INTRODUCTION

1. This Test Guideline (TG) 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 meets the requirements formulated by the United States, Canada, Europe (5, 6, 7) to test new products on larvae fed with spiked food under laboratory conditions in a tier 1 strategy.

3. The method enables determination of the lethal dose (72-h LD₅₀) following single exposure of larvae to a chemical (particularly pesticide active ingredient or formulation). These data should be used in an appropriate honeybee brood risk assessment scheme. The Test Guideline complement OECD TG 213 (Honeybee, Acute oral Toxicity test) and TG 214 (Honeybee, Acute Contact Toxicity test) 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. Synchronised larvae at the stage L1 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. The LD₅₀ 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 substance should be known. Useful information on the test substance including structural formula, purity, stability in water and light, octanol-water partition coefficient (Kow) should be reported. The physical appearance 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.

REFERENCE CHEMICAL

6. The toxic reference chemical is dimethoate (CAS RN. 60-51-5). The toxic reference is tested as a means to ensure that the test system and conditions are responsive and reliable. A dose of 8.8 microg a.i./larva, dissolved in 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 \( \leq 15\% \) across replicates;
   
   - In the toxic reference chemical treatment, larval mortality (after correction) should be \( \geq 50\% \) at D7.

DESCRIPTION OF THE TEST

Apparatus

8. Larvae are reared in crystal polystyrene grafting cells (ref CNE/3, NICOPLAST Society) having an internal diameter of 9 mm. The cells are firstly sterilised e.g. by immersing for 30 min in a solution of a product used for baby bottle cold sterilization (e.g. a solution of 25 ml of Milton sterilising fluid in 2.5 l of water) or other hypertonic saline/hypochlorite solution, and then dried in a laminar-flow hood. Each cell is placed into a well of a 48-well tissue culture plate, which is previously half filled with a piece of dental roll wetted with 500 microl of the same solution (Figure 1), to maintain the top of the grafting cell at the level of the plate.

![Figure 1: Larval cell in a tissue culture well.](image)

Test organisms

Origin of the larvae

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 K2SO4 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, and as close as possible within that range for the duration of the test.

10. Larvae are collected from three different colonies, each representing a replicate (see paragraph 22). Colonies should be adequately fed, healthy, as far as possible disease-free, with known history and physiological status.

11. Tests are realised with summer larvae, i.e. from eggs laid in a period starting mid-spring to end of summer. In case of sanitary treatment, the date of application to the colony and the product identity are provided. No treatment is allowed within the 4 weeks preceding the start of the test.

12. At D-3 (Figure 2), in order to ensure the production of larvae from three colonies, the queens of minimum three colonies are confined in their own colony in an excluder cage containing a comb with emerging worker brood and empty cells (Figure 2). The excluder cage is placed close to combs containing larvae. 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 to reduce isolation time in order to minimise 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).

Collection of larvae

13. At D1, the comb containing first instars larvae (Figure 2) is carried from the hive to the laboratory in an insulated container in order to avoid temperature variation and then maintained at ambient temperature. 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 to select newly hatched larvae that have not yet formed a “C” shape, and to randomize 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.

14. This randomization may be done at D4, just before administration of the chemical treatment.

Preparation of rearing material

Larval food

15. 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.

16. If some aggregates remain in sugar solutions they are crushed before mixing with the royal jelly. A “fresh royal jelly” is a royal jelly collected during the preceding 12 months; it is divided into
5g aliquots, and stored in a freezer at \( \leq -10^\circ C \). 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 to conduct a multi-residues analysis of each royal jelly batch in order to verify the absence of contaminant (mainly antibiotics and insecticides).

17. The diets are prepared freshly prior to each test and stored in a fridge at \( \leq +5^\circ C \) (but not frozen) during the whole duration of the test.

18. The micropipettes used to provide the diet into the cells are equipped with disposable tips. The larvae are collected with appropriate grafting tools or a thin paintbrush (e.g. N° 3/0).

**Test solutions**

19. The test chemical is normally dispersed in water. For poorly soluble chemicals, a solvent (e.g. acetone maximum 5% of the diet volume, or as low as possible) may be used to prepare the stock solution. In such case, a solvent-control is tested in addition to the regular diet-control.

