Proposal of a new OECD guideline for the testing of chemicals

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Honey bee (*Apis mellifera* L.) homing flight test, using single oral exposure to sublethal doses of test chemical

INTRODUCTION

1. This Test Guideline aims at assessing effects of test chemical on the homing ability of forager honey bees (also referred to as "bees" throughout the Guideline) following exposure to sublethal doses under controlled conditions. The success of the homing flight is measured in exposed versus non-exposed foragers simulating field realistic conditions over the short term. This method is based on previous work of the French research and development Unit (UMT PrADE) summarized in various publications (1) (2) (3), as well as by the French working group of the Biological Assay Commission (4). The methodology was internally tested in 2014 and ring tested for the first time in 2015 by eleven participating European laboratories. From 2016 to 2019 the ring testing was continued with twelve participating laboratories. The Test Guideline presented here corresponds to the ring test protocol tested in 2019 with minor adjustments that resulted from this last validation exercise.

2. Pollinators, such as honey bees, may be exposed to sublethal doses of plant protection products (PPP) or other chemicals while foraging in contaminated areas (consuming residue-containing food). These residues may not cause direct lethal toxicity in foragers, but may negatively affect honey bee behaviour, which could interfere with functions at the colony level. To address the potential risk of sublethal doses of chemicals on the foraging behaviour of free foraging honey bees, the homing flight test presented here can be used.

3. During the process of risk assessment and evaluation of toxicological characteristics of chemicals, a specific test assessing effects of an exposure to sublethal doses on honey bee behaviour might be requested, especially when results of Tier 1 toxicity tests suggest that further evaluation is needed. In the guidance document (5), the EFSA highlights the evaluation of sublethal effects as a current data-gap. The present homing flight test is carried out in order to determine effects of acute oral exposure to sublethal doses of a test chemical on the foraging behavior of worker honey bees. The test's endpoint 'homing success' covers both multiple physiological and cognitive functions that are involved in homing ability under field conditions (e.g. navigation, memory, flight muscle contraction and energetic metabolism). Chemicals used in the test system can be either an active substance (a.s.) or a formulation containing more than one active substance and potentially auxiliary substances.

4. The data should be included in an appropriate honey bee risk assessment scheme.

DEFINITIONS

5. The definitions, relevant for the purpose of this guideline, are given in Annex 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

6. The Test Guideline proposes a method that uses forager bees (focusing on pollen collectors), which are already familiar with their environment. To ensure this, returning forager

honey bees preferably carrying pollen are collected at the entrance of the colony hive and marked with a coloured powder. These marked bees are then taken to a starting site situated at 1 km distance (+/- 100 m) from the colony and released. Returning coloured bees are then re-captured at the entrance of the hive and transferred to the laboratory before starting the chemical single exposure experiment. An alternative method, using a field of *Phacelia* (drilled specifically for the test) was also explored during the first ring test in 2015. Foragers carrying the readily identifiable purple pollen loads from this crop were captured for the test. This method is more complicated to implement but could be useful and equally acceptable as an alternative. Accordingly, this "*Phacelia* method" is briefly described in Annex 7.

7. The homing flight test should not be performed under very high temperatures, which may impact the bee's behaviour. Temperatures may vary according to temperate or Mediterranean climate, but in any cases, they should not be more than 37°C at the release time.

8. When considering the testing of mixtures, difficult-to-test chemicals (e.g. unstable), or of test chemicals not clearly within the applicability domain described in this Guideline, upfront consideration should be given to whether the results of such testing will yield results that are scientifically meaningful, or not. If the test guideline is used for the testing of a mixture, a UVCB (substances of unknown or variable composition, complex reaction products or biological materials) or a multi constituent substance, its composition should, as far as possible, be characterized, e.g., by the chemical identity of its constituents, their quantitative occurrence and their substance-specific properties.

PRINCIPLE OF THE TEST

9. This test method measures the effect of a single oral exposure to sublethal doses of a test chemical (under controlled conditions) on the homing success of forager honey bees (under simulated field realistic conditions). Foragers are then released 1 km (+/- 100 m) away from the colony. Thereby, the homing success of chemically-exposed versus non-exposed foragers is compared. This is achieved by monitoring the experimental bees with radio-frequency identification (RFID) tagging technology.

10. Prior to tagging with a RFID-chip, foragers, preferably carrying pollen are selected at the hive entrance as test bees, are coloured with non-toxic powder and released 1 km from the colony. Returning coloured bees are collected. In order to ensure sufficient test bees for at least three treatment groups (non-exposed controls, test chemical and reference substance treatment groups) an excess of those bees required are coloured and released, prior to re-capture for tagging.

11. In the laboratory, the previously coloured bees are individually labelled with a RFID tag, assigned to treatment groups and are subsequently exposed to at least three doses of the test chemical, the control solution (water or solvent, preferably acetone) and to the reference substance. All solutions are administered orally (diet) and collectively to the honey bees (in small groups of ten bees) using 20 µL to 40 µL of a 30 % w/v aqueous sucrose solution per bee.

12. Honey bees are then released on the site that they are familiar with (i.e. the site at which they were released after colouring). The homing success is recorded automatically with the tag at the hive's entrance. This monitoring continues for 24 hours after the release. Homing failure is defined as the absence of a record at the entrance of the hive during this period. The homing rates are evaluated and compared between all treatment groups. The objective is to determine a 'No-Observed Effect Dose' (NOED) from the doses of the chemical tested. The duration of the homing flight can be used as an additional endpoint.

