Organisation for Economic Co-operation and Development

ENV/CBC/MONO(2021)7

Unclassified English - Or. English

16 July 2021

ENVIRONMENT DIRECTORATE
CHEMICALS AND BIOTECHNOLOGY COMMITTEE

GUIDANCE DOCUMENT ON HONEY BEE (APIS MELLIFERA L.) HOMING FLIGHT TEST, USING SINGLE ORAL EXPOSURE TO SUBLETHAL DOSES OF TEST CHEMICAL

Series on Testing and Assessment,
No. 332

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JT03479488

SERIES ON TESTING AND ASSESSMENT

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Environment Directorate
ORGANISATION FOR ECONOMIC COOPERATION AND DEVELOPMENT
Paris 2021

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Foreword

This guidance document presents a standardised test evaluating the ability of honeybees (Apis mellifera) exposed to a single dose of test chemical to return to the hive; it is a semi-field test, where dietary exposure to the test chemical and bee tagging occur in the laboratory prior to release in the field until the return to the hive. The project to develop this homing flight test was led by France; the project started in 2016 at the OECD. Several phases of inter-laboratory testing were organised between 2016 and 2019; reports are compiled in a document No. 333 published in the OECD Series on Testing and Assessment.

This guidance document was reviewed by a group of international experts in 2020 and approved by the Working Party of the National Coordinators of the Test Guidelines Programme in April 2021. The guidance document is published under the responsibility of the Chemicals and Biotechnology Committee of the OECD.

1 INTRODUCTION

1. This Guidance Document aims at assessing effects of test chemicals on the homing ability of forager honey bees (also referred to as “bees” throughout the Guidance Document) following oral 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 (6) for the first time in 2015 by eleven participating European laboratories. From 2016 to 2019 the ring testing was continued with 8 to 11 participating laboratories. The method described in this Guidance Document corresponds to the protocol ring-tested in 2019, with minor adjustments that result from the outcomes of this validation exercise. The test could be adapted for contact exposure. However, this exposure route has not been validated.

2. Pollinators, such as honey bees, may be exposed to residues of plant protection products (PPP) or other chemicals while foraging. Oral exposure to these residues may not cause direct lethal toxicity in foragers or may not induce visible signs of intoxication, 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 can be requested, especially when results of Tier 1 toxicity tests suggest that further evaluation is needed. In its guidance document (5), EFSA highlights that the Working Group concluded that a homing study can provide useful information on a range of sublethal parameters. The present test is carried out in order to determine effects of acute oral exposure to sublethal doses of a test chemical on the homing flight 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 one or more active substances and potentially auxiliary substances.

4. The data of the homing flight test should be included appropriately in the honey bee risk assessment.

2 DEFINITIONS

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

3 INITIAL CONSIDERATIONS AND LIMITATIONS

6. This Guidance Document describes a method that uses forager bees which are already familiar with their environment. To ensure this, returning forager honey bees 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 recaptured at the entrance of the hive and transferred to the laboratory before starting the chemical single exposure experiment.

7. The homing flight test requires training to acquire a level of technical proficiency to generate a valid study. Photographs in annexes and videos for critical phases (e.g. labelling phase) have been produced to illustrate guidance provided in this document and ensure the success of the test in the best possible conditions [link to video : https://youtu.be/LpKLCgdCYVQ].

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 Guidance Document, upfront consideration should be given to whether the results of such testing will yield results that are scientifically meaningful, or not. If the Guidance Document is used for the testing of a mixture other than pesticide formulations, 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.

4 PRINCIPLE OF THE TEST

9. This test method measures the effect of single sublethal oral doses of a test chemical (under controlled conditions) on the homing success of forager honey bees (under simulated field realistic conditions). Foragers are released 1 km (+/- 100 m) away from the colony. 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. Results from three separate test runs are required for interpreting results of a full homing flight test.

10. Prior to labelling with a RFID tag, returning foragers (mix of nectar and pollen foragers) are collected at the hive entrance, coloured with non-toxic powder and released 1 km (+/- 100 m) from the colony. Returning coloured bees are collected at the hive entrance and selected as test bees. In order to ensure a sufficient number of test bees for all treatment groups, an excess of bees required are coloured and released, prior to recapture 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 one of the possible treatments corresponding to at least three dosing solutions of the test chemical, the control solution(s) (water control, or water and solvent controls; if solvent is used it should be preferably acetone up to 1 %) and to the reference substance solution. All solutions are administered orally and collectively to the honey bees (in small groups of ten bees) using 20 µl to 30 µl of a 30 % w/v aqueous sucrose solution per bee.

12. RFID-tagged honey bees are 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 at the hive's entrance as returning tagged bees pass through the RFID reader. The monitoring period has a duration of 24 hours from the release of the test bees. 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) on homing success from all the doses of the chemical tested.