20. Dilutions of the stock solutions are made preferably with osmosed water, just before administration to the larvae, using disposable pipette tips equipped with a filter. The rate of the tested solution in the diet does not exceed 10% of the final volume, which remains constant.

**PROCEDURE**

**Conditions of exposure**

21. 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 toxic reference substance. For each test, the following treatments and control(s) are used:

   - control without solvent (minimum 12 larvae X 3 colonies=36 larvae)
   - control with solvent if necessary (36 larvae minimum)
   - 5 treatments, i.e. 5 increasing concentrations to test (36 larvae minimum) in a geometric series, with a factor not increasing 3, and covering the LD\( _{50} \); alternatively, when a limit test is performed (see paragraph 24), a single dose of 100 microg a.i./larva or the maximum achievable solubility, whichever is higher, may be tested;
   - Toxic reference substance, dimethoate 8.8 microg/larva (36 larvae minimum)

22. A total of seven to eight (if solvent is used) well-plates are used per test. Each group 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.

**Range-finding test**

23. In order to assess the adequate LD\( _{50} \) range, it is recommended to run a preliminary experiment where doses of the test chemical vary according to a geometrical ratio from 5 to 10.

**Limit test**

24. In some cases (e.g. when a test substance is expected to be of low toxicity or when a chemical is poorly soluble) a limit test may be performed, using 100 microg a.i./larva or the maximum achievable solubility, whichever is higher, in order to demonstrate that the LD\( _{50} \) is greater than this value. Five replicates 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, a full study should be conducted.
**Grafting and feeding of larvae**

25. The diet is warmed in the incubator before use. 20µl of diet A is dropped into each cell, and one larva is delicately transferred from the comb to each cell, on the surface of the diet, using a grafting tool or a wetted paintbrush. When a plate is completed with a minimum of 12 larvae from each colony, it is placed into the hermetic container, and then into a ventilated incubator at 34 to 35°C, and as close as possible within that range for the duration of the test.

26. All larvae are fed once a day (except at D2), on a warming plate, with a sterilised pipette tip following the schedule of Figure 3. Care should be taken to avoid touching the larvae when feeding them. Food is dropped next to the larva, along the wall of the grafting cell. The presence of uneaten food at termination of the test should be reported.

**Single administration of the chemical in the test solution**

27. On D4, a minimum of twelve well-fed larvae are selected and treated with the diet C containing the test solution at the suitable dose. The mixing of the test solution with the diet is performed just before administration on D4.

<table>
<thead>
<tr>
<th>Diet</th>
<th>A</th>
<th>-</th>
<th>B</th>
<th>C + test solution</th>
<th>C</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume of diet/larva (µl)</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
</tr>
</tbody>
</table>

Figure 3: Steps of an *in vitro* test.
Termination of the test

28. At D7, mortalities are counted and the test is terminated by freezing the plates at $\leq -10^\circ\text{C}$.

Observations

29. 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.

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

31. Other observations and abnormal behaviour 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. Mortalities are expressed in percentage of the reference populations after an adjustment according to the Abbott formula (8):

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

raw mortalities

$$M = \frac{(%P - %T)}{100 - %T} \times 100$$

percent mortalities

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

P: mortality due to the treatment

T: control mortality

S: surviving number in control

%P: mortality percentage due to the treatment

%T: control mortality percentage

34. Data are 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. 24h, 48h and 72h after administration of chemical treatment, respectively). Mortality data are analysed by appropriate statistical methods (e.g. probit analysis, moving-average, binomial probability) (8)(9). Plot dose-response curves at each recommended observation time (i.e. 24h, 48h and, 72h after administration of chemical) and calculate the slopes of the curves and the median lethal doses (LD$_{50}$) with 95% confidence limits. LD$_{50}$ should be expressed in $\mu$g of test substance per larva.
The test report should include the following:

Test substance:
- 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.

Test conditions:
- 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
- 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;
- explanation for any deviation from the Test Guideline and whether these deviations affected the results.

LITERATURE


