OECD/OCDE 247
Adopted:
9 October 2017

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

BUMBLEBEE, ACUTE ORAL TOXICITY TEST

INTRODUCTION

1. This test guideline is a laboratory test method, designed to assess the acute oral toxicity of pesticides and other chemicals to adult worker bumblebees. This test is based on OECD 213 – Honeybees, Acute Oral Toxicity Test (1), van der Steen *et al.* (1996) (2) and Hanewald *et al.* (2013) (3). The test method was ring tested first by an international ICPPR (International Commission for Plant-Pollinator Relationships) ring-test group in 2014 and second by an international OECD ring-test group in 2015. In the ring-tests, the following species were successfully used: *Bombus terrestris* and *Bombus impatiens*. The test is also applicable to other bumblebee species, but has not been documented.

2. Pollinators, such as bumblebees, may be exposed to residues of plant protection products or other chemicals either via contact (directly or via indirect transfer) or consumption of residue-containing food. To address the potential risk of oral exposure to a chemical, an acute oral study can be conducted in the laboratory by exposing adult worker bumblebees to the respective chemical.

3. Before use of the test guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

INITIAL CONSIDERATIONS AND LIMITATIONS

4. In the assessment and evaluation of toxic characteristics of chemicals, determination of acute oral toxicity in bumblebees may be required when exposure of bumblebees to a given chemical is likely. The acute oral toxicity test is carried out to determine the intrinsic toxicity of pesticides and other chemicals to bumblebees. The results of this test should be used to determine whether further evaluation is needed. In particular, this method can be used in step-wise programs for evaluating the risks of chemicals to

This Guideline was adopted by the OECD Council by written procedure on 9 October 2017 [C(2017)97].

pollinators, based on sequential progression from laboratory toxicity tests to semi-field and field experiments. Chemicals can be tested as active substance or as formulated products.

5. The method aims at the determination of the LD_{50} (see Annex for definitions) following a single exposure of adult worker bumblebees to a test chemical. The data should be used in an appropriate pollinator risk assessment scheme. This Test Guideline on bumblebees should be seen as a lower tier test in the context of an overall risk assessment scheme for pollinators.

PRINCIPLE OF THE TEST

6. Adult worker bumblebees are exposed to 50 % (w/v) aqueous sucrose solution containing the test chemical. The test duration is at least 48 h. If the mortality rate increases by ≥ 10 % between 24 h and 48 h in at least one treatment whilst control mortality remains at an accepted level, i.e. ≤ 10%, the duration of the test has to be extended up to 96 h. Mortality is recorded daily and compared with control values. Results are analysed in order to calculate the LD_{50} and NOED, if possible, at 24 h & 48 h and furthermore at 72 h & 96 h in case the study is prolonged.

VALIDITY OF THE TEST

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

- mortality in the water control should be ≤ 10 % at the end of the test. If included, also solvent control mortality should be ≤ 10 % at the end of the test.
- mortality in the toxic reference substance group should be ≥ 50 % at the end of the test.

DESCRIPTION OF THE METHOD

Test organism

8. The oral acute toxicity test is conducted using adult bumblebee workers (*Bombus* spp.).

9. Medium sized bumblebee colonies, having brood at all stages of development and a laying queen, containing ~60-80 bumblebee workers should be used to collect bumblebees for the test. It is recommended to use colonies within one week counted from the date of delivery.

Test cages

10. Bumblebees are kept individually in cages – "single housing". Single housing prevents hierarchy fights (among the queen-less bumblebee workers) potentially introducing mortality and it allows a precise assessment of affected or non-affected bumblebees. Furthermore, bumblebees do not share food via trophallaxis and need to be fed individually.

11. 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, wooden cages, etc. The size of the cages should be appropriate to the size of the bumblebees (minimum size 15 cm^3).

12. For further information, please refer to ANNEX 2.

Collection and Randomization of bumblebees

13. Adult bumblebee workers are either collected (without being anaesthetised) from the colony under red light or -chilling before they are transferred to test cages. Only those bumblebee workers should be used which can be collected without removing the cotton layer (if present) - for further details please refer to ANNEX 2 - in the colony. Very small and particularly very large bumblebees should be excluded from the test by visual inspection. The use of recently emerged bumblebees, recognizable by their greyish fur, as well as drones and queens are not to be used in this test.