VALIDITY OF THE TEST

13. The following validity criteria apply:

- For each test run, mortality in individual replicate of control and treatment groups should be less than or equal to 20 % from the exposure phase to the release phase in the field. Average mortality across replicates for the total number of controls and in all treatment groups, including the one in the group of bees exposed to the reference substance, should be less than or equal to 15 % from the exposure phase to the release phase in the field.

- For each test run, average homing success rate across replicates for the total number of control honey bees should be greater than or equal to 70 % over a 24-hour period after release.

- Homing success of the bees exposed to thiamethoxam should be statistically different from control bees and should be reduced to at least 20 % compared to control bees (from data of the test runs pooled for statistical analyses).

A full homing test comprises three test runs. In the case where one over three runs does not meet the validity criteria enumerated above or if results statistically differ from those of the two others runs (see paragraph 62), the run is ignored. A fourth run is then performed with another colony to replace the unsuccessful one. This must be done maximally one week after the last original test run.

5 REFERENCE SUBSTANCE

14. A reference substance should be included in each test run to ensure that the test system and corresponding conditions are responsive and reliable. From the ring test results with European honey bees, technical grade thiamethoxam showed to be appropriate for that purpose and is recommended at a nominal dose of 1 ng to 1.5 ng per bee. In the case where another dose of thiamethoxam would be tested according to bee subspecies, it must be justified.

6 TEST CONDITIONS TO MEET THE VALIDITY CRITERIA

15. From the literature and ring test experience (6), the test conditions that should be applied to fulfill the validity criteria are summarized in Table 1. This Table is referenced all along the method description.

Table 1: Summary of the test conditions to be fulfilled to meet the validity criteria

Considerations		Conditions
Colonies	**Avoidance of bee drift** (8), (9)	**- Colonies (at least 4) isolated from the rest of the apiary** (experimental site restricted to the tested colonies) **- Hives arranged in an apiary layout** (e.g. triangle, square arrangement…according to the number of hives) **- Hives separated by at least 10 meters**
	Colony size	**Colonies are checked before the test (one to three days before) to be composed of**: - A mated queen laying eggs - At least 15,000 adult worker bees - A proportion of 0.5 to 0.7 brood frames (all stages), 0.2 to 0.3 food frames (honey, nectar and pollen) and 0.1 to 0.2 empty frames for food storage. *To illustrate with a 10-frame configuration hive*: *5 to 7 brood frames, 2 to 3 food frames and at least 1 empty frame.*
	Hive configuration	**To allow a good circulation of the bees in and out of the RFID-equipped hive and to avoid trophallaxis between inside and outside bees at the hive floor:** - **Under cool temperatures**: hives with a full floor (no mesh) - **Under high summer temperatures**: hives with modified floor for the thermoregulation and ventilation of the colony. For the ring test, two mesh floors fixed together and separated by few centimeters with a super were used The hive volume can also be increased by adding one super on the top of the hive.
	Foraging activity	**Colonies providing enough foragers for the test**: The day of the test in the morning, the foraging activity of the colony must allow the capture of at least 600 foragers (mix of pollen and nectar collectors) returning to the hive

	Health status of the colonies (12)	**No visible symptoms of disease** **Varroa count** ideally as low as possible but in any case **≤ 3 mites per 100 bees** for each colony. (e.g. counting using the powder sugar method (Annex 2) or other relevant Varroa counting method)
Performance of the RFID system		**Control of the RFID system** (Annex 4): **-**Before the system is fitted to the hive -Before each test run
Weather conditions at the time of release of the labelled bees (11), (12)		**- Temperature range**: 18 to 31°C. Maximum may be of 35°C under hot climates (e.g. Mediterranean climate) **- Wind speed**: ≤ 4 of Beaufort scale representing 5.6 to 7.8 m/s **- No rain**

7 DESCRIPTION OF THE METHOD

The honey bees and the preparation of the colonies

16. Three different colonies are tested in separate test runs. They contain mated queen of known origin, but the use of sister queens should be avoided. Queens are not older than two years. No chemical treatments (such as anti-Varroa treatments, antibiotics…) must have been applied 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. Health status of the colonies including Varroa mite counts (as number of Varroa per 100 bees) should be recorded. Indeed, Varroa may negatively impact the homing performances of the bees (6) (7). To perform the counting, bees are sampled from a brood frame with opened and capped brood. Test colonies should be comparable regarding their colony strength, brood activity and food storage. Considerations for the size, hive configuration and health status of the tested colonies are stated in Table 1.

17. Colonies used for the test are installed at the experimental site, at least one week before the first test run and if possible with favourable weather conditions for foraging activities, to allow an acclimation period and the bees to familiarise themselves with the environment. It is advised to install at least 4 colonies if a supplementary test run must be performed (see paragraph 13). Importantly, if the colonies are placed at the same time on the site drift of bees between colonies should be avoided when applying the conditions stated in Table 1.

