Organisation de Coopération et de Développement Economiques
Organisation for Economic Co-operation and Development

31-Aug-2007

English - Or. English

ENV/JM/MONO(2007)22
Unclassified

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

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GUIDANCE DOCUMENT ON THE HONEY BEE (APIS MELLIFERA L.) BROOD TEST UNDER SEMI-FIELD CONDITIONS

JT03231272

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Environment Directorate

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FOREWORD

This document is a Guidance Document on the Honey Bee Brood Test. The Honey Bee Brood Test is conducted under semi-field conditions and enables the quantitative assessment of adverse effects of plant protection products on the development of the honey bee brood under conditions close to the real world. The test is required for the assessment of pesticides, in particular insect growth regulators, in the European Union.

At the 17th Meeting of the Working Group of National Coordinators of the Test Guidelines Programme (WNT) in 2005, a Standard Project Submission Form was presented by Germany to develop a Test Guideline on Honey Bee Brood Test. The project proposal was approved and included on the workplan. Despite the completion of a limited ring-test in 2002, it turned out that the reproducibility and repeatability of the test method had not been thoroughly investigated. After discussions with Germany, it was agreed that the project should focus on the development of a Guidance Document on how to conduct honey bee brood tests, with the expectation that in the future sufficient data can be collected to document the reproducibility of the test.

In February 2006, the Secretariat circulated the initial draft Guidance Document to the WNT and to the Working Group on Pesticides, for comments. Comments were received from Denmark, France, Germany, Netherlands, United Kingdom, United States, and BIAC. In light of the comments made and after discussion between the Secretariat and Germany, the Secretariat organized a consultation with experts from Germany (lead country) and France, given that most comments were from French experts. The consultation took place in Paris in November 2006.

Following this consultation, the draft Guidance Document was revised taking into account all comments, and circulated again in December 2006 to those experts who had provided comments in the first round. Further comments were provided in January 2007. A final draft Guidance Document, prepared by Germany in February 2007, was agreed by the WNT at its 19th meeting, in March 2007.

This document is published on the responsibility of the Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology.

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INTRODUCTION

1. According to the currently established decision-making scheme for the environmental risk assessment of plant protection products (OEPP/EPPO, 2001) a honey bee brood test is required if a product is likely to affect bee brood development. A honey bee brood test is a reasonable approach when a product is known to act as an Insect Growth Regulator (IGR) and is applied in to the flowering crop or a product is applied in to the flowering crop and the active is known to be transferred into the bee hive in considerable amounts or a product is applied as a seed treatment or soil granule and the active is known to be a systemic compound found in pollen or nectar. Several laboratory test methods have been presented, e.g. by Rembold and Czoppelt (1982), Czoppelt (1993), Wittmann (1981 and 1982) and discussed so far. However, these methods are considered too unstable to fulfil basic requirements of standard laboratory tests under GLP. Recently a new larval in vitro test has been introduced by Aupinel et al. (2005), and is currently being ring-tested. However, due to the fact that data on exposure of larvae are scarce and doubtful, it is not admitted to relate larval toxicity with predicted environmental concentrations (PEC) such as field application rates or residue values for a final risk assessment as is usually done for TER (Toxicity/Exposure-Ratio) calculations. The "In-hive field test" published by Oomen *et al.* (1992) is a qualitative test method and therefore does not provide a realistic risk assessment, based on a realistic exposure scenario. The purpose of the Guidance Document is to introduce a test method under semi-field conditions for the quantitative assessment of adverse effects of plant protection products on the honey bee brood under conditions close to the real world. The proposed test method is not a mechanistic study, it is a study to assess the impact of plant protection products on the development of the honey bee brood.

SEQUENTIAL TESTING STRATEGY

2. The Guidance Document acknowledges the assumption that the most reliable risk assessment is based on data collected under conditions which most resemble normal plant protection and bee-keeping practice, whereas laboratory tests might be considered convenient basic assessment tools which may in addition be used to clarify specific scientific issues. Field test results should be regarded as decisive when conclusions from laboratory or tunnel tests conflict with those from field tests.

3. Preliminary screening can be made by using an in vitro bee brood-feeding test. Therefore, if any effects are detected in a laboratory feeding test, or in a qualitative tunnel or field test as described by Oomen *et al.* (1992), a 2nd tier brood test as described in the Guidance Document might allow for a more quantitative assessment of the effects on the honey bee brood. As demonstrated by the use of Insegar (Fenoxycarb) potential effects on pupae and adult worker bees can be detected as well. However, finally only field tests including the check of the brood effects might deliver an acceptable degree of reality as well as certainty.