VALIDITY OF THE TEST

13. The following validity criteria apply:

- For each test run, control mortality and mortality in all treatment groups, including mortality in the group of bees exposed to the reference substance should be less than or equal to 15 % before release.

- For each test run, the homing rate of the control honey bees should be greater than or equal to 60 % over a 24 hours period after release at the site 1km away from the honey bee colony. The cumulative homing rate of three test runs (i.e. representing a full homing flight test) for control honey bees should be above or equal to 70 %.

- The homing success rate of the reference substance-exposed bees should be statistically significantly lower than that of non-exposed control bees.

REFERENCE SUBSTANCE

14. The reference substance is included to ensure that the test system and corresponding conditions are responsive and reliable. According to the ring test results, a nominal dose of 1 to 1.5 ng per bee of technical grade thiamethoxam showed to be appropriate for that purpose.

DESCRIPTION OF THE METHOD

The honey bees and the preparation of the colonies

15. It is preferable to use pollen foragers for the test. Foragers from three different healthy colonies with non-related queens are tested in separate test runs. These colonies should be queen-right honey bee colonies in good conditions, e.g. disease-free and with low *Varroa mite* infestation (below 5 *Varroa mites* per 100 bees), consisting of a queen of known origin which is not older than two years. Treatments against disease (e.g. against *Varroa*) must have been completed at least one month before the start of the test. A colony inspection by an experienced/trained beekeeper is carried out shortly before test start (i.e. one to three days before) on each experimental colony to verify its health status. Only strong and actively foraging, queen-right colonies consisting of at least 15,000 honey bees are suitable for the test. Test colonies should be as homogenous as possible regarding their colony strength, brood activity and food storage. For hives with a ten frame configuration, colonies composed of five to seven brood combs with all brood stages visible (eggs, uncapped larvae and pupae), two to three combs with nectar, honey and pollen stores, and at least one empty frame should be chosen for the experiment (Annex 4).

16. Colonies used for the test are installed at the experimental site, at least one week before the first test run, to allow an acclimation period and the honey bees to familiarise themselves with the environment. Importantly, if the three colonies are placed at the same time on the site, they are separated spatially by at least 10 meters and placed in a staggered configuration to minimise the chance of drift of bees between the colonies.

Honey bee identification

17. Use of RFID (Radio Frequency Identification) technology is recommended as it has been proven to be an efficient method to measure effects of chemicals like plant protection products (e.g. thiamethoxam) on the homing flight ability of foragers (1) (2) (6); this was also demonstrated

by ring test results. The use of individually labelled honey bees and hive mounted readers allows continuous monitoring and recording of the return of the test bees to the hive.

RFID device

18. The RFID technology (1) (7) allows detection each time a tagged bee passes the reader located at the hive entrance (working distance of 3 mm). The principle depends on the emission of a radio signal from the reader, which is reflected by the tag on the bee's thorax and provides the identification of the bee. RFID tags (2.0 x 1.7 x 0.5 mm) are glued dorsally on the thorax of the bees. The maximum weight of an individual tag is 3 mg, which is equivalent to 3% of the body weight of a worker bee. This is significantly less than the weight of pollen (between 8 and 29 mg) or nectar loads (between 40 and 80 mg) carried routinely by workers during a foraging flight (8) (9). Each tag has a unique identity (UID).

19. Four to five RFID readers are installed at the hive entrance in a parallel arrangement by means of an interface (constructed of plastic or wood) between hive and readers (Annex 2). Readers must be separated from each other by at least 40 mm to avoid interference during RFID recording. Using real-time recording, the reader sends the data scanned from the RFID tag along to a data storage system, which is automatically saved to a database. The tag identification code and the exact time of the event (date, hour, minute and second) are recorded in electronic form. In the ring tests, the MAJA 13.56 MHz RFID system (Microsensys GmbH, Erfurt, Germany) was used. Each reader spanned a tunnel of 14 × 21.5 mm (7 mm high) (Annex 2) to ensure that bees can go in and out of the hive without disrupting their natural behaviour. To maintain continued recording, a constant power supply is required, either via battery or an external power supply.

20. The reading rates of the RFID system should be known and should cover at least 95 % of the bees' passing. To achieve this, measures should be taken both, before the system is fitted to the hive, as well as before the start of the experiments by simulating honey bees passing (e.g. using tags that are glued on small plastic or wooden sticks). A protocol for controlling the performance of the RFID system is proposed in Annex 3.

Hive equipment

21. The first experimental hive is equipped with the RFID device at least two days before the start of the test. It is, however, necessary to check that the bees correctly circulate through the RFID readers in and out of the colony (i.e. no clustering of bees at the hive entrance) before the test and that no trophallaxis between inside and outside bees occurs at the bottom of the hive. One to two supers can be added on top of the hive in order to increase hive's volume and allow good thermoregulation during hot climatic conditions.

22. If all the colonies are installed at the test site at the same time and if the test runs are performed one after another within a short time, the colonies used for the next test runs should each be equipped with other RFID devices or a blank wooden or plastic system that mimics the RFID readers with the interface. This blank system is also placed at the hive entrance to allow the forager bees to familiarise themselves with the entrance style prior to fitting the RFID readers for the experiment. The blank system has also the function to impede foragers to enter unequipped hives (i.e. minimises honey bees drifting).

Tag batches

23. Pre-numbered 'Tag batches' are used to tag the bees. Each batch contains a number of RFID tags which must be read and identified before the experiment. A RFID pen reader is used to

identify the unique identification codes (UIDs) of the tags in a particular batch. The batches are then allocated to the corresponding treatment group. This allows the UIDs and hence the bees and batches to be tracked. At least three batches of ten tags are prepared per test run and treatment level; each batch of 10 tags is allocated to a replicate of 10 bees.

