

## **OECD GUIDELINES FOR THE TESTING OF CHEMICALS**

### **Honeybees, Acute Contact Toxicity Test**

#### **INTRODUCTION**

1. This test guideline is a laboratory test method, designed to assess the acute contact toxicity of pesticides and other chemicals to adult worker honeybees. It is based principally on the European and Mediterranean Plant Protection Organization (EPPO) Guideline for evaluating side effects of plant protection products on honeybees (*Apis mellifera*) (1). The suggestions for improvements of the EPPO test, made by the International Commission for Plant-Bee Relationships (ICPBR) at its fifth International Symposium on the Hazards of Pesticides to Bees in Wageningen (the Netherlands) in 1993 have also been taken into account (2). Other existing guidelines have also been considered (3)(4)(5).

#### **INITIAL CONSIDERATIONS**

2. In the assessment and evaluation of toxic characteristics of substances, determination of acute contact toxicity in honeybees may be required, e.g. when exposure of bees to a given chemical is likely. The acute contact toxicity test is carried out to determine the inherent toxicity of pesticides and other chemicals to bees. The results of this test should be used to define the need for further evaluation. In particular, this method can be used in step-wise programmes for evaluating the hazards of pesticides to bees, based on sequential progression from laboratory toxicity tests to semi-field and field experiments (6). Pesticides can be tested as active ingredients (a.i.) or as formulated products.

3. A toxic standard should be used to verify the sensitivity of the bees and the precision of the test procedure.

4. Definitions used are given in the Annex.

#### **PRINCIPLE OF THE TEST**

5. Adult worker honeybees are exposed to a range of doses of the test substance dissolved in appropriate carrier, by direct application to the thorax (droplets). The test duration is 48h. If the mortality rate is increasing between 24 and 48h whilst control mortality remains at an accepted level, i.e.  $\leq 10\%$ , it is appropriate to extend the duration of the test to a maximum of 96h. Mortality is recorded daily and compared with control values. The results are analysed in order to calculate the  $LD_{50}$  at 24 and 48h (see Annex for definitions), and in case the study is prolonged at 72h and 96h.

### **VALIDITY OF THE TEST**

6. For a test to be valid the following conditions apply:
- the average mortality for the total number of controls must not exceed 10 per cent at the end of the test;
  - the LD<sub>50</sub> of the toxic standard meets the specified range.

### **DESCRIPTION OF THE METHOD**

#### **Collection of bees**

7. Young, adult worker bees of the same race should be used, i.e. bees of similar age, feeding status etc. Bees should be obtained from adequately fed, healthy, as far as possible disease-free and queen-right colonies with known history and physiological status. They could be collected in the morning of use or in the evening before test and kept under test conditions to the next day. Bees collected from frames without brood are suitable. Collection in early spring or late autumn should be avoided, as the bees have a changed physiology during this time. If tests have to be conducted in early spring or late autumn, bees can be emerged in an incubator and reared for one week with “bee bread” (pollen collected from the comb) and sucrose solution. Bees treated with chemical substances, such as antibiotics, anti-varroa, etc., should not be used for toxicity test for four weeks from the time of the end of the last treatment.

#### **Test cages**

8. Easy to clean and well-ventilated cages are used. Any appropriate material can be used, e.g. stainless steel, wire mesh, plastic, disposable wooden cages, etc. Groups of ten bees per cage are preferred. The size of test cages should be appropriate to the number of bees, i.e. providing adequate space.

#### **Handling and feeding conditions**

9. Handling procedures, including treatment and observations may be conducted under (day) light. Sucrose solution in water with a final concentration of 500 g/l (50% w/v) should be used as food and provided *ad libitum* during the test time, using a bee feeder. This can be a glass tube (ca 50 mm long and 10 mm wide with the open end narrowed to about 2 mm diameter).

#### **Preparations of bees**

10. The collected bees may be anaesthetized with carbon dioxide or nitrogen for application of the test substance. The amount of anaesthetic used and times of exposure should be minimised. Moribund bees should be rejected and replaced by healthy bees before starting the test.