4. The method described in this guidance document was designed to assess the effects of plant protection products (PPPs), and has been validated using the active Fenoxycarb, which is known to act as an insect growth regulator (IGR), to the honey bee brood (*Apis mellifera* L.) under semi-field conditions (tunnel conditions followed by field conditions). The method can be used to address concerns regarding the impact on the brood development in honey bee colonies which are exposed to treatments of PPPs in agricultural crops. The aim of this test is to fill an identified gap or complement the sequential testing scheme with the development of a test method under semi-field conditions and to produce quantitative data that can be used as the basis for the evaluation of IGRs and other larvicidal compounds. The method is based on the studies of Oomen et al. (1992), Mühlen (1996), Tornier (1999), Schur *et al* (2003) and the recent version of EPPO-guideline No. 170.

APPLICABILITY OF THE TEST

5. The test allows the assessment of data regarding side effects of plant protection products sprayed onto the flowering crop on the honey bee brood, as honey bees are likely to be exposed to these chemicals. However, PPPs of different types, by which honey bees may be contaminated, can be tested according to this test method as long as the test substance is taken up by the worker bees and transferred to the larvae. The method is suitable for all types of bee hives with movable frames, but not for the use of skeps.

6. Compared to laboratory experiments with honey bee brood the method shows some advantages:

- The brood is growing up in its natural environment in the hive and is not disturbed by artificial test conditions.
- The bees and their brood are put into an acceptable worst case situation by the test design.
- Because of the practical conditions of the test design the application of nearly all types of formulations and treatments will be possible, i.e. sprays, wettable granules and powders, products for soil application and seed treatment. Different modes of application require appropriate adaptation of the study design.
- It is possible to observe the effects of the test substance to the bee brood and the corresponding changes in the colony within the hive comprising a whole bee brood cycle.

7. Limitations of the test:

- The test can not be performed under adverse climatic conditions.
- Low temperatures during daytime (< 15°C) prevent a sufficient flight activity of the bees in the crop.
- High temperatures during daytime (> 30°C) may stop the nectar secretion and raise the gas phase of the test substance. By that a sufficient flight activity in the crop may also be prevented.

- Rainy periods should be avoided for the performance of the test. The test substance may be washed down from the crop and is not more available for a sufficient contamination of bees and brood. Moreover the flight activity in the crop during rainy periods normally is low.

DESCRIPTION OF THE TEST

Principle of the test

8. Small healthy honey bee colonies are initially placed in tunnel tents (herein after named "tunnels") shortly before full flowering of the crop, a few days before application of the test chemical. Following exposure of the bees in the tunnel for the period of flowering of the crop (e.g. at least 7 days after application of the product), the hives are placed outside the tunnel for the remaining of the study and are free to forage in the field. It is important to check that the neighbouring environment within a radius of 3 km is free from bee attractive main crops (e.g. sunflower, maize, oil seed rape, fruit orchards) as well as the test substance or likewise compounds. Mortality of honey bees, flight activity, and condition of the colonies and development of the bee brood are evaluated several times over a period of at least 4 weeks after the initial brood assessment. Results are evaluated by comparing the treated colonies with the water-treated colonies and with the reference chemical-treated colonies.

