PROPOSAL FOR A NEW GUIDELINE FOR THE TESTING OF CHEMICALS

HONEY BEE (*APIS MELLIFERA* L.), CHRONIC ORAL TOXICITY TEST 10-DAY FEEDING TEST IN THE LABORATORY

INTRODUCTION

1. This Test Guideline describes a chronic oral toxicity test on adult worker honey bees under laboratory conditions over an exposure period of 10 days. The test is based on the OECD TG 213- Acute Oral Toxicity Test (1998) (1),(2). The test was validated by a German ring test group in 2013, by a 1st international ring test in 2014 and a 2nd international ring test in 2015 (3)(4),

2. Pollinators like honey bees may be exposed to residues of plant protection products (PPP) or chemicals for a prolonged period of time, either *via* contaminated food, stored and consumed by the bees in the hive, or by foraging on contaminated areas. To address this potential risk, a chronic toxicity study can be conducted in the laboratory by exposing young adult bees to treated food (sucrose solution) over a period of 10 days.

INITIAL CONSIDERATIONS

3. Test chemicals are typically PPPs that can either be tested as active substances or as formulations.

4. The bees used in this test should be young worker bees (max. 2 days old) in order to start the test with bees of a similar age.

5. A reference chemical should be used to verify the sensitivity of the bees and the reliability of the test system.

PRINCIPLE OF THE TEST

6. Young bees (max. 2 days old) are exposed to 50 % (w/v) aqueous sucrose solution containing the test chemical by continuous and *ad libitum* feeding over a period of 10 days. Mortality and behavioural abnormalities are observed and recorded daily during the 10 day test period. The chronic effects of the test

chemical are evaluated by comparing the results of the test chemical treated group to those of the respective control group. The test is designed for the determination of the following endpoints:

- LC_{50} (median lethal concentration) and the LDD_{50} (median lethal dietary dose) values after 10 days of exposure

- NOEC (no observed effect concentration) and NOEDD (no observed effect dietary dose).

In some cases (*e.g.* when a test chemical is expected to be of low toxicity or when a test chemical is poorly soluble) a limit test may be performed, in order to demonstrate that the NOEDD is greater than or equal to the limit dose tested, and the LDD_{50} is greater than the limit does tested, if no effects are observed in the study.

VALIDITY OF THE TEST

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

- The average mortality across replicates for the untreated control and solvent control groups is ≤ 15 % at the end of the test (10 days following start of exposure); when a solvent control is included, the average mortality across replicates for the solvent control should also be ≤ 15 %.

- The average mortality in the reference chemical treated group is ≥ 50 % at the end of the test (10 days following start of exposure).

DESCRIPTION OF THE METHOD

Collection of the bees

8. Young bees (max. 2 days old) from queen-right colonies without symptoms of diseases and which are adequately maintained with known history and physiological status should be used for the test. No chemical substances (such as antibiotics, anti *varroa* treatments, *etc.*) should have been used in the hive for at least one month prior to the test. If one colony cannot provide the appropriate number of bees, comb(s) from several colonies may be used. In this case it is ensured that the bees are equitably distributed across the treatments. Brood frames with capped cells which are expected to hatch on the same day can either be incubated in a climatic chamber or kept without nurse bees in a worker excluder box returned to the hive until hatch. In the first case sufficient food supply should be ensured either by honey and pollen which is on the same brood comb or by an additional comb containing food. One day before the test starts, the bees can be collected from the combs and distributed into the test cages. Anesthetisation should be avoided during collection. Bees should be acclimated to test conditions for about one day (after a hatching period of one day). Bees are to be fed with sucrose solution *ad libitum* but no additional feeding of pollen and water is necessary during acclimation and test period. No starvation period is necessary before test start.

Test cages

9. Easy to clean or disposable and passively ventilated cages are used. Any appropriate material can be used, *e.g.* stainless steel, cardboard, wire mesh, plastic, disposable wooden cages, *etc.* Groups of 10 bees per cage are used, since it allows a precise assessment of affected *vs.* non affected bees. The size of the cages should be sufficient considering the number of bees *i.e.* providing adequate space (*e.g.* minimum size 200 cm^3).