14. Bumblebees used in the test are weighed individually. Subsequently bumblebees are randomly allocated to the different treatment groups.

Handling and Feeding

15. Handling procedures, including treatment and observations may be conducted under red or artificial light. For all treatment groups feeding solutions are prepared by dissolving sucrose in water with a final concentration of 50 % w/v (e.g. 500 g sucrose / L). During the exposure phase, bumblebees are fed a diet containing the test- or reference substance. During the observation period all bumblebees across the different treatments are fed untreated 50 % (w/v) aqueous sucrose solution *ad libitum*. Feeding solutions are offered to the bumblebees using an appropriate feeder e.g. a commercially available plastic syringe with a volume of 2 mL; the tip (bippus) should be removed.

Preparation of the test organism

16. Bumblebees should be acclimatised to the test conditions (including single housing) for at least 8 h with access to an untreated 50 % (w/v) aqueous sucrose solution *ad libitum*. As moribund bumblebees may occur, these must be discarded and replaced by healthy bumblebees before starting the test. Therefore, it is necessary to cage and acclimatise bumblebees in excess to the number that is needed for the test. An advisable number would be 5% of the total number of animals entering the test (Please note: Compensation for non-feeders – paragraph 32 - should be considered additionally).

17. To ensure that the treated diet is consumed within max. 4 h bumblebees are starved 2 – 4 h prior to the exposure period.

Preparation of test doses

18. A stock solution of the test chemical is prepared or the test chemical can be directly mixed with 50 % (w/v) aqueous sucrose solution (treated feeding solution). In case of good water solubility, water is used as solvent. For test chemicals of low water solubility, an organic solvent can be used (e.g. acetone). The concentration of solvent used depends on the solubility of the test chemical and should be the same for all treatment levels and the solvent control. If acetone is used as solvent, the maximum acetone concentration in the final feeding solutions should be kept as low as possible, but should not exceed 5 % (v/v). Any other solvent, solubiliser or thickener can be used (e.g. to improve the homogeneity of the feeding solution) as long as the validity criterion of the solvent control group is met.

19. The final feeding solutions are prepared from the stock solution or dilution of higher concentrated solutions with 50 % (w/v) aqueous sucrose solution.

20. Appropriate control solutions should be prepared if a solvent, solubiliser, dispersant, etc. is used. In this case, two separate control groups should be used: one water containing 50 % sucrose solution, and one containing the solvent, solubiliser, dispersant, etc. at the same concentration as in the test chemical dose(s).

Analytical Verification

21. Once during the experimental phase at least one aliquot of the lowest concentration and one aliquot of the highest concentration of the feeding solutions should be taken and stored directly after preparation in a freezer at a temperature below or equal to -18°C for analytical determination of the actual concentration of the test chemical. If a stock solution has been used for the preparation of feeding solutions take one additional sample of this stock solution for the analytical determination as well.

22. 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.

TEST PROCEDURE

Test and control groups

23. The number of doses and replicates tested should meet the statistical requirements for determination of LD_{50} with 95 % confidence limits. Normally, five doses in a geometric series, with a scaling factor not exceeding 2.2, and covering the dose range for LD_{50} are required for the test. The number of doses has to be determined in relation to the slope of the toxicity curve (dose versus mortality) and considering the statistical method chosen for the analysis of the results. In case of unknown toxicity of the chemical, a range-finding test is recommended first, to choose appropriate dose values.

24. In case of a dose response test a minimum of 30 replicates (cages), each containing one bumblebee should be used per treatment. In case of low toxicity of the test chemical a limit test can be performed with 50 replicates (cages) for each of the control and the test chemical treatment and with at least 30 replicates for the toxic reference substance.

25. Please note: one colony is not sufficient to perform a dose – response design test. Therefore, worker bumblebees from several (at least three) colonies are needed. Ensure that bumblebees from different colonies are randomly allocated to the different treatment groups to avoid any colony effect within a treatment group.

Treatment of controls when a solvent is used

26. If a solvent is used, two controls, a water control group and a solvent control group are included in the test. Both water control and solvent control are tested for statistically significant differences. If there is no statistical significant difference, both controls may be pooled for further statistical evaluations. In case of a statistical difference, the solvent control is used for LD_{50} calculation and for mortality corrections of the test chemical treatments.