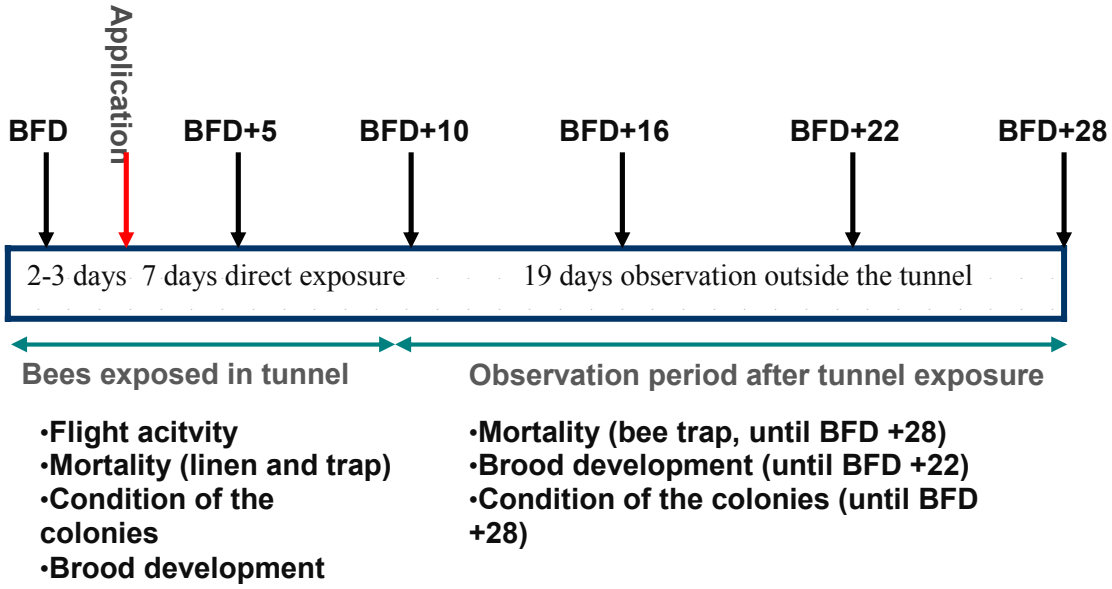


Figure 1: Timescale of the test and assessments made (BFD = Brood area Fixing Day)

9. The time period in the tunnels takes approx. 2-3 days before the treatment to acclimatise and further 7 days after application for direct exposure. After the exposure in tents the colonies are placed in areas where no attractive main crops are available ideally within a radius of 3 km to ensure that the contaminated food in the test colonies will be assimilated by the colony. In order to prevent starvation of the colonies, these should be kept in accordance with good bee keeping practice.

Experimental conditions

10. Worker honey bees of a small queen-right colony are forced to forage in a tunnel containing an attractive flowering crop treated with the test chemical. The test chemical has to be applied during full bee flight (e.g., for phacelia, an average of at least 10 bees/m^2 should be counted at a given time t), to ensure that the colony is exposed to the test chemical. The application of the reference chemical and water in the control tunnels has to be done at the same time period as in the test chemical tunnels, in order to ensure the same conditions (weather conditions, flight activity) for application for a direct comparability of the treatments.

Design of the test

11. Each test should include 3 treatments:

- Test chemical: An IGR or other plant protection product with possible/potential insect growth regulating or larvicidal properties should normally be applied at the highest recommended field rate (ml or g/ha).

- Reference chemical or positive control: An IGR known to produce adverse effects on honey bee brood (e.g. Fenoxycarb (CAS. 121-75-5)). The product Insegar should be applied at a rate of at least 600 g/ha corresponding to 150 g Fenoxycarb/ha.

- Control: The plants are treated with tap water. For example, a water volume of 200-400 L/ha is recommended for the application on *Phacelia.*

12. All spray applications should be done at the same water volume.

13. It is suggested to run the test with at least three replicates for better statistical analysis.

Preparation of the colonies

14. Small healthy honey bee colonies (e.g. Mini Plus, nuclei) should be used for the test. All colonies of one set have to be produced at the same time from colonies headed by sister queens to guarantee that the colonies in all variants are uniform as far as possible. Sister queens are the progeny of the same queen, which are mated at the same place in order to minimise genetic variability. The size of the colonies should be chosen based on the available crop area per tunnel. In the following example the colony size is described based on a crop area of 40 m^2: The quantity of (uncontaminated) food (especially pollen) stored which are in the hive at the beginning of a study should be kept at a minimum necessary to keep up colony viability and brood status, in order to ensure chemical exposure of the brood and to prevent that larvae feed on uncontaminated food. The ratio brood to food (nectar and pollen) should not exceed 4:1. A colony should consist of approximately 3000 brood cells respectively 750 cm^2 with brood in all stages, 1 food comb with honey and pollen and approximately 800 g worker bees (approximately 6000 worker bees). All colonies should be well balanced with regard to stores and strength before the start of the study. Bees should be free of clear clinical symptoms of disease (e.g. *Varrosis*, *Nosemosis*, *Amoebiosis*, Chalkbrood, Sacbrood, American or European foulbrood) or pests (*Varroa destructor*). Colonies should be free of unusual occurrences (e.g. presence of dead bees, dark "bald" bees, "crawlers" or flightless bees, unusual brood patterns or brood age structure). All hives are equipped with a dead bee trap (Illies *et al*., 2002) at the entrance to count the number of dead bees. The colonies should be set-up in the tunnels shortly before full flowering of the crop and at least three days before application. The colonies should be exposed in the tunnels for a period of at least 7 days after the application.