Feeding solution

24. The feeding solutions for the control, test and reference substance are prepared with 30 % (w/v) aqueous sucrose solution. It was previously demonstrated that a more concentrated 50 % (w/v) aqueous sucrose solution led to a much lower food consumption in some cases less than half of the test chemical feeding solution after a 40 min post-exposure starvation (10).

Preparation of the stock and final feeding solutions

25. In case of good water solubility, the stock solution of the test chemical is prepared in deionised water. If the test chemical has low water solubility, then acetone solvent is used instead. The concentration of organic solvent used depends on the solubility of the test chemical and should be the same for all the test chemical treatments tested. Considering the results of tests performed during the ring tests 2015 and 2016, the acetone concentration of the feeding solution should not exceed 1 % (v/v). Additional substances as solvents, solubilizer or thickener might be used if it can be demonstrated that the validity criteria for the control group are met. The stock solution can be stored appropriately (*e.g.* in tightly closed containers) in cool conditions in the dark (in the refrigerator). If the stability of the product is known, stock solutions can be prepared in advance of the test.

26. The final feeding solutions are prepared from the stock solution or dilution of intermediate solutions with 30 % (w/v) aqueous sucrose solution (treated feeding solutions). The final feeding solutions can be stored in the refrigerator.

27. The feeding solution should be freshly prepared for each test run.

28. If additives, e.g. a solvent, solubilizer or thickener, are added to the test sucrose solutions, appropriate, similar controls should be prepared. In this case, an additional deionised water control is prepared. However, if reliable data showing no effects of the additive are available, the water control need not be included.

Analytical verification

29. The test runs should be conducted with the same chemical batch.

30. For each test run, an adequate aliquot of (e.g. 5 mL) of each feeding solution is taken at the day of the test and frozen at ≤ –18°C for further analytical determination of the actual concentration in the feeding solutions and dose per bee. The stock solution will also be stored at ≤ –18°C the day of the test for analytical verification, if needed. Details on analysis of the feeding solutions and their stock solutions must be stated clearly in the final report of the study.

Test cages

31. The cages must be ventilated and of a suitable size for the number of the foragers captured. Cages can be constructed of stainless steel or made of plastic for single use only. Test cages should be designed so that the bees can be observed during the exposure period and to allow monitoring

of the uptake of the test solutions, either having transparent panels or being completely transparent for this purpose. They should also open easily to allow insertion and release of the bees.

TEST PROCEDURE

32. All the laboratory phases are performed in a room where temperature should be of 23 ± 3°C. The experimental unit is a cage (i.e. replicate) containing a group of ten individuals. There will be at least three cages or replicate per treatment. Exposure to the test chemical treatment is collective for the 3 replicates of the same treatment level. Each test run consists of an untreated control (water and/or organic solvent, preferably acetone), the test chemical comprising at least three sublethal doses, and the reference substance. The sublethal dosing solutions are spaced by a constant factor not exceeding three. These doses may be set by selecting the No Observable Effect Dose (NOED) or the LD_{10} (the dose at which 10% of the individuals tested will die) as the highest dose. If these data are not available, in order to determine the NOED, a preliminary test should be performed to establish this value. This can be done by conducting a modified OECD TG 213 acute toxicity test (Annex 4).

33. The homing flight test comprises three test runs performed at the same experimental site. Each test run is conducted with bees from one honey bee colony (one colony per test run is tested). Each test run is performed independently from the others, at different days. The data of the three test runs are pooled and analyzed in order to determine a NOED on homing success (see paragraph 57 to 62 “Data treatment”).

34. If possible, the homing flight test is conducted during periods of high forager bees activity and when food resources (nectar and pollen) are abundantly available from spring to summer. Tests might be conducted in late summer and early autumn too, if blooming and attractive flowers are available in the neighbourhood. However, a higher variability in weather conditions and higher *Varroa* infestations of the colonies (11) should be considered and avoided if performing tests in these periods (Annex 5). Under these circumstances it is advisable to report *Varroa* mite counts per colony.

Capture and preparation of “coloured” foragers

35. It is preferable to collect returning foragers carrying pellets of pollen at the hive entrance in the morning when foraging activity starts. Different methods can be used to collect foragers:

- Foragers collected one by one with entomological forceps

The collected bees are placed in groups of 100 to 300 individuals in boxes (e.g. plastic food trays of 600 to 2000 cm^3; 11 x 15 x 12 cm), each closed with a lid. Bees are introduced in each box via a hole in the lid closed with appropriate material (e.g. cork, piece of paper)

- Foragers collected with aspirator or similar (e.g. insect aspirator or modified vacuum)

The collected bees are captured in plastic bottles (e.g. 1000 cm^3) closed with a lid. Bees are kept in plastic bottles or transferred to boxes (e.g. plastic food trays of 600 to 2000 cm^3). When bees are not counted during the collection phase, containers (bottles or boxes) are pre-weighed when empty and then weighed again following of the bees captured using a field precision balance (e.g. max 500 g, precision 0.1 g). The resulting weight of bees is converted into a number of bees captured (weight of bees / mean weight per bee). To do so, a mean weight per bee may be estimated by weighing a group of 20 foragers of the experimental colony.

36. For each test run, a minimum of 600 bees from the same colony are captured and held in a minimum of collection units (i.e. a maximum of 200 bees per box). The bees may be fed during the collection with candy (e.g. Apifonda®) *ad libitum*.

