PROPOSAL FOR A NEW GUIDELINE FOR THE TESTING OF CHEMICALS

Bumblebee, 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 bumblebees. This test is based on OECD 214 – Honeybees, Acute Contact Toxicity Test (1), van der Steen *et al.* (1996) (2) and Hanewald *et al.* (2013) (3). The test method was ring tested by a 1st international ICPPR (International Commission for Plant-Pollinator Relationships) ring-test group in 2014 and a 2nd international OECD ring-test group in 2015.

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 contact with a chemical, an acute contact study can be conducted in the laboratory by exposing adult worker bumblebees to the respective chemical.

INITIAL CONSIDERATIONS AND LIMITATIONS

3. In the assessment and evaluation of toxic characteristics of chemicals, determination of acute contact toxicity in bumblebees may be required when exposure of bumblebees to a given chemical is likely. The acute contact 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 test chemicals to pollinators, based on sequential progression from laboratory toxicity tests to semi-field and field experiments. Test chemicals can be tested as active ingredients (a.i.) or as formulated products.

4. 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 is in addition to OECD TG 214 (1) and should be seen as a lower tier test in the context of an overall risk assessment scheme for pollinators (4)

PRINCIPLE OF THE TEST

5. Adult worker bumblebees are exposed to the test chemical dissolved in an appropriate carrier, by direct application to the dorsal thorax (droplet). The test duration is at least 48 h. If the corrected mortality rate increases by $\geq$ 10 % between 24 h and 48 h in at least one treatment whilst control mortality remains at an accepted level, i.e. $\leq$ 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

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

- mortality in the untreated control and if used, in the solvent control ≤ 10 % at the end of the test;
- mortality in the toxic reference group should be ≥ 50 % at the end of the study.

DESCRIPTION OF THE METHOD

Test organism:

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

8. 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

9. 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.

10. 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).

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

Collection and Randomization of bumblebees:

12. Adult bumblebee workers are either collected (without being anaesthetised) from the colonies under red light or by 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 colonies. 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, should be avoided.

13. Bumblebees that enter the test are weighed individually. The bumblebee weight is used as a gauge of an equal distribution of bumblebee sizes among the different treatment groups. Subsequently bumblebees are randomly allocated to the different treatment groups.

Handling and Feeding

14. Handling procedures, including treatment and observations, may be conducted under day, red or artificial light. For all treatment groups, feeding solutions are prepared by dissolving sucrose in water with a final concentration of 500 g/L (50 % w/v). The 50 % (w/v) aqueous sucrose solution should be provided *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

15. 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 should 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 entering the test.

Preparation of test doses

16. The test chemical is applied as a solution in a carrier, i.e. an organic solvent or a water solution, containing an appropriate surfactant (reducing surface tension for an equal distribution of the test chemical on the animal). 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. Any other solvent can be used as long as the validity criterion of the solvent control group is met.

17. 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 non-dosed control group, and one containing the solvent, solubiliser, dispersant, etc. at the same concentration as in the test chemical dose(s).

TEST PROCEDURE

Test and control groups

18. 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 have 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.

19. 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 the control and the test chemical treatment and with at least 30 replicates for the toxic reference chemical.

20. Please note: one colony is not sufficient to perform a dose – response design test. Therefore, worker bumblebees from several 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

21. If a solvent is used, two controls, a non-dosed control group and a solvent control group should be included in the test. The solvent control should be used for LD_{50} and mortality corrections of the test chemical treatments, whereas the toxic reference chemical is compared to the non-dosed control.

Reference substance

22. 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. 10 µg a.i. Dimethoate / bumblebee has been shown to be suitable to achieve a mortality of ≥ 50 % following an acute contact exposure (5). However, other toxic reference substances would be acceptable where sufficient data can be provided to verify the expected sensitivity of bumblebees.

Exposure

23. Anaesthetized bumblebees are weighed and individually treated by topical application. A volume of 2 µl of solution containing the test chemical at the suitable dosage should be applied with a micro-applicator or pipette to the dorsal side of the thorax of each bumblebee. After application, the bumblebees are returned to their individual test cages and supplied with aqueous 50 % (w/v) sucrose solution *ad libitum*.