Technique for honey bee identification

18. Use of RFID (Radio Frequency Identification) technology is proposed as it has been proven to be an efficient method to measure the effects of chemicals like plant protection products (e.g. thiamethoxam) on the homing flight ability of foragers (1) (2) or foraging behaviour (13). This was also demonstrated by the ring test results. The use of individually RFID-tagged honey bees and hive mounted readers allows continuous monitoring and recording of the return of the test bees to the hive. Other technology may be used if proven to be efficient and allowing continuous identification of returning bees.

RFID device

19. The RFID technology (1) (14) allows detection each time a tagged bee passes the reader located at the hive's entrance (e.g. working distance of 3 mm). The principle is based 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. Then, using real-time recording, the reader sends the data scanned from the RFID tag to a storage system, which are 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. The RFID tags (e.g. 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 (without the glue) should not be more than 5 mg, which is equivalent to about 5 % of the body weight of a worker bee. This is also 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 (15) (16). Each tag has an unique identification code (UID).

20. Any RFID system and design can be used for the test according to the available technology. In the ring tests 2015-2019, the MAJA 13.56 MHz RFID system (Microsensys GmbH, Erfurt, Germany) was used. Based on the ring test results, four to five RFID readers should be installed in parallel at the hive entrance by means of an interface (constructed of plastic or wood) between hive and readers (Annex 3). The interface must impede the bees to enter/exit the hive except through the readers. Readers were separated from each other by a minimum of 30 mm to avoid interference during RFID recording. Each reader spanned a tunnel of 14 × 21.5 mm (7 mm high) to ensure that bees can go in and out of the hive without interrupting their natural behaviour. This tunnel allows detecting any RFID-tagged bees regardless of their position when crossing a reader, because of antennas positioned up and down the reader. To maintain continued recording, a constant power supply is required, either via battery or an external power supply.

21. The reading rates of the RFID system should be known and should cover at least 95 % of the bees' passing. To achieve this, measurements should be taken both before the system is fitted to the hive as well as before the start of each test run by simulating honey bees passing (e.g. using tags that are glued on small plastic or wooden sticks) (Table 1). An example of a protocol for controlling the performance of the RFID system is described in Annex 4.

Hive equipment

22. The experimental hive is equipped with the RFID device at the entrance at least two days before the test. Before fitting the system on the hive, it is necessary to modify the hive configuration for a good thermoregulation and bees' circulation through the readers (i.e. no clustering of bees at the hive entrance) but also to avoid trophallaxis between inside and outside bees at the bottom of the hive. Considerations for the hive configuration are stated in Table 1.

23. If all the colonies are installed at the test site at the same time and if the test runs are performed one after the other within a short time (maximum one week between the tests), 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. It also functions to impede entry of foragers into unequipped hives (i.e. minimises honey bees drifting).

Tag batches

24. 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 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 10 tags are prepared per test run and treatment group. Each batch of 10 tags is allocated to a replicate of 10 bees.

Feeding solution

25. The feeding solutions for the control, test and reference substance are prepared with 30 % (w/v) aqueous sucrose solution. Previous studies have demonstrated that a more concentrated 50 % (w/v) aqueous sucrose solution led to a much lower food digestion during the post-exposure starvation. In some cases, the bees assimilated less than half of the test chemical feeding solution after a 40 min post-exposure starvation (17). The feeding solution should be prepared at maximum one day before for each test run.

Preparation of the stock and final feeding solutions

26. 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 organic solvent (e.g. acetone) can be used instead of water. 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 such as solubilizer or thickener might be used if it can be demonstrated that the validity criteria for the control group are met. The stock solution should be stored appropriately (*e.g.* in tightly closed containers) in cool conditions in the dark (in the refrigerator). New stock solutions are prepared for each test run. If the product is assumed to be stable in the aqueous or solvent solution, stock solution can be prepared in advance of the test. If not, it has to be prepared freshly the day of the test run.

27. 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 are prepared maximum one day before each test run according to the stability of the product and should be stored in the refrigerator.

28. If organic solvent or additives (e.g. solubilizer or thickener) are added to the test sucrose solutions, appropriate controls should be prepared in addition to the deionised water control.

Analytical verification

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

30. For each test run, an adequate aliquot (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. The stock solution should also be stored at ≤ –18°C the day of the test for additional analytical verification, if problem with the feeding solution analysis arises. Details on analysis of the feeding solutions and their stock solutions must be stated clearly in the final report of the study.

31. The measured concentration should be within +/- 20% of the nominal. If this is the case, the results can be expressed as nominal or measured concentrations, if not, the results should be expressed as measured concentrations. When using measured concentrations, it should still be possible to statistically differentiate between the different treatment groups tested.

Test cages

32. The cages must be ventilated and of a suitable size for the number of the foragers captured in field or exposed after labelling in laboratory. 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. If different cages than those for the capture of the bees are used for the exposure phase after labelling, the size should be at least 200 cm^3 to provide adequate space for 10 bees per cage.