37. Hydrophobic bright coloured powder is added to each box containing captured bees (at a rate of 30 mg powder per 100 bees). For the ring tests from 2016 to 2019, hydrophobic industrial bright red fluorescent pigments (e.g. Pigment Laser Red Fluorescent A3 – T series, COLOREY SAS, France) were used with an amount of approximately 0.3 mg per bee (i.e. 30 mg powder per 100 bees). No toxic effect on the bees at this rate was observed. Appropriate powder aliquots can be prepared in advance. The powder is introduced through the hole of the box lid or carefully into the bottles (care must be taken to prevent bees from escaping). Boxes are gently shaken in order to distribute the colour powder over the bees to mark them.

38. The coloured bees are transported to the release site 1 km (+/- 100 m) away from the experimental colonies. It has been demonstrated that bees regularly forage at distances of 1 km from the hive (12) (13) (14). The boxes with coloured bees are then all placed in close proximity to each other on a suitable flat surface. The containers are all then opened as simultaneously as possible and the bees allowed to exit. If necessary, the bees can be emptied out manually. In hot climates, it is recommended to perform this in the shade to avoid heat stress or suffocation of the bees.

Recapture of the coloured foragers at the hive entrance

39. To facilitate the recapture of coloured foragers, it is recommended to block the entrance of the RFID readers (e.g. with pieces of sponge) during release of the powdered bees at the release site. Coloured bees returning to the hive are collected off the flight board. The collection period should not exceed 2 hours following release of the bees (Annex 5). These bees will now have at least one homing experience to the hive from the release site and therefore, a prior knowledge of the way back to the colony. Bees are grouped into cages with *ad libitum* food. Candy (e.g. Apifonda®) can be used. If necessary, water can also be provided once the bees are recaptured. Cages with collected bees are kept in darkness in an isolated box (e.g. cooler) without cold blocks. A damp cloth can be placed inside the box in order to avoid overheating and to maintain high humidity.

40. The number of coloured bees recaptured depends on the number of treatment groups. A sufficient number of coloured foragers must be recaptured to obtain at least 30 forager honey bees to be labelled with a RFID tag per treatment group for each test run, before exposure in the laboratory (i.e. 150 to 180 labelled bees needed for three treatment groups, one control (water) or two controls (water + solvent) groups and one reference substance group). It is also advised to recapture extra coloured bees to avoid any problems (i.e. escaping bees, mortality...).

Feeding ad libitum

41. Recaptured foragers are transferred to the laboratory. They will be provided with food *ad libitum* (candy: e.g. Apifonda®) but no water for one hour in order to synchronize their dietary state. During this feeding period, cages are placed in dark conditions at 23 ± 3°C (e.g. half opened isolation box covered with a wet towel to avoid dehydration).

Labelling phase

42. After the feeding period, the bees undergo a starvation period of 90 minutes. During this period, the bees are transferred one by one from the cages to a holding cage where a sponge

plunger allows them to be immobilised without damage and labelled with a RFID tag (e.g. queen marking device, Annex 2). The tag is mounted on the dorsal part of the thorax of the foragers using a small drop of glue such as "dental cement". Care should be exercised to avoid gluing or hindering the movement of the wings (Annex 5). Dental cement (e.g. Temposil®, Coltene) is non-corrosive and has proven useful, as it dries very quickly in less than two minutes. During the labelling phase, the dental cement equipment is placed in crushed ice when not used to avoid the dental cement to dry immediately in the tip of the syringe.

43. The labelling is performed without using anaesthetic on the bees. The RFID tags are recorded and distributed by treatment beforehand (see paragraph 23 "Tag batches"). After labelling, the foragers are transferred in small groups of ten bees into cages (appropriately labelled with treatment group. A minimum of three replicates (cages) of ten bees per treatment is required. After labelling, the cages are placed in the dark before exposure to the test chemical-treated or control feeding solutions. Cages can be kept in an isolated box slightly opened, covered with a damp cloth in order to keep the bees cool and maintain suitable humidity.

44. It is recommended to label the bees per small groups, e.g. 10 by 10, and to alternate the order of bees' labelling according to treatment from one replicate to another (randomization, see Annex 5). If some bees die or lose their tag before the exposure phase, these bees may be replaced by newly labelled ones. Any tags becoming dislodged or not adhering to the bees can be re-used to label new test bees. In this case, the number of bees in the cages should be readjusted before the exposure phase.

Exposure phase

45. The foragers are fed with 20 µl per bee (200 µl per group of 10 bees) of the feeding solution containing the test chemical at the different concentrations, the control solutions and the reference substance. If 100 % pollen foragers are used, 40 µl per bee (400 µl per group of 10 bees) is recommended[*]. The volume of feeding solution is distributed using a feeder system enabling contact with the food only through the mouth parts (e.g. the bevelled tip of a micropipette, Annex 2). The bees in a cage will share the feeding solution by trophallaxis and thus an even treatment dose distribution to all bees is assumed. The exposure phase is completed once the bees have consumed all the offered volume. To facilitate the calculation of food uptake in the event that the bees do not consume the entire dose it is recommended that the feeders are weighed as they are filled.

46. The exposure period is one hour, in dark conditions in order to limit stress. If bees from some cages do not consume all the feeding solution within one hour, the exposure phase will be prolonged up to a maximum of 30 minutes or until all the bees have consumed the sucrose solution within all treatment groups (with a maximum exposure phase of 1.5 hours). The start and end time of exposure are recorded. If after the maximum exposure period of 1.5 hours there is still food remaining, the feeders will be re-weighed in order to calculate the consumption and hence the actual dose per bee, per cage received.