Feeding Solutions

10. The feeding solutions for the control, test chemical and reference chemical treatments are prepared with 50 % (w/v) aqueous sucrose solution. All feeding solutions have to be homogeneous without obvious signs of precipitation throughout one feeding interval (about 24 hours).

Preparation of the Stock and Treated Feeding Solutions

11. A stock solution of the test chemical is prepared or the test chemical can be directly mixed with 50 % (w/v) sucrose solution (treated feeding solution). In case of good water solubility, deionized water is used as a solvent. For test chemicals of low water solubility, acetone can be used as a solvent. The concentration of organic solvent used depends on the solubility of the test chemical and should be the same for all concentrations tested. The maximum acetone concentration in the final feeding solutions can be up to 5 %. Any other solvent, solubiliser or thickener can be used (*e.g.* to improve the homogeneity of the feeding solution during the 24 hours feeding interval) as long as the validity criterion for the control groups is met. Depending on the stability of the test chemical in the solution, the stock solution can be prepared only once for the entire test period and stored appropriately *e.g.* in tightly closed containers under cool conditions in the dark (refrigerator, *ca.* 6 ± 2°C). If the test chemical is assumed to degrade quickly in the aqueous or acetone solution, the stock solution has to be prepared freshly every day or at adequate time intervals.

12. The final feeding solutions are prepared from the stock solution or dilution of higher concentrated solutions with 50 % (w/v) aqueous sucrose solution. The final feeding solutions have to be prepared at intervals not exceeding four days and kept in the fridge (*ca.* 6° ± 2°C).

13. If acetone (or another solvent, solubiliser or thickener) is used as solvent, two control groups are required, *i.e.* one with pure 50 % (w/v) aqueous sucrose solution and one with 50 % (w/v) aqueous sucrose solution containing the same concentration of acetone (or any other solvent, solubiliser or thickener) as in the test chemical group.

Analytical Verification

14. If the feeding solutions are prepared daily, one sample of the lowest concentration and one sample of the highest concentration of the feeding solutions should be stored directly after preparation in a freezer at a temperature below or equal to -18°C for further analytical determination of the actual concentration of the test chemical; this should be done once during the experimental phase. In case a stock solution has been used for preparation of feeding solutions, it is recommended to take one additional sample for the analytical determination of the stock solution as well.

15. If the stock solution or the feeding solutions are not prepared daily, analytical determination is equally required, *i.e.* once during the experimental phase after preparation and additionally once at the end of the maximum storage period, for both the lowest and the highest concentrations of the feeding solutions and the stock solution. A maximum storage of the feeding solutions of 4 days should not be exceeded and it is recommended to store the feeding solutions in the fridge.

16. Likewise, if a new batch of the test chemical needs to be used during the test phase, one additional sample of the lowest and highest concentrations is required for analytical verification of each new batch of the test chemical. Ideally studies should be conducted with the same chemical batch.

PROCEDURE

Test and control groups

17. The number of concentrations and replicates tested should meet the statistical requirements for the determination of NOEC/NOEDD values, the LC_{50}/LDD_{50} (or LCx where applicable) with 95 % confidence limits at the end of the test period. Normally at least five test concentrations with a factor not exceeding 2.5 covering the range for the LC_{50} are required for the test.

18. In case of unknown toxicity, a range-finding test can be performed to derive appropriate concentrations in the final test.

19. In case of a dose-response test a minimum of three replicates (cages), each containing 10 bees should be used per treatment. Limit tests should be performed with five replicates (cages) for the control and the test chemical treatment groups, and at least 3 replicates for the reference chemical group.

Reference chemical

20. A reference chemical group should be included in the test. The preferred reference chemical is dimethoate (technical material or formulated product; CAS No. 60-51-5). One concentration of the reference chemical, which leads to an expected mortality of ≥ 50 % at the end of the test period should be used to demonstrate the sensitivity of the bees and the reliability of the test system. One concentration between 0.5 and 1.0 mg a.i./kg feeding solution has been shown to be suitable to achieve a mortality of ≥ 50 % following chronic exposure.