15. Within at least 4 weeks before the start of the test no medical treatments of the colonies are allowed.

Test conditions

16. Tunnels with a minimal size of 40 m^2 floor space should be used. The minimum height of the tunnels should be 2.5 m, to guarantee an unhindered flight of the bees. The covering gauze should have a maximal mesh size of 3 mm. The test crop should be attractive to honey bees. Suitable are for example *Phacelia tanacetifolia*, *Sinapis arvensis* and *Brassica napus*. The test crop should be drilled according to the regulations for good agricultural practice in order to guarantee a sufficient plant density according to soil and climatic conditions. During the whole testing period the colonies should be supplied with water. A water feeder should be placed into each tunnel as water supply for the bees. During the application the water feeder should be removed from the tunnel.

Application of treatments

Test product

17. Use formulated products only.

Mode and time of application

18. The products should be applied by a spray boom with calibrated nozzles according to good agricultural practice. Spraying of the covering gauze should be avoided.

19. Application should normally be performed at the time of full flowering of the crop and during full bee flight or, when required (e.g. for testing of residual or delayed action), in accordance with the intended use pattern of the product (normally late morning). The wind speed should not exceed 2 m/sec outside the tunnel.

Dosing

20. Test products should normally be applied at the highest field rate (ml or g/ha) intended for the registration of the product in order to produce a worst-case exposure for the bees.

Assessments

Duration of the study

21. The total observation period of the colonies is at least 28 days. Based on pre-application data, information should be available on when high flight activity should be anticipated.

Meteorological data

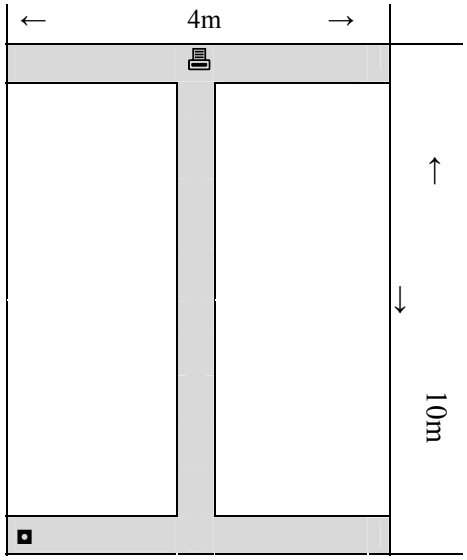
22. During the whole testing period the following meteorological data should be recorded daily (ideally inside the tunnel):

- temperature (min, max and mean);
- relative humidity (min, max and mean);
- rainfall (total daily);

- wind speed (only during application inside and outside the tunnel);
- cloudiness (during assessment).

Mortality of honey bees

23. The assessments of the number of dead bees should be carried out at approximately the same time in the morning. Mortality of honey bees should be assessed on sheets suitable for the collection of bees (e.g. linen sheets) which are spread out in front of the hives and at the front, middle and back of the tunnels. Before the start of the test, paths will be created in each tunnel by removing of the plants and by smoothing the ground. Subsequently, the path will be covered with the aforementioned sheets (area covered approx, 8 m^2) in order to facilitate the collection of the dead bees in the crop area. Additionally the dead bees will be noted and counted in the dead bee traps which are fixed at the entrance of the hives. The assessments will be done according to the time table presented in Table 1. The number of dead bees should be separated into adult worker bees, larvae, pupae and males.



Legend:

= Linen Sheets

= Water supply

= Hive

Figure 2: Location of the linen sheets, bee hive and water supply in the tunnel tents

Table 1: Evaluation of mortality of honey bees

Time of the test	Evaluations of mortality *
over at least two days before application	once a day at the same time of the day in the morning
on the day of application	• shortly before application • 2 h after application • in the evening after daily flight activity of the bees
during exposure period in the tunnels	once a day at the same time of the day in the morning
up to day +28 after BFD (out of the tunnels; only in bee traps)	once a day at the same time of the day in the morning

* Remark: At each evaluation day the dead bees have to be counted and removed.
BFD = Brood area Fixing Day: One or two days before application a brood comb is taken from each colony for marking areas with at least 100 cells containing eggs.

Flight activity

24. Flight activity should be recorded on a 1 m^2 area, at 3 different places in each tunnel according to the time table presented in Table 2. At each assessment time the number of bees that are both foraging on flowering plants and flying around the crop will be counted for a short time period (for example 10-15 seconds depending on the crop) per marked area.