47. After exposure phase, the treated bees undergo another 40 minutes starvation period. During this period, cages are kept in dark and humid conditions, e.g. by placing them in a half-

[*] Pollen collectors generally have a lower crop content of nectar than nectar collectors and may need to consume more sucrose solution. The volume of sucrose solution may be increased to 40 µl per bee but will depend upon the requirement of the bees.

opened isolated box with a wet towel, to avoid dehydration and reduce stress to the test animals. After the 40 minutes starvation period to the release phase, no food is provided.

48. At the end of the exposure phase, the dead bees and those that have lost their tag, are recorded. They are collected during the release phase. The tags can be identified according to their UIDs and excluded from the study. From the exposure phase to the release phase in the field, the number of dead bees is used to calculate the mortality rate per treatment for each test run.

Honey bee release

49. It is recommended to pass a tag through each RFID reader just before release, in order to give a precise record of the time of release (this must of course be co-ordinated with the release team).

50. Following the exposure phase, the bees are then transported to the original release site (at 1 km (+/- 100 m) distance from the hive equipped with the RFID system), from where they were released following colouring. Safe keeping of the bees must be ensured during transport. The transport of the bee cages in cooling boxes containing a damp cloth has proved to be useful, particularly if the release site is far away from the laboratory.

51. The cages from all treatment and control groups are put in the same place, on a flat surface at least few cms off the ground (e.g. cages can be placed on a small table), and then opened simultaneously to release the bees. If necessary, the bees are emptied out. The behaviour of test bees should be observed until release. During release, bees should display normal behaviour and be able to fly when exiting the cages. Any physically damaged bees or any bees for which the tagging process has inhibited wing movement should be recorded and removed from the study.

52. A period of at least two hours is allowed between the release time and sunset to ensure that foragers are able to fly back to the hive. The release time (hour and minutes) is recorded. A thermo-hygrometer or other suitable device is used to measure local weather conditions (temperature and relative humidity (%)) during the release phase. Cloud coverage and wind strength (null, average, high) are also qualitatively recorded. Weather conditions must be favourable to foraging (wind below 5 of Beaufort scale, temperatures > 15 °C and no rain), this is particularly important for the release time to sunset, this period being the critical period for the foragers' homing flight.

Test schedule

53. For each test run, bee colouring, capture, labelling with RFID-tags, exposure and release phases take place over one day. The RFID recording of the labelled foragers' homing flight to the hive start immediately after the release and last 24 hours. A sequential pattern is proposed in Annex 6.

Observations

54. The data recorded with the RFID readers for the bees returning to the hive are the tag/chip ID number, the reader number and the reading time (date, hour, minute and second). These raw data are recorded continuously for 24 hours in electronic form in the storage system equipped with the appropriate software. Recorded raw data are collected 24 hours after the release.

55. The weather conditions (temperature and relative humidity) are recorded hourly using a data logger on the experimental site situated near the test hives, or can be provided by a local

weather station. If possible rainfall (in mm per day) and wind speed (m/s per hour) can also be recorded using the proposed weather station.

56. The geographic (GPS) coordinates of the release site and the location of the experimental colonies should be recorded.

DATA AND REPORTING

Data treatment

57. After labelling and before release in the field, the number of dead bees is used to calculate a mortality rate per treatment for each test run.

58. The homing flight is characterized by two variables:

- The homing success (main variable), which is a binomial variable with a value of 1 if the honey bee returns to the hive over the 24-hours period; or 0 if it does not return.

- The homing time 24 hours after release (complementary and optional variable), which is a quantitative variable. For each bee, it is defined as the time between the release and the first recording when entering the hive.

59. Homing success and its duration are determined from three data files: firstly from the honey bees released (with tag ID number), secondly from the information at the release site (date, hour and minutes of release) and thirdly from the RFID recording at the hive entrance. During the 24 hours of RFID recording, a bee can be recorded several times when it passes the RFID reader (in or out the hive) for foraging activities. Therefore, several data points may be recorded and can be calculated for the same bee. The shortest homing time per run, which corresponds to the first record at the hive after release, is retained for the calculation of the homing time.

60. The results should be presented as homing rate to the hive over the 24-hour period per treatment groups and test run. For the statistical analysis, data (e.g. number of bees released and the number of returning bees) from the three test runs should be pooled to maximize the number of bees and the statistical power of the test. The analysis performed per run, on smaller sample sizes, may present statistical limitations. Homing performances per treatment level may be illustrated (e.g. percentage of returning bees over the 24 h period). Results may include homing duration analysed in the same way as homing performances and also illustrated (e.g. boxplots with medians and quartiles).

61. Statistical analyses are performed using an appropriate statistical method. From the data of the three test runs pooled, the homing rates to the hive obtained over the 24-hour period for each treatment level may be compared using an exact binomial test or a Chi-Squared test. If homing duration data are analysed, data normality and homogeneity of variance will be tested first (e.g. Shapiro-Wilk test associated with a Bartlett test). When data show a normal distribution and variance homogeneity, homing durations obtained for each treatment are compared with a parametric test (e.g. linear model, one-way ANOVA). If not, data are compared using an appropriate non-parametric test (e.g. Kruskal-Wallis test followed by Mann-Whitney tests for paired comparisons). For homing rates and duration, an adjusted significance threshold is applied for paired comparisons with an appropriate method (e.g. Bonferroni, Dunn-Sidak method).