8 TEST PROCEDURE

Test design

33. The full homing test comprises three test runs performed at the same experimental site. Each test run is conducted with bees from a different colony (one colony per test run is tested) (Table 2). For logistical reasons it is recommended to perform each test run independently from the others, at different days (maximum one week between the tests). One test run includes one day of manipulation (e.g. capture, labelling, exposure and release) and 24-h RFID recording of the homing success. The data from the three test runs are pooled, if not statistically different (see paragraph 62), and analyzed in order to determine a NOED on homing success.

34. All the laboratory phases are performed at 24 ± 2°C. The replicate is a cage containing a group of ten adult forager bees. There are at least three replicates (cages) per test group. Each experimental test run consists of one or two untreated control (water or water and solvent), at least three sublethal doses of the test chemical, and the reference substance as test groups (Table 2).

Table 2: Summary of the test design

Considerations	Conditions
Number of test runs and colonies for the homing test	3 test runs with one different colony for each test run
Number of test groups	For each test run: - 1 or 2 untreated controls (water or water and solvent) - At least 3 sublethal doses of the test chemical - The reference substance thiamethoxam at the nominal dose of 1 ng to 1.5 ng per bee
Number of replicates	For each test run: -At least 3 replicates (cages) per test group ➔ the replicate is the cage containing a group of 10 bees
Total number of cages	For each test run with 5 test groups (e.g. 1 water control, 3 sublethal doses of the test chemical, 1 sublethal dose of the reference substance): - At least 15 cages containing each 10 labelled bees
Number of bees labelled and exposed	For each test run: At least 30 bees per test group with 10 labelled bees per cage

Tested doses

35. The sublethal dosing concentrations of the test chemical are spaced by a constant factor not exceeding three. To select the tested doses, mortality of exposed bees should not statistically differ from those of control bees and the doses should not induce severe signs of intoxication (e.g. knock down, affected or trembling bees) that may prevent the bees from flying. The dose range may be set by selecting the LD_{10} obtained from previous acute toxicity tests (OECD TG 213) (18) divided by two or a NOED on mortality as the highest dose. If data on sublethal effects are not available, a preliminary test should be performed to establish the NOED (mortality, 48 h), by conducting a modified OECD TG 213 acute oral toxicity test (Annex 5). As an option for the dose range, if relevant data on environmental concentrations of the test chemical are available, the test might be performed by testing at least one environmental dose to which bees have a risk to be exposed to (e.g. 90^{th} percentile exposure case) instead of experimental dose(s).

Capture and preparation of "coloured" foragers

36. Returning foragers (mix of nectar and pollen foragers) are collected at the hive entrance in the morning when foraging activity starts. Different methods can be used to collect foragers. Two examples of methods are proposed below:

- Foragers collected one by one and counted (e.g. use of 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), 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 with captured bees 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 colonies.

37. Any other method may be used if it proves to be suitable to collect enough returning foragers for the test without hurting them.

38. For each test run, a minimum of 600 bees from the same colony are captured and held in a minimum of collection units (see also Table 1). Then, containers with captured bees should be placed in the shade and in an insulated box (e.g. cooler) to protect them from heat stress. The bees are also fed during the collection with candy (e.g. Apifonda®*) *ad libitum* and may be provided with water.

39. Hydrophobic bright coloured powder is added to each box containing captured bees. Colouring only helps to recognize and recapture the bees of interest returning to the hive after release. For the ring tests 2016 to 2019, hydrophobic industrial bright red fluorescent pigment (Table 3) was used with an amount of approximately 0.3 mg per bee (i.e. 30 mg powder per 100 bees). At this rate, recaptured bees could clean and remove powder easily when grouped in cages and no toxic effect alone or in interaction with thiamethoxam was observed from a pre-test (6) and ring test results as a whole. Other hydrophobic fluorescent pigments can be suitable (preferably pink, orange, red). These can be obtained from providers of pigments and dyes for the industrial sector. Nevertheless, preliminary experiments need to be performed to check the suitability of these other pigments (safe for the bees and providing a good colouring). 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.

Table 3: Characteristics of the hydrophobic industrial bright red fluorescent pigment used for the ring tests 2016 to 2019

Name of powder	Technical characteristics
Pigment Laser Red Fluorescent A3 (T series, COLOREY SAS, France)	- **Aspect**: powder - **Granulometry / Average particle size**: 5-6 µm - **Density (at 20 ° C)**: 1.37 g / cm3 - **pH (at 25°C)**: approximately 6-7.5 in 5% aqueous dispersion

* Composition of Apifonda® :

- Saccharose: max. 83.0 %,
- Fructose : about 5 %,
- Glucose : about 5 %,
- Other sugars : about 7-8 %

	- **Solubility**: insoluble in water, hydrocarbons and most common solvents - **Decomposition point**: 190°C

40. The coloured bees are transported in the isolated box (e.g. cooler) and in darkness 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 (19) (20) (21). 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 (e.g. summer conditions), it is recommended to perform the release in the shade to avoid heat stress or suffocation of the boxed bees. Release of the bees in front of hedges, forests or buildings (distance from the release site ≤ 20 m) should be avoided not to disturb homing flight. Foragers should also not cross highways or surface waters (e.g. impoundments, rivers) during the travel back to the colony. The release site should be the same for all test runs of a full homing flight test. Any spraying event in the close environment (i.e. between the release site and the home apiary, and about 300 meters around the anticipated flight path) of the release site and at the time of the bees' release should also be avoided. In such a case, the test should be postponed. The release site may be changed if spraying event occurs during the first test run.