Table 2: Evaluation of flight activity

Time of the test	Evaluations of flight activity
over at least two days before application	once a day during the flight activity of the bees
on the day of application	• shortly before application • 2-4 times in the first hour after application in order to observe special effects such as repellence • 2 h after application • 4 h after application • 6 h after application
on the day following application	three times during the flight activity of the bees (morning, midday, evening)
during exposure period in the tunnels	once a day during the flight activity of the bees

25. All abnormal behaviour of the bees should be reported.

Brood assessments

Condition of the colonies

26. The condition of the colonies will be assessed once before the application and six times after the application according to the following table:

Table 3: Assessment days for evaluation of the condition of the colony

Assessment days
• BFD
• Application at +2 days (± 1 day) after BFD
• + 5 days (± 1 day) after BFD
• + 10 days (± 1 day) after BFD
• + 16 days (± 1 day) after BFD
• + 22 days (± 1 day) after BFD
• + 28 days (± 1 day) after BFD

27. For the condition of the colonies the following parameters will be assessed in order to record effects of the test chemical:

- Strength of the colony (through estimation of comb area covered with bees),
- Presence of a healthy queen,
- Comb areas with pollen and nectar,
- Comb areas containing eggs, larvae and capped cells,

28. The coverage of a comb is estimated assuming that a comb is covered by 120 bees per 100 cm^2 if bees are sitting very close to each other (Imdorf *et al.*, 1987) The estimations will be done for all combs (both sides) in each hive. Other methods are possible and should be reported in the study record if used.

29. The assessment of the areas containing brood and food will be done by estimating subareas of 100 cm^2. Afterwards the number of cells per brood stage/food stock is calculated assuming that 100 cm^2 of the comb comprise 400 cells (Imdorf *et al*., 1987). These estimations will be done for all combs (both sides) in each hive. Other methods are possible and should be reported in the study record if used.

Development of the bee brood

30. The time schedule of the brood assessment days was chosen in order to check the bee brood at different expected stages during the development (see Table 4). The application in the tunnels will be performed 2 days (± 1 day) after BFD.

Table 4: Assessment of the development of the bee brood

Assessment day	**Determined brood stage in marked cells**
BFD	egg
Assessment day	**Expected brood stage in marked cells**
+ 5 days (± 1 day) after BFD	young to old larvae
+ 10 days (± 1 day) after BFD	capped cells
+ 16 days (± 1 day) after BFD	capped cells shortly before hatch
+ 22 days (± 1 day) after BFD	empty cells or egg containing cells

31. The development of the bee brood in individual marked cells will be observed by using acetate sheets. At the assessment before the application (= BFD) one brood comb will be taken out of each colony to select areas with at least 100 cells containing eggs. The first acetate sheet is used as a positioning device for all sequent assessments. The sheet should be fixed with needles on the wooden frame and the position on the frame will be marked. This procedure allows placing sequent sheets exactly in the same position on each of the following observation days. The position of the first 100 marked cells is fixed on the positioning device and copied to all sequent evaluation sheets. The growth stage of the brood in each cell will be noted on the acetate sheet. Thereby the development of each individually marked cell throughout the duration of the study can be determined (pre-imaginal developmental period of worker honey bees is normally 21 days). For the different brood stages, when assessing single cells, the following symbols and colors presented in Table 5 are suggested. However, other methods could be used and described in the study report (e.g.: photos and measurement on the frame for benchmarking).

32. The acetate sheet is removed between observation periods.

Table 5: Coding of the brood stages

Brood stage	**Symbol**	**Colour**
eggs	•	blue
young larvae (L1 – L2)	•	green
old larvae (L3 – L5)	•	red
pupae (capped cells)	•	black
nectar	x	blue
pollen	x	green
dead larvae/pupae	⊕	black
empty	x	black

33. For the evaluation of the different brood stages of single marked cells, the recorded growth stages are transferred into values counting from 0 to 5 and listed in tables as follow:

0: termination of the development (e.g. nectar or pollen found in a cell, if in the previous assessments the presence of brood was recorded);

1: egg stage

2: young larvae (L1 – L2)

3: old larvae (L3 – L5)

4: pupal stage (capped cell)

5: empty after hatching or again filled with brood (eggs and small larvae)

N: cell containing nectar

P: cell containing pollen

34. Cells filled with nectar and pollen after the termination of the brood in the respective cell (counted 0) may identified by an "N" and "P" in the following assessments; the respective cells have to be excluded from further calculations, but will be included in the overall evaluation in the end.