#### **Preparation of doses**

11. The test substance is to be applied as solution in a carrier, i.e. an organic solvent or a water solution with a wetting agent. As organic solvent, acetone is preferred but other organic solvents of

low toxicity to bees may be used (e.g. dimethylformamide, dimethylsulfoxide). For water dispersed formulated products and highly polar organic substances not soluble in organic carrier solvents, solutions may be easier to apply if prepared in a weak solution of a commercial wetting agent (e.g. Agral, Citowett, Lubrol, Triton, Tween).

12. Appropriate control solutions should be prepared, i.e. where a solvent or a dispersant is used to solubilise the test substance, two separate control groups should be used, one treated with water, and one treated with the solvent/dispersant.

## **PROCEDURE**

### **Test and control groups**

13. The number of doses and replicates tested should meet the statistical requirements for determination of LD<sub>50</sub> with 95% confidence limits. Normally, five doses in a geometric series, with a factor not exceeding 2.2, and covering the range for LD<sub>50</sub>, are required for the test. However, the number of doses have to be determined in relation to the slope of the toxicity curve (dose versus mortality) and with consideration taken to the statistical method which is chosen for analysis of the results. A range-finding test enables the choice of the appropriate doses.

14. A minimum of three replicate test groups, each of ten bees, should be dosed with each test concentration.

15. A minimum of three control batches, each of ten bees, should be run in addition to the test series. If an organic solvent or a wetting agent is used three additional control batches of each ten bees for the solvent or the wetting agent have to be included

### **Toxic standard**

16. A toxic standard should be included in the test series. At least three doses should be selected to cover the expected LD<sub>50</sub> value. A minimum of three replicate cages, each containing ten bees, should be used with each test dose. The preferred toxic standard is dimethoate for which the reported contact LD<sub>50</sub>-24h is in the range 0.10-0.30 µg a.i./bee (7). However, other toxic standards would be acceptable where sufficient data can be provided to verify the expected dose response (e.g. parathion).

### **Exposure**

#### **Administration of doses**

17. Anaesthetized bees are individually treated by topical application. The bees are randomly assigned to the different test doses and controls. A volume of 1 µl of solution containing the test substance at the suitable concentration should be applied with a microapplicator to the dorsal side of the thorax of each bee. Other volumes may be used, if justified. After application, the bees are allocated to test cages and supplied with sucrose solutions.

**Test conditions**

18. The bees should be held in the dark in an experimental room at a temperature of  $25 \pm 2$  °C. The relative humidity, normally around 50-70 %, should be recorded throughout the test.

**Duration**

19. The duration of the test is 48h. If mortality increases by more than 10 per cent between 24h and 48h, the test duration should be extended up to a maximum of 96h provided that control mortality does not exceed 10 per cent.

**Observations**

20. Mortality is recorded at 4 h after dosing and thereafter at 24h and 48 h. If a prolonged observation period is required, further assessments should be made at 24 hours intervals, to a maximum of 96 hours, provided that the control mortality does not exceeding 10 per cent. All abnormal behavioural effects observed during the testing period should be recorded.

**LIMIT TEST**

21. In some cases (e.g. when a test substance is expected to be of low toxicity) limit test may be performed, using 100 µg a.i./bee in order to demonstrate that the LD<sub>50</sub> is greater than this value. The same procedure should be used, including three replicate test groups for the test dose, the relevant controls, and the use of the toxic standard. If mortalities occur, a full study should be conducted. If sublethal effects are observed (see paragraph 20), these should be recorded.

**DATA AND REPORTING****Data**

22. Data should be summarised in tabular form, showing for each treatment group, as well as control and toxic standard groups, the number of bees used, mortality at each observation time and number of bees with adverse behaviour. Analyse the mortality data 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, if relevant, 72h, 96h) and calculate the slopes of the curves and the median lethal doses (LD<sub>50</sub>) with 95% confidence limits. Corrections for control mortality could be made using Abbott's correction (9)(10). LD<sub>50</sub> should be expressed in µg of test substance per bee.