Exposure (feeding)

21. The feeding solutions are offered *ad libitum* to the honey bees via feeders (*e.g.* plastic syringes, minimum content of 2 mL; tip removed). The bees in one replicate share the feeding solution (trophallaxis) and thus can be expected to all be exposed. The feeding solution is replaced daily by changing the feeders. Each feeding interval is 24 h (± 2h). The amount of feeding solution(s) consumed is determined daily by initially weighing the feeders before and after feeding using a calibrated balance.

Evaporation

22. It is necessary to adjust for possible evaporation of test solutions from the feeders with additional test cages which are set up at the main test. These cages contain no bees, only pre-weighed feeders

containing diet of untreated control and/or solvent control (each tested with min. 3 replicates). These should be placed in the test environment alongside the test units. At the daily feeder exchange the feeders are re-weighed and replaced with new feeders. This evaporation figure can then be subtracted from the calculated food consumption to give the corrected food consumption accounting the loss by evaporation.

Test conditions

The bees should be kept in constant darkness under controlled climatic conditions at a target temperature of 33°C with maximum deviations of ± 2°C and a relative humidity of 50 – 70 %. Short-term deviations (≤ 2 hours per day) from the recommended test conditions are unavoidable and should not affect the integrity or outcome of the test.

23. Temperature and humidity should be recorded continuously with appropriate and calibrated equipment.

Duration

24. Bees are continuously exposed to the feeding solutions over a period of 10 days.

Observations

Mortality should be recorded daily at about the same time of the day (every 24 h ± 2h), starting 24 ± 2 hours after start of the test period (initial feeding).

25. Additionally, behavioural abnormalities should be recorded daily at the same time as the assessments of mortality.

26. Behavioural abnormalities should be quantitatively observed according to the following categories:

m = moribund (bees cannot walk and show only very feeble movements of legs and antennae, only weak response to stimulation; *e.g*. light or blowing; bees may recover but usually die),

a = affected (bees still upright and attempting to walk but showing signs of reduced coordination; hyperactivity; aggressiveness; increased self-cleaning behaviour; rotations; shivering),

c = cramps (bees contracting abdomen or entire body),

ap = apathy (bees show only low or delayed reactions to stimulation *e.g.* light or puff of air; bees are sitting motionless in the unit).

v = vomiting

27. Any behavioural abnormalities which are not included in the list should be noted and clearly described.

After 10 days of exposure the final assessments of mortality and food consumption are done and thereafter the test is terminated by freezing the test cages including the bees at ≤ -10°C (preferably lower) or by using other humane methods.

DATA AND REPORTING

Data

28. The data should be summarized in tabular form, showing the number of bees tested, mortality and number of bees with adverse behaviour assessed at each observation time. Data on mortality are analysed by appropriate statistical methods (*e.g.* regression analyses, moving-average interpolation, binominal probability) in order to calculate the LC_{50} (expressed in mg/kg) and LDD_{50} (and LCx if applicable) (expressed in µg or ng/bee/day) values with 95 % confidence limits and the NOEC/NOEDD at the end of the test. Correction for control mortality could be made using standard procedures (e.g. Abbott,5):

29. Data on food consumption should be calculated and displayed as:

- mean consumption of feeding solution per bee for each day (mg/bee); the number of living bees at the beginning of each feeding interval is taken for this calculation;
- overall mean daily consumption of feeding solution per treatment over the test period (mg/bee/day);
- overall mean daily consumption of feeding solution per replicate over the test period (mg/bee/day);
- mean uptake of test chemical per bee per day (µg or ng a.i./bee/day);
- accumulated uptake of test chemical per bee over the test period (µg or ng a.i./bee).

30. It is necessary to adjust for possible evaporation of test solutions from the feeders. In case the subtraction of the evaporation figure from the calculated food consumption leads to a negative value, the food consumption of the respective day will be considered to be "0" (no food consumption).