35. Based on the numbering described above, mean values (indices) can be calculated for each colony and assessment day. Assuming that at the first assessment only eggs will be marked, the index is 1.0. An increase of the brood index (see paragraph 40) during the following assessment can be observed, if a normal development of the brood is presumed. This increase is caused by the development from eggs to larval stages, to the pupae and finally to the adult, emerged bee and furthermore due to the rising numbers which are assigned to the brood stages. Details of the evaluation of the results are presented by Schur *et al*. (2003).

Evaluation of the test results

36. The evaluation of the results will be done by comparing the results in the test chemical treatment to the water treated control and to the reference chemical treatment, and furthermore by comparing the pre- and post-application data regarding:

- mortality in the dead bee traps and on the linen sheets (number of dead adult bees, pupae and larvae)
- flight activity in the crop
- condition of the colonies
  - strength of the colonies (through estimation of comb area covered with bees)

- brood development
  - average brood areas per hive
  - detailed brood assessment in single cells

37. The test results allow further calculations such as:

Brood termination-rate

38. Based on the brood termination-rate the failure of individual eggs or larvae to develop is quantitatively assessed. For the calculation of the brood termination-rate the observed cells are split into 2 categories:

- The bee brood in the observed cell reached the expected brood stage at the different assessment days or was found empty or containing an egg after hatch of the adult bee on BFD +22 → successful development
- The bee brood in the observed cell did not reach the expected brood stage at one of the assessment days or food was stored in the cell during BFD +5 to +16 → termination of the bee brood development

39. For the final calculation the number of cells, where a termination of the bee brood development was recorded, is summed up for each treatment and colony, is multiplied by 100 and divided by the number of cells observed in order to obtain of the brood termination-rate in %.

Brood-index

40. The brood-index is an indicator of the bee brood development and facilitates a comparison between different treatments. The brood-index is calculated for each assessment day and colony. Therefore the brood development in each cell will be checked starting from BFD 0 up to BFD +22. The cells are classified from 1 to 5 as described in paragraph 33, if the cells contain the *expected* brood stage at the different assessment days. If a cell does not contain the expected brood stage or food is stored in the cell during BFD +5 to +16 (see Table 4) the cell has to be counted 0 (see Table 5) at that assessment day and also on the following days, irrespective whether the cell is filled again with brood. This might require a further transformation of a value as described in paragraph 33. For the final calculation the values of all individual cells in each treatment, assessed at the same day, are summed up and divided by the number of observed cells in order to obtain the average brood-index.

Compensation-index

41. The compensation-index is an indicator for recovery of the colony and will also be calculated for each assessment day and colony. The cells are classified from 1 to 5 as described in paragraph 33, solely based on the identified growth stage on the assessment days. By that the compensation of bee brood losses will be included in the calculation of the indices. For the final calculation the values of all individual cells in each treatment, assessed at the same day, are summed up and divided by the number of observed cells in order to obtain the average compensation-index.

Statistical Analysis

42. Specific statistical analysis for bee trials in semi-field and field conditions are still under development. In general it is recommended to follow the OECD guidelines (OECD, 2006).

Report

The report should contain the following data:

- the physical/chemical properties and further data needed for the identification of the test chemical,
- day of the preparation of the colonies,
- health status of the colonies,
- meteorological data,
- description of the tunnels,
- description of the test design,
- test duration and performance of the test,
- test results (mortality, flight activity, condition of the colonies and bee brood development)
  - Tabular and graphic presentation of results
  - Ecological significance of the observed effects
  - Population recovery (observed or inferred), with a discussion of relevance to natural recovery processes
  - Statistical methods used

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ANNEX I

Abbreviations

BFD	Brood area Fixing Day
IGR	Insect Growth Regulator
PEC	Predicted Environmental Concentration
TER	Toxicity/Exposure-Ratio

Glossary

Health Status Colonies will be checked for clinical symptoms of bee diseases like *Varroosis, Nosemosis, Amoebiosis,* Chalkbrood, Sacbrood, American and European foulbrood and for unusual occurrences (e.g. presence of dead bees, dark "bald" bees, "crawlers" or flightless bees, unusual brood patterns or brood age structure).

Brood termination-rate The brood termination-rate quantifies the failure of the brood development of a colony based on the examination of individual eggs, larvae or pupae.

Brood-index The brood-index is an indicator for the brood development of colonies based on the success of individual eggs or larvae to develop.

Compensation-index The compensation-index is an indicator for a colony to recover from an impact on brood development.