62. The analysed data will enable the estimation of a 'No Observed Effect Dose' (NOED) on the homing performance. The NOED is expressed as µg or ng of the test chemical per bee.

Test report

63. The test report should include the following information:

Test chemical:

- 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, expiration date for use;

- stability of the test chemical itself, if known;

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

Test system:

- Details on the test species (scientific name, race, age of the queen, health status of the colonies used and especially regarding *Varroa* mite infestation of the colonies, possible pre-treatments carried out).

Test conditions:

- Recording RFID system used for the homing flight and reading rate (paragraph 20);

- Place and dates when the test was conducted including geographical coordinates of the release site and the location of the experimental colonies;

- Number of test runs, control (water or solvent), number of tested doses, volume of solvent in the sucrose solution (where applicable), description of the test cages (type, material, size, feeding device, etc.), details on coloured powder used (name, provider, physical nature, chemical identification, relevant physical-chemical properties).

- Number of foragers captured before colouring, number of coloured bees recaptured after the first release, number of bees labelled and released a second time per treatment after exposure phase, number and percentage of dead bees per treatment and test run after exposure phase and before release, number of bees that lost their tags per test run after exposure phase and before release, RFID code of the bees released (dead bees or bees that lost their tags excluded) for each treatment and test run;

- Start and end time (hour and minutes) of feeding phase *ad libitum*, of starvation and exposure phases in the laboratory, time points of RFID recording: time (hour and minutes) of release in the field and the time point of the 24-hours recording;

- RFID data for each test run;

- Temperature and relative humidity conditions during the labelling and exposure phase in the laboratory, weather conditions during the release phase and the climatic conditions during the 24-hours period after release (at least hourly).

- Other observations such as feeder weight, pre-feeding and post feeding, if any of the treatment groups fail to completely consume the food amount offered. This allows calculation of the actual quantity consumed and hence the dose ingested.

Results:

- Results of the preliminary study determining the range of treatment doses, if conducted, or other justification for the dose selection (see Annex 4);

- Nominal doses used, measured doses of the test chemical in the feeding solutions for the three test runs, and analytical method used;

- Mortality rate of all treatment, control and reference substance groups before release;

- Consumption of the feeding solution as actual test chemical uptake per treatment group from feeders' weight. If all is consumed, the tested dose per bee is considered ingested.

- Statement regarding the validity of the study, with regard to the validity criteria;

- Homing rate (%) over the 24-hours period for each treatment and control per test run as well as for the data of the test runs pooled;

- Homing duration over the 24-hours period for each treatment and control per test run as well as for the data of the test runs pooled if performed;

- Description of the statistical analyses carried out; NOED (µg or ng per bee) on the homing success determined from the data of the pooled test runs;

- Any deviation from the guideline or any other relevant information (e.g. presence of blooming crops, flowers or trees at the time of the experiment to support foraging activity…).

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Annex 1: Definitions

Dose is the defined as the amount of test chemical consumed or applied. Dose is expressed as mass of test chemical per test animal. It is expressed in µg or ng active ingredient or formulated product per bee.

LD_{10}: is the lowest dose of the test chemical (µg or ng per bee) calculated or estimated from an acute toxicity test (OECD TG 213), that can cause death in a maximum of 10 % of the individuals when administered by the oral route. However, this dose does not significantly induce higher mortality when compared to the control bees, 48 to 96-h after exposure. It is therefore considered as the highest dose without effect on mortality for the homing flight test dose range.

NOED (No Observed Effect Dose):
1) In an acute toxicity test (Annex 4), the NOED is the highest dose (µg or ng per bee) that does not significantly induce higher mortality when compared to the control bees, at least 24-h after exposure.
2) In a homing flight test, the NOED (µg or ng per bee) is the dose that does not significantly induce higher homing failure when compared to the control bees 24-h after release.

Release point in the homing test is the site located at 1 km (+/- 100 m) away from the experimental colony where the forager bees are released **1)** the first time after being coloured with a powder to recognize and recapture the bees of interest at the hive entrance for the test, **2)** a second time after RFID labelling and exposure in the laboratory to follow the homing performance 24 h-after release.

RFID (Radio Frequency Identification) is a technology that allow detecting (scanning) each time a tag-equipped bee is passing a reader located at the hive's entrance (working distance of 3 mm). The principle depends on the emission of a radio signal by the reader which is reflected by the RFID tag (2.0 x 1.7 x 0.5 mm and 3 mg) mounted on the bee's thorax and provides the unique identification (UID) code of the bee. The tag has no power source (passive function) and obtains its operating power during the reading process from the RFID reader.

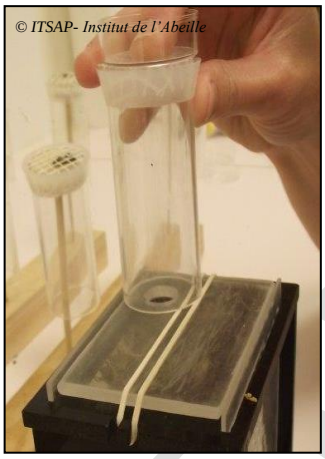
Test run is a homing test performed with the bees from one colony. For the homing flight test, three tests runs are performed on different days, each one with a different colony.

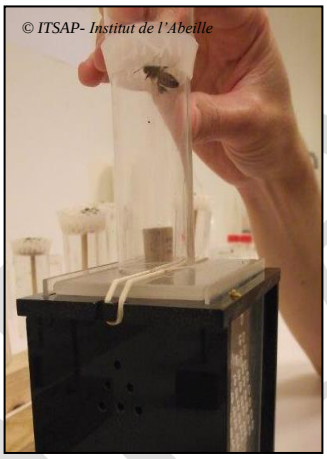
UID (Unique Identification) means the unique code for an RFID tag. This technology offers the possibility to have an unlimited number of codes that allows to individualize the bees.