**Test report**

23. The test report must include the following information:

## Test substance:

- physical nature and relevant physical-chemical properties (e.g. stability in water, vapour pressure);
- chemical identification data, including structural formula, purity (i.e. for pesticides, the identity and concentration of active ingredient (s)).

## Test bees:

- scientific name, race, approximate age (in weeks), collection method, date of collection;
- all relevant information on colonies used for collection of test bees, including health, any adult disease, any pre-treatment, etc.

## Test conditions:

- temperature and relative humidity of experimental room;
- housing conditions including type, size and material of cages;
- methods of administration of test substance, e.g. carrier solvent used, volume of test solution applied, anaesthetics used;
- test design, e.g. number and test doses used, number of controls; for each test dose and control, number of replicate cages and number of bees per cage;
- date of test.

## Results:

- results of preliminary range-finding study if performed;
- raw data: mortality at each concentration tested at each observation time;
- graph of the dose-response curves at the end of the test;
- LD<sub>50</sub> values, with 95% confidence limits, at each recommended observation time, for test substance and toxic standard;
- statistical procedures used for determining LD<sub>50</sub>;
- mortality in controls;
- other biological effects observed and any abnormal responses of the bees;
- any deviation from the Test Guideline procedures and any other relevant information.

**LITERATURE**

- (1) EPPO (1992). Guideline on Test Methods for Evaluation the Side-Effects of Plant Protection Products on Honeybees (No. 170). Bulletin OEPP/EPPO Bulletin, 22, 203-215.
- (2) Harrison E.G. (1993). Proceedings of the Fifth International Symposium on the Hazards of Pesticides to Bees, October 26-28, 1993. Plant Protection Service, Wageningen, The Netherlands. Report IUBBSS, 14 pp + Appendices 180 pp.
- (3) SETAC (1995). Procedures for Assessing the Environmental Fate and Ecotoxicity of Pesticides. Edited by Dr. Mark R. Lynch. Published by SETAC-Europe, Belgium. March 1995.

- (4) Stute, K. (1991). Auswirkungen von Pflanzenschutzmitteln auf die Honigbiene. Richtlinien für die Prüfung von Pflanzenschutzmitteln im Zulassungsverfahren, Teil VI, 23 - 1, Biologische Bundesanstalt für Land- und Forstwirtschaft (BBA), Braunschweig, Germany.
- (5) US EPA (1995). Honey Bee Acute Contact Toxicity Test (OPPTS 850.3020). Ecological Effects Test Guidelines. EPA 712-C-95-147, Washington DC, United States of America.
- (6) EPPO/Council of Europe. (1993). Decision-Making Scheme for the Environmental Risk Assessment of Plant Protection Products - Honeybees. EPPO bulletin, vol. 23, No.1, 151-165. March 1993
- (7) Gough, H.J, McIndoe, E.C, Lewis, G.B. (1994). The use of dimethoate as a reference compound in laboratory acute toxicity tests on honey bees (*Apis mellifera* L.) 1981-1992. Journal of Apicultural Research, 22, 119-125.
- (8) Litchfield, J.T. and Wilcoxon, F. (1949). A simplified method of evaluating dose-effect experiments. Jour. Pharmacol. and Exper. Ther., 96, 99-113.
- (9) Finney, D.J. (1971). Probit Analysis. 3rd ed., Cambridge, London and New York.
- (10) Abbott, W.S. (1925). A method for computing the effectiveness of an insecticide. Jour. Econ. Entomol., 18, 265-267.

ANNEXDEFINITIONS

Acute contact toxicity is the adverse effects occurring within a maximum period of 96 h of a topical application of a single dose of a substance.

Dose is the amount of test substance applied. Dose is expressed as mass ( $\mu\text{g}$ ) of test substance per test animal ( $\mu\text{g}/\text{bee}$ ).

LD<sub>50</sub> (median lethal dose) contact, is a statistically derived single dose of a substance that can cause death in 50 per cent of animals when administered by contact. The LD<sub>50</sub> value is given in  $\mu\text{g}$  of test substance per bee. For pesticides, the test substance may be either an active ingredient (a.i.) or a formulated product containing one or more than one active ingredient.

Mortality: an animal is recorded as dead when it is completely immobile.