Reference substance

27. One dose of the reference substance leading to an expected mortality of ≥ 50 % at the end of the test period should be used to demonstrate the sensitivity of the bumblebees and the reliability of the test system. 4 µg active ingredient. Dimethoate / bumblebee has been shown suitable to achieve a mortality of ≥ 50 % following an acute oral exposure (4). However, other toxic reference substances would be acceptable where sufficient data can be provided to demonstrate the expected sensitivity of bumblebees.

Exposure (feeding)

28. Each bumblebee should be provided with 40 µL of aqueous 50 % sucrose solution, containing the test chemical at the appropriate dose. Other volumes may be used, if justified. Syringes filled with the treatment - specific diet are weighed before and after the feeding in order to determine the exact diet consumption. The feeding period starts when the syringes with the treatment- specific diet are plugged into the test-cages and ends when these syringes are replaced with syringes containing untreated aqueous 50 % (w/v) sucrose solution. The duration of the exposure period should not exceed 4 h. Untreated aqueous 50 % (w/v) sucrose solution is then provided *ad libitum*.

29. At higher doses, for some chemicals, rejection of test chemical dose may result in little or no food being consumed. After a maximum of 4 h, unconsumed treated diet should be replaced with the sucrose solution alone.

30. So called "non-feeders" can be present in the test. These are single individual bumblebees not feeding on the offered sucrose solution, independently from the offered test chemical. As such, even in control groups, "non-feeders" will show a distinctly lower food uptake compared to other individuals under the same conditions / treatments. A "non-feeder" is an individual bumblebee that consumes < 80 % of the mean consumption of the respective treatment group within the maximum exposure (feeding) period. Due to the limited food uptake "non-feeders" have not been sufficiently exposed to the test chemical and should therefore not be considered in the derivation of endpoints. Otherwise, the limited exposure of "non-feeders" could possibly lead to an overestimation of LD_{50} values. If data points are excluded from the analysis due to low food uptake unrelated to repellence effects, this must be stated in the study report.

31. If necessary bumblebees will be classified as feeders and non-feeders after completion of the test. A full data record will be provided for both, feeders and non-feeders in the raw data and the final report. Non-feeders need to be marked as such in the data and will be excluded from endpoint calculation. However, in special cases (e.g. where anti-feeding effects of the test chemical are seen), individuals with a lowered consumption threshold can be included in analysis, if justified.

32. To compensate for non-feeders in each treatment group it is recommended to use more than thirty bumblebees per treatment and replace non-feeders by feeders (Please note: mortalities during acclimatisation need to be compensated additionally).

Test conditions

33. Between assessments and handling bumblebees should be kept in constant darkness under controlled climatic conditions, at a target temperature of 25 ± 2 °C and a relative humidity of 60 ±20 %. Climatic conditions should be recorded continuously with appropriate and calibrated equipment. Short-term deviations (≤ 2 h) from the recommended ranges are partly unavoidable (e.g. due to handling of the set-ups) and will normally not result in major disturbances of the test performance.

Duration

34. After exposure to the test chemical, bumblebees are observed for at least 48 h. If test chemical mortality increases by ≥ 10 % between 24 h and 48 h in at least one treatment group whilst control mortality remains at an accepted level ≤ 10 %, the test should be extended up to a maximum of 96 h.

Observations and measurements

35. Mortality is recorded within 4 - 5 h after start of the test chemical administration as well as after 24 h and 48 h. If a prolonged observation is required, further assessments should be made after 72 h and 96 h.

36. Additionally, sublethal effects should be recorded daily at the same time as mortality assessments. Sublethal effects will be recorded as follows:

unaffected = bumblebees show inconspicuous behaviour (including natural occurring phases of inactivity).

affected = bumblebees are still upright and attempting to walk but displaying signs of reduced coordination.

moribund = bumblebees are unable to walk, and show only very feeble movements of legs and antennae, only weak response to stimulation; e.g. light or blowing; bumblebees may recover but usually die.

LIMIT TEST

37. In some cases (e.g. when a test chemical is expected to be of low toxicity) it may be appropriate to conduct a limit test, using e.g. 100 µg a.i. or chemical / bumblebee in order to demonstrate that the LD_{50} is greater than this value. The above described procedure should be used (including relevant controls, and the use of the toxic reference substance), but instead of using 30 replicates per treatment group, 50 replicates are used, except for the toxic reference substance where at least 30 replicates are used.