Test conditions

24. 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

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

Observations and measurements

26. Mortality is recorded within 4 - 5 h after the start of 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.

27. 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

28. 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 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. 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

29. Data should be summarised in tabular form, showing for each treatment group (including all control and toxic reference 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. LD_{50} calculations need to be corrected for mortality occurring in the control group with appropriate methodology. Endpoints should be expressed in µg of test chemical per bumblebee (µg / bumblebee).

Test report

30. The test report must include the following information:

Test chemical and reference substance:

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

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical and environmental fate properties, measured or estimated (e.g. hydrolysis, vapour pressure, log Kow, log Koc, log Kd (soil), log Koa, air/soil partitioning coefficient, biodegradability in soil or other biodegradability information).

- chemical identification, such as IUPAC or Chemical Abstract (CA) Index name, CAS Registry 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, UVCB and mixture:

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

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 chemicals), 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, surfactant, etc.;
- volume of test solution applied;
- description of droplet-applicator;
- anaesthetics used;
- place and date of test.

Results:

- raw data: mortality in each tested dose at each observation time;
- graph of the dose-response curves at the end of the test, if available;
- mortality in controls;
- LD_{50} values, with 95 % confidence limits, at each recommended observation time for the test chemical and the reference substance;
- 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.214: Honeybees, acute contact 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) EC, 2009. Regulation (EC) No 1107/2009 of the European parliament and of the council concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. Official Journal L 309.1: 24.11.2009.

(5) Hanewald, N., Gladbach, D., Andrade T.O., Mastitsky, S. (2016). Report of the International Ring Test for the Standardisation of an Acute Oral and Contact Test on Bumblebees in the Laboratory in 2015 – in prep

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 contact toxicity is the adverse effects occurring after a topical application of a single dose of a test chemical within a maximum period of 96 h.

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

LD_{50} (median lethal dose) contact is a statistically-derived single dose of a chemical that can cause death in 50 % of animals when administered by contact application. 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.

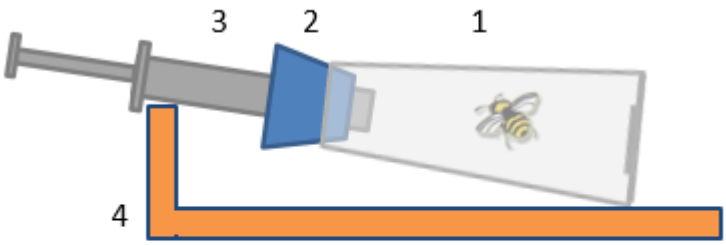
ANNEX 2

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

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.

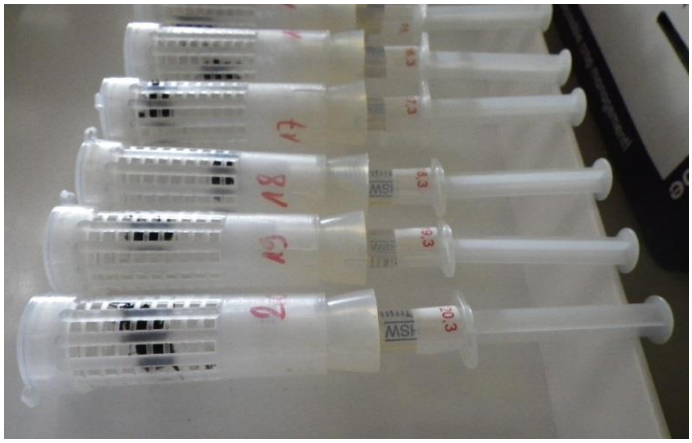


Figure 2: Illustration of a trial setup: several cages are placed next to each other to allow olfactory and visual contact between individuals. (picture: Bayer Cropscience)

Surfactant:

A surfactant is needed to ensure an equal distribution of the droplet applied to the bumblebee`s back. In 2014, in the 1st international ICPPR ring test, Tween 80 was the surfactant of choice. As most of the laboratories reported, Tween 80 was not appropriate for the hairy bumblebees. Consequently, in the 2015 workshop, it was decided to use Triton X for the OECD ring test. Triton X ensured homogenous distribution and is therefore recommended as surfactant for acute contact bumblebee testing.

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.