Test report

31. The test report includes the following information:

Test and reference chemical

- Chemical identification, such as IUPAC or CAS name, CAS number, structural formula, purity;
- source, lot number, expiration date for use, if available;
- stability of the test chemical itself, if known;

- solubility and stability of the test chemical in water and solvent (if used);

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Test system

Details on the test species (scientific name, race, age, incubation and collection method, and information on the colonies used like health status, pre-treatment etc.);

Test conditions:

Conditions during incubation, acclimatization (if applicable) and test period;

Description of the test cages (type, material, size);

Method and frequency of the preparation of the stock solution and the feeding solutions;

Test design (number of treatment groups (control(s), test chemical, reference chemical), number of replicates, number of bees per cage);

Date of the start and the end of the test;

Results

Mortality at each observation time for all treatments tested;

Consumption of feeding solution at each observation time for all treatments tested;

Nominal test concentrations used and measured concentrations of the test chemical in the feeding solutions, and analytical method used;

Evaporation figures;

LC_{50}/LDD_{50}, NOEC/NOEDD and/or LCx values if some of them are applicable with 95 % confidence limits for the test item at the end of the test; Description of all statistical procedures used in the study;

Any other biological effects observed *e.g.* behavioural abnormalities, anti-feeding effects;

Deviations from the guideline and any other relevant information.

LITERATURE

(1) OECD (1998). Guideline for the Testing of Chemicals, No. 213: Honey bees: acute oral toxicity test. Organisation for Economic Cooperation and Development, Paris.

(2) Kling, A. & Schmitzer, S. (2015): Proposal for a new OECD guideline for the testing of chemicals on adult honey bees (Apis mellifera L.) in a 10 day chronic feeding test in the laboratory and results of the recent ring test 2014. Hazards of pesticides to bees - 12th International Symposium of the ICP-PR Bee Protection Group, Ghent (Belgium), 15-17 September 2014. Julius-Kühn-Archiv, 450, pp. 69-74.

(3) OECD (20xx), Summary of the Results of the 1st International Ring Test for the Standardisation of a 10 Day Chronic Feeding Test ond Honey Bees (Apis mellifrea L.) in the Laboratory. ENV Publication, Series on Testing and Assessment No. XXX, Paris.

(4) OECD (20xx), Summary of the Results of the 2nd International Ring Test for the Standardisation of a 10 Day Chronic Feeding Test ond Honey Bees (Apis mellifrea L.) in the Laboratory. ENV Publication, Series on Testing and Assessment No. XXX, Paris.

(5) Abbott, W.S. (1925). A method for computing the effectiveness of an insecticide. Jour. Econ. Entomol., 18, 265-267.

ANNEX

DEFINITIONS

LC_{50} (median Lethal Concentration) is a statistically calculated concentration of a substance that can cause death in 50 % of the test organisms at the end of the test period. It is expressed in *e.g.* mg or µg active ingredient or formulated product per kg food.

LDD_{50} (median Lethal Dietary Dose) is a statistically calculated dietary dose of a substance that can cause death in 50 % of the test organisms at the end of the test period. It is expressed in *e.g.* µg or ng active ingredient or formulated product per bee per day.

NOEC (No Observed Effect Concentration) the highest tested concentration next below the LOEC (lowest effect concentration). In case that the LOEC cannot be determined, the NOEC will be considered to be greater than or equal to the highest concentration tested. If, in a limit test, the effect at the tested concentration is not significantly different statistically from the control the NOEC is considered to be greater than or equal to the tested concentration. It is expressed in *e.g.* mg or µg active ingredient or formulated product per kg food.

NOEDD (No Observed Effect Dietary Dose) the highest tested dose per bee per day, administered by chronic feeding exposure, next below the LOEDD (lowest effect dietary dose). In case that the LOEDD cannot be determined, the NOEDD will be considered to be greater than or equal to the highest dose tested. If, in a limit test, the effect at the tested dose is not significantly different statistically from the control the NOEDD is considered to be greater than or equal to the tested dose. It is expressed in *e.g.* µg or ng active ingredient or formulated product per bee per day.

LOEC/LOEDD (Lowest Observed Effect Concentration/Dietary Dose) is the lowest concentration out of the tested concentrations at which a significant difference statistically from the control group is observed.

LC_x (Lethal Concentration for x % effect) is defined as the concentration that causes an x % of an effect within a given exposure period when compared with a control.

OTHER EVALUATIONS

With the data generated in a 10-day chronic feeding test a calculation based on Haber´s law can be performed in order to determine possible accumulative toxicity of test items. Further guidance can be provided by using the publication/protocol of J. E. Cresswell, University of Exeter (reference to paper/URL of website).