Recapture of the coloured foragers at the hive entrance

41. 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 coloured 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. These bees will 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 containing candy *ad libitum* (e.g. Apifonda®). Water may also be provided. Cages for recapture are alternated every time a returning bee is collected. This allows the captured bees to be randomly allocated to cages. The cages with collected bees are kept in darkness in an insulated box (e.g. cooler) without any cold blocks. A damp cloth can be put inside the box in order to avoid overheating and to maintain high humidity.

42. 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 foragers to be labelled with a RFID tag per treatment group for each test run before exposure in the laboratory (e.g. 150 to 180 labelled bees for three treatment groups, one control (water) or two controls (water + solvent) groups and one reference substance group). It is advised to recapture a surplus of coloured bees for possible replacements (i.e. escaped or dead bees).

Feeding ad libitum

43. Recaptured foragers are transferred to the laboratory. They are kept with solid 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 24 ± 2°C (e.g. half opened insulated box covered with a damp cloth to avoid dehydration).

Labelling phase

44. After the feeding period, the bees undergo a starvation period of 1.5 hours. 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 (Annex 3). Sides of the tag are gently picked up with fine forceps and mounted on the dorsal part of the thorax of the foragers using a small drop of glue such as "dental cement" (see paragraph 46). Care should be exercised to avoid gluing or hindering the movement

of the wings but also to gently fix the tag with forceps in the glue without damaging its upper encoded part. 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 [link to video on labelling phase: https://youtu.be/LpKLCgdCYVQ].

45. The labelling is performed without using anaesthetic on the bees. The RFID tags are recorded and assigned to the treatment groups beforehand (see paragraph 24 “Tag batches”). The bees prepared for labelling are collected from different capture cages to randomly allocate them within and across treatments after labelling. The order of labelling of the bees is also randomized for each replicate and treatment group assigned. The tag batches used should be recorded once the bees are labelled.

46. Dental cement (e.g. Temposil®, Coltene) can be used to glue the tag. It is biocompatible with a good retention rate (tag loss of 3 % as a mean for labelled living bees over the five ring test years). It has also proven useful as it dries very quickly, in less than two minutes. During the labelling phase, when not in use, the dental cement must be placed on crushed ice to prevent the cement from drying in the tip of the syringe. Other glue may be used if it offers the same features. Two operators perform the labelling operation (one operator to apply the glue and another one to affix the tag) and a total of at least 4 to 5 operators are needed for the labelling phase with 5 to 6 treatment groups (Figure 1).

47. After labelling, the foragers are transferred in small groups of 10 bees into cages (appropriately labelled with replicate and treatment group). A minimum of 3 replicates (cages) of 10 bees per treatment is required (Table 2). Once the labelled bees have been transferred into a cage, the cage is placed in the dark (e.g. cages can be kept in an insulated box slightly opened, covered with a damp cloth in order to keep the bees cool and maintain suitable humidity before the exposure phase). 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. Attention should be paid not to mix a bee labelled with a tag assigned to one replicate and treatment group in another replicate or treatment group. If a tag cannot be re-assigned to one cage and treatment, it is discarded from the study.

Exposure phase

48. Each replicate of 10 foragers are exposed with 20 µl to 30 µl per bee (200 to 300 µl per group of 10 bees) either to one of 3 tested concentrations of the test chemical, to the control solution(s) or to the reference substance solution. 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 3). The bees in a cage share the feeding solution by trophallaxis and thus an even treatment dose distribution to all bees is assumed. To facilitate the calculation of food uptake in the event that the bees do not consume the entire dose, the feeders must be weighed as they are filled.

49. The exposure period is 1 hour and is performed in dark conditions to limit stress. The volume of food consumed by the bees is recorded at least every 30 mins during this phase. If bees from some cages do not consume all the feeding solution within one hour, the exposure phase is prolonged for all cages 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 exposure phase is completed once the bees have consumed all the offered volume of feeding solution. 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 must be re-weighed in order to calculate the consumption and hence the actual dose per cage and per bee received. Any evidence of possible regurgitation during exposure phase should be reported and the bees from these cages should be discarded as the actual dose received per bee cannot be calculated.

50. After the 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-opened insulated box

with a damp cloth to avoid dehydration and reduce stress to the test animals. After the 40 minutes starvation period to the release phase, no food is provided.

51. From the exposure phase to the release phase in the field, 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. The number of dead bees is used to calculate the mortality rate per treatment for each test run.

