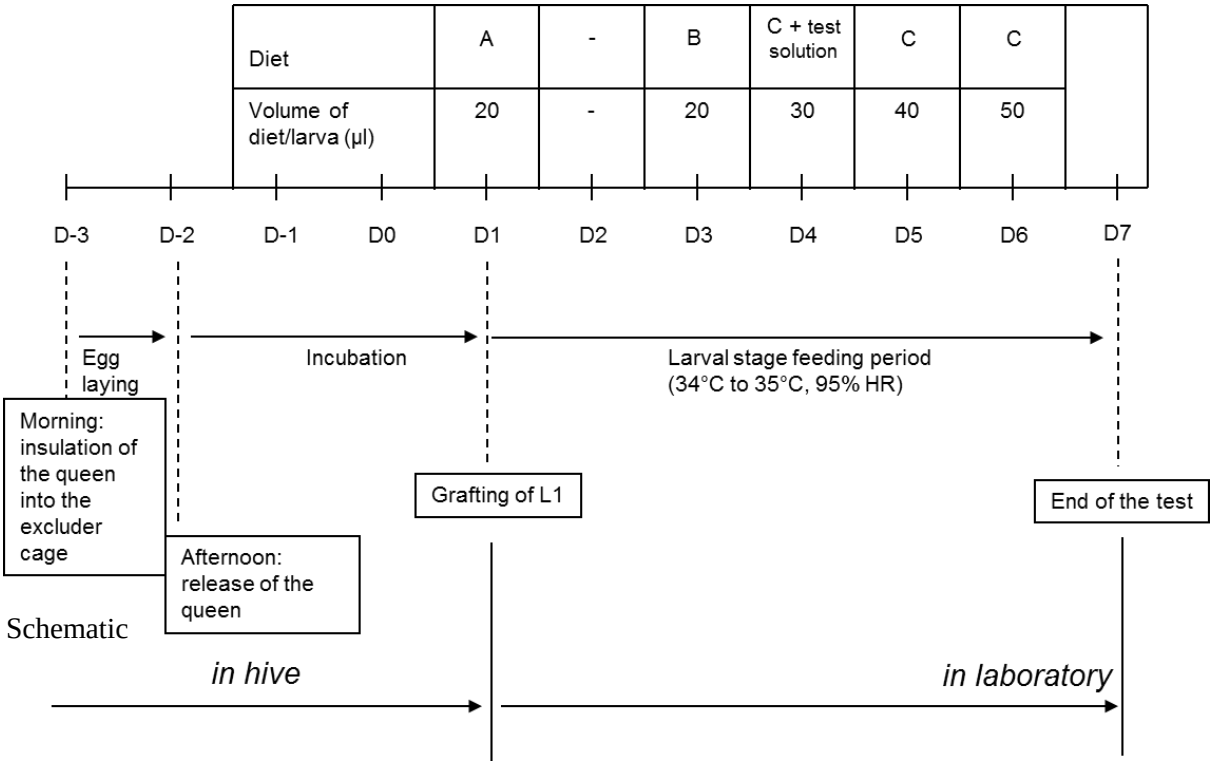

Figure 3: Schematic representation of the important steps of the larval toxicity test (D=day)

Observations

30. Following the chemical exposure on D4, mortalities are checked and recorded at the time of feeding on D5 and D6 and at termination of the test on D7. An immobile larva or a larva which does not react to the contact of the grafting tool or paintbrush is noted as dead.

31. At the feeding time, dead larvae are systematically removed for sanitary reasons.

32. Other observations should be recorded to aid in the interpretation of mortality. The presence of uneaten food on D7 should be (qualitatively) reported.

DATA AND REPORTING

Data and statistical analysis

LD_{50} calculation

33. Mortality is expressed in percentage of the initial population after an adjustment according to the Abbott formula (11):

$$M = \frac{(P-T)}{S} \times 100$$ raw mortality

$$M = \frac{(\%P - \%T)}{100 - \%T} \times 100 \quad \text{percent mortality}$$

M: adjusted mortality expressed in percent of the initial population, i.e. initial number of larvae

P: number of dead larvae in the treated group

T: number of dead larvae in the control group

S: number of surviving larvae in the control group

%P: mortality percentage due to the treatment

%T: control mortality percentage

34. Data is summarised in tabular form, showing for each treatment group, as well as controls and reference chemical groups, the number of larvae used, mortalities at D5, D6 and D7 (*i.e.* 24 h, 48 h and 72 h after administration of chemical treatment, respectively). Mortality data are analysed by appropriate statistical methods (e.g. probit analysis, moving-average, binomial probability) (8)(9). The dose-response curves are plotted at each recommended observation time (*i.e.* 24h, 48h and, 72h after administration of chemical) and the slopes of the curves and the median lethal doses (LD_{50}) are calculated with 95% confidence limits. LD_{50} should be expressed in μg of test chemical per larva.

Test report

35. The test report should include the following:

Test chemical:

- physical nature and relevant physical-chemical properties;
- chemical identification data, including purity.

Test species:

- source, species and sub-species of honeybee, supplier of source (if known) and the culture conditions used;
- health condition of the hive used in the test.

Test conditions:

- place and date of the test;
- description of the test system: type of well-plates used, number of larvae per treatment level and controls, solvent and concentrations used (if any), test concentrations used for the test chemical;
- incubation conditions: temperature (mean, standard deviation, minimum and maximum values) and relative humidity.

Results:

- the number and percentage of larvae considered dead at each treatment level, control(s) and toxic reference chemical (dimethoate);

- nominal test concentrations used and measured concentration in the stock solution. The measured concentration should be within 20% of nominal;

- the mortality at D5, D6 and D7 and the overall 72 h-LD_{50} at D7 with a 95% confidence interval and a graph of the fitted model, the slope of the concentration-response curve and its standard error; statistical/mathematical procedures used for the determination of the LD_{50};

- other observations, including the presence of uneaten food at test termination.

Any deviation from the Test Guideline and relevant explanations.

LITERATURE

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