38. If statistically significant mortality occurs, a full dose-response study should be conducted. If sublethal effects are observed, these should be recorded as mentioned above.

DATA AND REPORTING

Data treatment

39. Data should be summarised in tabular form, showing for each test group (including all control-, toxic reference- and chemical treatments) the number of bumblebees used, mortality at each observation time and number of bumblebees showing sublethal effects. The mortality data should be analysed using appropriate statistical methods (e.g. Probit analysis, Weibull, binomial probability, fitting dose-response model). Plot dose-response curves at each recommended observation time (i.e. 24 h, 48 h and, if relevant, 72 h, 96 h) and calculate the slopes of the curves and the median lethal doses (LD_{50}) with 95 % confidence limits. Correction for control mortality could be made using standard procedures (e.g. Abbott, [5]).

Endpoints should be expressed in µg of test chemical per bumblebee (µg / bumblebee) based on actual consumption.

TEST REPORT

40. The test report must include the following information:

Test chemical and reference substance

Test chemical and reference substance:

- Mono-constituent substance:

  physical appearance, water solubility, and additional relevant physico-chemical properties; chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc. (including the organic carbon content, if appropriate).

- Multi-constituent substance, UVCBs (substances of Unknown or Variable composition, Complex reaction products or Biological materials) and mixtures:

  characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physico-chemical properties of the constituents;

- source, batch and/or lot number, if available;

- solubility of the test chemical in water or solvent, if available;

- physical appearance and additional relevant physicochemical properties

- chemical identification, such as chemical substance name, IUPAC or CAS number;

Test system:

- scientific name, species of bumblebee, supplier, approximate colony age in weeks (if available), collection method, date of collection, weight of each bumblebee used in the test;

- all relevant information on colonies used for collection of test bumblebees, including health certificate, any adult disease, any pre-treatment, etc., if available.

Test conditions:

- description of the test design: number of treatment groups (including controls and reference substances), number of replicates for each treatment group, tested doses of the test chemical;

- temperature and relative humidity during experimental phase and acclimatisation;

- light sources during assessments and handling;

- description of test cages (type, material, size, feeding device, etc..)
- preparation of test chemical doses: used solvent, solubiliser, dispersant, etc., used sugar;
- volume of test solution offered and time needed for consumption of the test chemical treatments;
- anaesthetics used;
- place and date of test.

Results:

- raw data: mortality in each tested dose at each observation time;
- Nominal test concentrations used and measured concentrations of the test chemical in the feeding solutions, and analytical method used;
- consumption of the test chemical will be reported as actual test chemical uptake per treatment group, calculated on the basis of the mean food intake per treatment group;
- number of non-feeders and feeders for each treatment group;
- graph of the dose-response curves at the end of the test, if available;
- mortality in controls and reference substance group;
- LD_{50} values, with 95 % confidence limits, at each recommended observation time for the test chemical;
- NOED, if possible;
- statistical procedures used for determining LD_{50} and NOED;
- sublethal effects observed;
- any deviation from the Test Guideline and any other relevant information.

LITERATURE

(1) OECD (1998). OECD guideline for testing of chemicals, No.213: Honeybees, acute oral toxicity test. Organisation for Economic Cooperation and Development, Paris.

(2) Steen. J.J.M. van der, Gretenkord, C. Schaefer, H. (1996). Methods to determine the acute oral and contact LD50 of pesticides for bumble bees (Bombus terrestris L.) Proceedings ICPBR 6th Symposium on the Hazard of Pesticides to Bees 1996 Braunschweig, Germany

(3) Hanewald, N., et al. (2013). Optimizing laboratory toxicity test methods for Bumblebees (Bombus terrestis L.) (Presented by BASF SE on the SETAC Conference in Glasgow 2013)

(4) OECD (2017). Report of the International Ring Test for the Standardisation of an Acute Oral and Contact Test on Bumblebees in the Laboratory in 2015. Series on Testing and Assessment No.269 ENV Publications. OECD, Paris.

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

Recommended literature for data treatment:

(6) OECD (2006) Current approaches in the statistical analysis of ecotoxicity data: a guidance to application. OECD Environment Health and Safety Publications, Series on Testing and Assessment. No. 54, 147 p.

ANNEX 1

DEFINITIONS:

Acute oral toxicity is the adverse effects occurring after an oral exposure to a single dose of a test chemical within a maximum period of 96 h.