Honey bee release

52. Following the 40 min post-exposure starvation period, 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 cages in cooling boxes containing a damp cloth has proved to be useful, particularly if the release site is not next to the laboratory (maximum 10 minutes by car). A thermometer can be added in one cooling box to monitor potential heat stress (see Table 1 for temperatures range). No cold block is used during transport.

53. It is recommended to pass a tag through/below 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).

54. The cages from all treatment, reference and control groups are put in the same place, on a flat surface at least few centimetres 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 release time (hour and minutes) is the time point when all the cages are opened and is recorded. As for the first release, it is recommended to perform the release in the shade in hot climates to avoid heat stress or suffocation of the boxed bees. During release, labelled bees should be able to fly well when exiting the cages. All dead bees from the exposure phase to the release phase, bees with visible signs of intoxication and/or inhibited wing movements, and bees with unstuck tags should be recorded and discarded from the study. For each test run, the number of released bees in all groups should be on average greater than or equal to 70% of the tagged bees of each group.

55. 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 (e.g. 16.10 to 17.10 pm, see experimental timing in Figure 1). This period is also critical for the foragers' homing flight. Generally, a great majority (≥ 90 %) of the released bees return to the hive two hours after release. A thermo-hygrometer and a handheld wind meter or other suitable device are used to measure local weather conditions (temperature and relative humidity (%), wind speed in m/s) during the release phase. Weather conditions must be favourable to foraging (wind speed ≤ 4 of Beaufort scale representing 5.6 to 7.8 m/s, temperature 18 – 35°C and no rain, Table 1). Cloud coverage is also qualitatively recorded (e.g. null, average, high).

Test schedule

56. 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 starts immediately after the release and lasts 24 hours. A sequential pattern for each test run with timing and number of operators needed for 5 or 6 test groups is proposed in Figure 1.

Observations

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

58. 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 weather station nearby the hives (e.g. maximum 300 m from the hives). Rainfall should also be recorded (at least in mm per day) with the weather station or rain gauge. If possible, wind speed (m/s per hour) is also recorded from the weather station.

59. The geographic (GPS) coordinates of the release site, the location of the experimental colonies and landscape type (e.g. agricultural, sub-urban area) should be recorded. Additional information including a map of the study site depicting land cover (e.g., forested, agricultural, grass land, hedges, surface waters) within 1 km around the colonies should be included particularly with respect to blooming plants (e.g. crops, *Robinia pseudoacacia*, *Castanea sativa)*.