Annex 2: Illustrations

Capture at the hive entrance
Hive is equipped with a RFID system
(Microsensys, GmbH, Erfurt, Germany)

Coloured bee on the flight board





RFID tag glued on the bee thorax
with glue
(dental cement, Temposil®)

Transfer of a forager to a holding cage for labelling with a RFID tag

Collective exposure phase followed by
a 40 mins-post starvation period

Honey bee release in field conditions

Tagged bee on the flight board
RFID reader with tunnel to scan labelled bees
going in and out the hive

Annex 3: Protocol to control the performance of the RFID system

- 6 « test » tags glued onto small plastic or wooden sticks. UIDs of the tags are first recorded.

- Each tag is passed five times through each of the four to five readers ➔ Twenty to twenty-five readings per tag and an expected total of 120 to 150 readings for the 6 test tags

- Tested tag should be read at least one time each time it passes through a reader

- Reading rates (%) = (recorded data/expected data) x 100

Expected data are 120 or 150 readings according to the number of readers.

The acceptance criterion for the performance of the RFID system is that at least 95% of the reading of the tags should be recorded.

Annex 4: Preliminary test procedure

- The homing flight test must be conducted at sublethal doses. If data on sublethal effects are not available (e.g. LD_{10}) from acute toxicity tests, a preliminary test in the laboratory may be conducted on forager bees. Mortality must be ≤ 15% as a validity criterion. Care should be taken in choosing the tested dose, as the threshold between lethal and sublethal doses can sometimes vary considerably depending on factors related to the bees (genetic, food stress, health, stomach content) and the climate. The preliminary test is based on OECD TG 213 procedure and allows a NOED ('No Observed Effect Dose') to be determined on mortality at least 24 hours after exposure. This dose corresponds to the maximum experimental dose not causing significantly higher mortality than that of the control.

Amendments to OECD TG 213 are:

- Use of foragers, preferably pollen foragers, as test bees. They are captured at the hive entrance in the morning when foraging begins to be active and grouped into cages (10 bees per cage) with food *ad libitum* such as candy (e.g. Apifonda®). The captured bees may be collected from different colonies with individuals of one colony per cage (bees of different colonies must not be mixed),

- Three to five test concentrations with a factor not exceeding 3. The number of doses and the progression of the dose range must be specified,

- Exposure condition: in laboratory, the bees undergo a 1.5 hours starvation period before exposure. Then, they are exposed by giving them 20 or 40 µl per bee (200 µl for 10 bees) of the feeding solution containing the test chemical at different concentrations or the control solution,

- 30 % (w/v) aqueous sucrose solution for feeding solutions and feeding *ad libitum*,

- Mortality is recorded until at least 24 hours,

- The number of dead bees at least 24h after exposure may be compared using an exact binomial test or a Chi-Squared test. An adjusted significance threshold is applied for paired comparisons with appropriate method (e.g. Bonferroni, Dunn-Sidak method).