Dose is the amount of test chemical consumed or applied. Dose is expressed as mass of test chemical per test animal (μg / bumblebee).

LD_{50} (median lethal dose) oral is a statistically-derived single dose of a chemical that can cause death in 50 % of animals when administered orally. The LD_{50} value is given in μg of test chemical per bumblebee. For pesticides, the test chemical may either be an active ingredient (a.i.) or a formulated product containing one or more active ingredient(s).

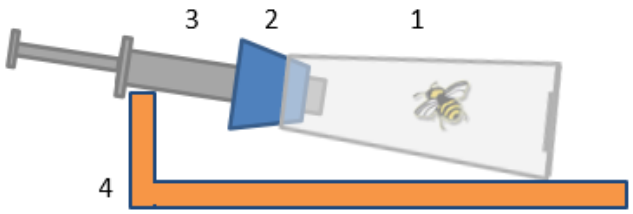
NOED (no observed effect dose) the dose that is not statistically significant different in mortality when compared to the control.

ANNEX 2

GENERAL RECOMMENDATIONS OF THE RING TEST GROUP:

Test cages:

- Each bumblebee should be housed in an individual cage for the duration of the test.
- The ring-test group proposes Nicot® queen breeding systems (see pictures attached) with 2 mL plastic syringes with tips cut off to enlarge the feeding opening for the bumblebees. Individual cages are placed next to each other to allow olfactory and visual contact between individuals.



1 = „Nicot“ system cage
2 = pierced rubber plug
3 = clipped off 2 mL syringe (feeding source)
4 = rack
5 = bippus

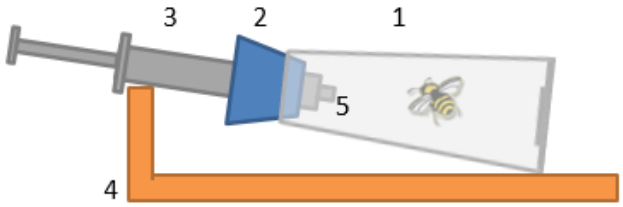


Figure 1: Illustration of a single housing cage: bumblebees are individually housed in Nicot® cages and fed via syringes. The system is slightly inclined to the syringes side to ensure that the diet flows to the opening of the syringe (particularly during ad libitum feeding). Syringes were kept in position by means of a rubber plug with

a drilled hole in the middle of the plug. Please note that on the upper illustration the tip of the syringes (bippus) was removed for ad libitum feeding. During the test chemical feeding (exposure) the bippus should remain as shown on the lower illustration.

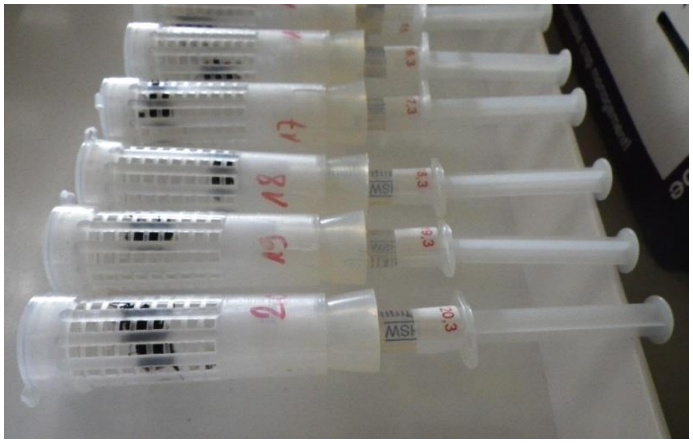


Figure 2: Illustration of a trial setup: several cages are placed next to each other to allow olfactory and visual contact between individuals. (picture: Bayer Cropscience)

Timing of the test:

- Although, colonies are commercially available in Central Europe all year round, experience from the ring test participants (communication during the workshops in 2014, 2015 & 2016) showed higher variability in food uptake and mortality of bumblebees during winter months. Therefore, it is recommended to conduct tests only from March to October in order to gain higher reliability and reproducibility of the test.

Supplement to Collection and Randomization of bumblebees:

- It is highly recommended to use bumblebee colonies covered with a cotton layer. According to experiences in the laboratory, workers are the first ones crawling on top of the cotton layer whereas very young or male bumblebees remain in the nest. This facilitates the selective choice of worker bumblebees.