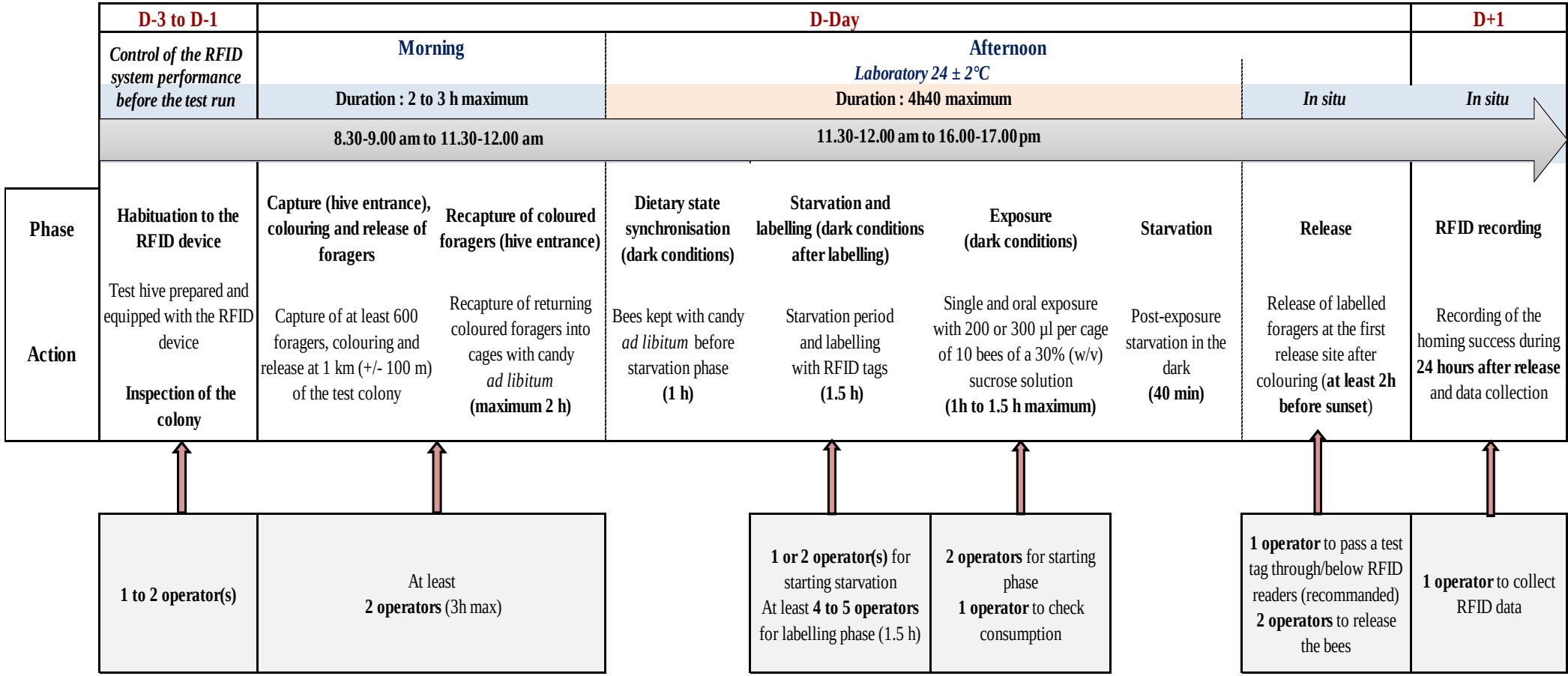

Figure 1: Sequential pattern of a homing flight test run. Timing of the manipulation phases the day of the test (D-Day) as well as number of operators needed for 5 to 6 test groups (3 dosing solutions of the test chemical, 1 or 2 control solution(s) (water or water + solvent) and the reference substance solution) are indicated. The sequential pattern is repeated for the other test runs.

9 DATA AND REPORTING

Data treatment

60. After exposure and before release in the field, the number of dead bees is used to calculate an average mortality rate (across replicates) per treatment for each test run.

61. The homing success or failure is assessed by comparing the honey bees released (with UIDs of the tags) and the release time (date, hour and minutes of release) to the RFID data recorded at the hive entrance when bees return to the hive.

62. Homing success rate over the 24-hour period is calculated per treatment group and valid test run. If a solvent control is integrated, this control is used for statistical comparison with the test item groups. It must be first ensured that mortality and homing success don't statistically differ between water and solvent control bees. Any statistical differences for bees returning or not to the hive between the test runs (all replicates considered) is first tested. If no differences in returning bees are recorded, data (e.g. number of bees released and the number of returning bees) from all the replicates and test runs are pooled for further statistical analyses to maximize the statistical power of the test. In the case of a statistical difference, the data of the run that differ cannot be pooled. Then, an additional fourth trial is conducted with another colony to perform the data analyses from three test runs. This must be done maximally one week after the last original test run. Therefore, data need to be evaluated quickly after their collection to check if they can be pooled. Homing success per treatment group may be illustrated (e.g. percentage of returning bees over the 24 h period).

63. Statistical analyses are performed on pooled data (all the replicates and test runs). The homing rates to the hive obtained over the 24-hour period for each treatment group (control and three treatment groups) are compared using an exact binomial test, a Chi-Squared test or a Cochran-Armitage step-down trend test. An adjusted significance threshold is applied for paired comparisons (e.g. Holm-Bonferroni, Dunn-Sidak method).

64. The analysed data enable the estimation of a 'No Observed Effect Dose' (NOED) on the homing success. The NOED is expressed as µg or ng of the test chemical per bee. If treated diet was not completely consumed by the bees, the dose of the consumed test substance per group should be determined prior to NOED estimation.

Test report

65. 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, plant protection product, 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, possible pre-treatments carried out at least or more than 4 weeks before the test);
- Colony size information (e.g., colony strength, status of brood and food store, see Table 1);

Test conditions:

- Recording RFID system used for the homing flight and the overall reading rate calculated before the test and before each test run;
- Place and dates when the test was conducted including geographical coordinates of the release site, the location of the experimental colonies and landscape type (e.g. agricultural, sub-urban area), map depicting land cover 1 km around the colonies with respect to blooming plants (e.g. crops, *Robinia pseudoacacia*, *Castanea sativa*).
- Number of test runs, control (water or water + solvent), number of tested doses, percentage in 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, UIDs 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 with a check of diet consumption every 30 mins in the laboratory, time points of RFID recording: time (hour and minutes) of release in the field and the time point of the 24-hour 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 (data per hour);

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. Any evidence of possible regurgitation during exposure phase should also be reported and individuals from the cage(s) excluded from the study.

Results:

- Results of the preliminary study determining the range of treatment doses, if conducted, or other justification for the dose selection;
- Nominal doses used, measured doses of the test chemical in the feeding solutions for the three test runs, and analytical method used with limit of detection (LOD) and limit of quantification (LOQ);
- Mortality rate of all treatment, control and reference substance groups after exposure and 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;
- Homing rate (%) over the 24-hour period for each treatment and control per test run as well as for the data of the test runs pooled;

- 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 Guidance Document or any other relevant information.

10 LITERATURE

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Annex 1: Definitions

Dose is defined as the amount of test chemical consumed or offered. 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.

Foragers: For the homing flight test, foragers returning to the hive after food resources collection and carrying nectar and pellets of pollen are captured at the hive entrance to be coloured for the test.

Homing success is the number of RFID labelled bees released that successfully fly back to the hive 24h-after release. These bees are recorded with the RFID system installed at the hive entrance. For each treatment group, the homing success rate is calculated as the number of released bees that successfully fly back to the hive 24h-after release on the number of released bees multiplied by 100.