795 **Annex 5: Recommendations for the homing flight test performance**
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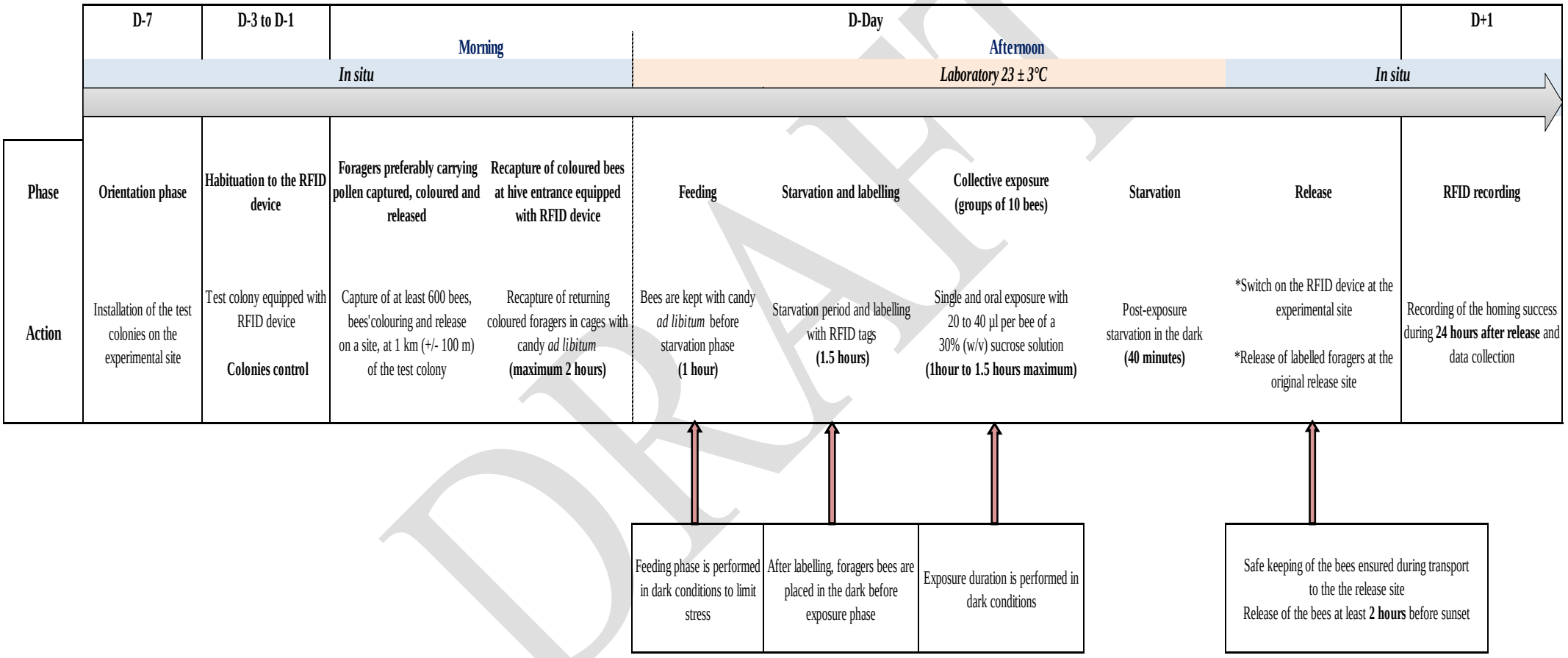
Considerations	Recommendations
Homing flight test performance	1- Practical training is required before performing the homing flight test for the first time.
Weather conditions & food resources	1- As far as possible, the test should be conducted during periods of high bee activity, when weather conditions are favourable and when food resources (nectar and pollen) are available: **spring and summer**. 2- Tests should not be performed under very high temperatures, which may impact the bee's behaviour. 3- Tests may be conducted in late summer - early autumn season if blooming and attractive flowers are available. Problems may occur due to the higher variability in weather conditions and because of varroa infestation of the colonies. It is advised to check for Varroa load in such cases. Varroa count per colony should be below 5 varroa mites per 100 bees.
Colonies	1- Prepare the colonies according to local conditions (strong and active colonies with sufficient brood and food resources) 2- Check for good activity of the colony by monitoring the foraging activity and especially pollen foragers at the hive entrance in the morning prior to starting a test. 3- Pay attention that bees can pass through the RFID readers to enter and exit the colony (e.g. no clustering of bees at the hive entrance) and that no trophallaxis between inside and outside bees occurs. One to two supers can be added on top of the hive in order to increase hive's volume and assist thermoregulation during hot climatic conditions.
Coloured bees recapture	1- From experience of the ring test participants, all or almost all the required coloured bees will return to the hive within the first hour (maximum recapture time should be up to a maximum of 2 hours after release). 2- As an indicator, if few coloured bees are collected within the first hour after release (e.g. less than 100 bees), it's more appropriate to perform the test again another day.
Labelling phase	1- A labelling training session with metal tags is recommended (using blank or damaged RFID tags, which may be available from the manufacturer on request) before performing the test, as experience gluing and fixing tags is crucial. 2- The number of operators needs to be adapted to enable the labelling of all the bees in 90 min. (minimum of 30 bees per treatment and run). 3- Attention should be paid not to damage bees or attach tags in such a way that the bees' wings are damaged or their movement is impeded. 4- For each test run, replicates of 10 bees (total of at least 30 bees per treatment level) will be labelled randomly for all the treatments during the labelling session. Sequential labelling of replicates of 10 bees from the same treatment level must be avoided for a successful randomisation.

Annex 6: Sequential pattern of the homing flight test



Annex 7: Alternative method to the bees colouring using a Phacelia field

This annex presents specific information on the use of a Phacelia field, as an alternative to the bees colouring, capture and release. The other steps of the test follow this Test Guideline.

1) Plantation of a specific Phacelia crop:

A plot of blooming Phacelia approximately 1 hectare in size is planted so that the nearest border is 1 km (+/- 100 m) away from the experimental colonies. The length of the shortest plot edge should not be less than 30 m. Due to its specific and easily recognisable pollen colour, this plant allows easy identification of bees foraging on this crop. No chemical treatments must be applied to the Phacelia prior or during the testing period.

If it is not possible to have a 1-ha field of Phacelia, the plot area can be a reduced if necessary, as the objective is only to collect a sufficient number of foragers carrying pellets of Phacelia pollen. However, attention must be paid to other crops blooming; the phacelia must remain attractive to the bees to allow the collection of a sufficient number of Phacelia foragers. It is recommended not to have a Phacelia plot less than 0.5 ha in size.

There must be no other plot of blooming Phacelia within 4 km around the experimental hive (about twice the average distance covered by the pollen foragers in a simple agricultural landscape, (12)). High density of hive (more than 50 hives) in a 1-km radius away from phacelia plot should be avoided.

It is recommended to start the trial when at least 50% of the plot of Phacelia is blooming, but it's possible to start testing before this point if bees are actively foraging on the crop.

2) Honey bees capture at the hive entrance for the test

In the morning, only foragers carrying purple pellets of Phacelia pollen, returning from the Phacelia plot specifically planted, are captured on the flight board when foraging begins to be active and grouped into cages with food *ad libitum* according to this Test Guideline procedure.

➔ To facilitate the recruitment and capture of sufficient numbers of foragers of Phacelia pollen for the test, forager honey bees, carrying pellets of pollen (or not), can be collected from the flight board into cages and released at the Phacelia plot early in the morning one or two times. This may encourage foraging and recruitment of 'phacelia foragers' and thus increase numbers of desired bees needed for the study.

3) Laboratory phase and release

After the capture, the Phacelia pollen forager bees are transported to the laboratory to be labelled and exposed according to this Test Guideline procedure. At the release time, the Phacelia field is the release point of the RFID tagged bees (the bees are familiar with the site).