LD_{10}: is a dose of the test chemical (µg or ng per bee) calculated or estimated from an acute toxicity test (OECD TG 213) (20) that caused mortality of 10 % of the tested individuals when administered by the oral route.

Mortality: in the homing flight test, the mortality is considered and calculated on the bees in cages after exposure to treated solutions and before release in the field.

NOED (No Observed Effect Dose):
1) In the preliminary test procedure (Annex 5), the NOED is the dose (µg or ng per bee) immediately below the LOED that does not induce significantly higher mortality when compared to the control bees within a stated exposure period (48-h after exposure here for this test).
2) In a homing flight test, the NOED (µg or ng per bee) is a dose that does not induce significantly higher homing failure when compared to the control bees 24-h after release.

Release site 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 success 24 h-after release. The release site should be the same for all test runs of a full homing flight test.

Replicate in the homing test is the cage containing a group of 10 bees. Each test run considers at least 3 replicates (or cages) and at least 30 bees per test group.

RFID (Radio Frequency Identification) is a technology that allows detecting (scanning) each time a tag-equipped bee passes 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 (e.g. 2.0 x 1.7 x 0.5 mm and maximum 3 mg) glued on the bee's thorax and provides the unique identification (UID) code of the bee. To maintain continuous recording, a constant power supply is required for the system (e.g battery, external power supply).

Test run is a homing test performed with the bees from one colony. One test run includes one day of manipulation (e.g. capture, labelling, exposure and release) and 24-h RFID recording of the homing success. 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 almost unlimited number of codes that allows to individualize the bees.

UVCB are substances of Unknown or Variable composition, Complex reaction products or Biological materials. UVCB may be substances from biological, chemical or mineral origin and resulting from a synthesis or refinement process.

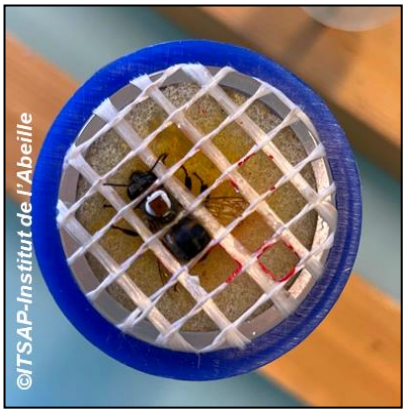
Annex 2: Example of Varroa Mites counting using the powder sugar method

https://wildflowermeadows.com/wp-content/uploads/2018/12/Powdered-Sugar-Roll-Text.pdf

Annex 3: Illustrations of the main steps of the homing flight test

Capture at the hive entrance
Hive is equipped with the RFID system
(MAJA 13.56 MHz, Microsensys, GmbH, Germany)

Coloured bee on the flight board



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

Exposure phase
per group of 10 bees

Exposure occurs in the dark. The light is for taking a photo only

Honey bee release

It is recommended to perform the release phase in the shade in hot climates.

Tagged bee on flight board
RFID reader with tunnel to scan labelled bees entering/exiting the hive
(MAJA 13.56 MHz, Microsensys, GmbH, Germany)

Annex 4: Example of protocol to control the performance of the RFID system

The control of the RFID system performance should be done both before the system is fitted to the hive as well as before the start of each test run. The control before each test run may be conducted with a lighter procedure (e.g. 3 tags passed 2 times through/below each reader).

Example of procedure for MAJA 13.56 MHz RFID system (Microsensys GmbH, Erfurt, Germany):

- 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.

In order to be used in this guidance document, the RFID system should allow to record at least 95% of the reading of the tags.

Annex 5: Preliminary test procedure

The homing flight test must be conducted at sublethal doses. If data on sublethal effects are not available (e.g. $LD_{10}/2$) with LD_{10} coming from acute toxicity tests (OECD TG 213 (18)), a preliminary test in the laboratory may be conducted on forager bees. Mortality in control bees must be ≤ 10 % as a validity criterion. The preliminary test is based on OECD TG 213 procedure (18) and allows a NOED ('No Observed Effect Dose') to be determined on mortality 48 hours after exposure. This dose should not cause significantly higher mortality than that of the control and should not induce visible signs of intoxication (e.g. knock down, affected or trembling bees) that may affect flying abilities of the bees.

Amendments to OECD TG 213 are:

- Use of mix of returning nectar and pollen foragers as test bees. They are captured at the hive entrance in the morning when foraging begins 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 the same colony per cage (bees of different colonies must not be mixed in a cage). Bees from the different colonies are then used as different replicates for each treatment tested,
- Three to five test concentrations spaced by a constant factor not exceeding 2. The number of doses and the 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 to 30 µl per bee (200 to 300 µl for each group of 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* after exposure,
- Mortality is recorded 24 and 48 h-after exposure,
- The number of dead bees 48h-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. Holm-Bonferroni, Dunn-Sidak method).