2.8 Guidance document on the honeybee (*Apis mellifera*) brood test under field conditions

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1. Introduction

According to the EU Regulation 1107/2009, the effects of a plant protection product (PPP) on honeybees have to be investigated. 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 practices. Field test results should be regarded as complementary studies to the laboratory or tunnel tests. However, field tests including assessments of the effect of PPP on the brood might deliver an acceptable degree of reality and certainty and should be seen as higher tier study in the context of an overall risk assessment scheme for bees.

The purpose of this Guidance Document is to introduce a new test method aiming to assess the adverse effects of PPP on honeybee (*Apis mellifera*) brood development and on foraging and mortality of honeybees under *field* conditions.

1.1 Test Guidance

This methodology is based on the recommendations from:

- CEB guideline n°230, Part 6: field study, updated in 2011. Méthode d'evaluation en plein champ des préparations phytopharmaceutiques employées en application foliaire ou en traitement des semences ou du sol.

- OECD guidance document n°75 (2007): Guidance document on the honey bee (*Apis mellifera* L.) brood test under semi-field conditions.

- EPPO guideline 170(4), Side effects on honeybees.

1.2 Applicability

The test allows the assessment of data regarding side effects of PPP sprayed onto the flowering crop on the honey bee brood, as honey bees are likely to be exposed to these chemicals. However, PPP applied before the flowering period, by which honey bees may be contaminated during exposure periods, can be tested according to this test method as long as the test substance is taken up by the worker bees and transferred to hives.

Compared to the current studies on brood effect, this methodology in field conditions shows some advantages:

- The brood is growing up in its natural environment in the hive and is not disturbed by the enclosure under insect-proof tunnels.
- The bees and their brood are put into realistic conditions.
- PPP is applied in realistic conditions according to the intented Good Agriculture Practices.
- Because of the experimental conditions of the test design, any type of formulation (liquid and solid) and application (foliar and soil application and seed treatment) can be tested. Different modes of application require only appropriate adaptation of the study design.
- It is possible to assess the effects of products on bee brood development during at least one whole bee brood cycle.
- The test can be delayed in case of adverse climatic conditions.

- In case of low or high temperature during daytime (<10°C or >32°C) bees will continue to take care of brood and forage in the surrounding environment.

2. Study Layout

2.1 Test Item

The PPP under evaluation should be used at the recommended dose and according to the intended Good Agricultural Practices.

2.2 Control

The control is represented by an untreated or water treated object of similar area. It is used for the test validation and as a comparison for the potentially occurring effects of the test item.

2.3 Test Organism

Species: Apis mellifera L.

Choice of the test species: This species is recognised by scientist community for the observations and assessments to be carried out in this study and it is readily available in appropriate individual numbers. Furthermore, *A. mellifera* is an important pollinator of numerous flowering plants and an economically important species.

Origin of the beehives and colonies: Beehives, each with a colony of approx. 20,000 bees, will be provided by a local beekeeper. The colonies will have queens of the same maternal origin and the same age (not older than 2 years). They should be homogeneous regarding population size, colony strength, food storage, and brood.. The hives will consist of 10 or 12 frames comprising 5-6 frames for broods of all ages and 3-4 storage frames. Super (boxes above brood chamber) are placed on top of hive if apiarist conditions require.

Bees shoud be free of clear clinical symptoms of disease.Treatments against *Varroa* can be carried out up to 4 weeks before the beginning of the study.

2.4 Definition and Numbers of Treatments, Replicates, and Test Units

The number of treatments is at least 2, one untreated control and one study item during flowering out of foraging activity. This number can rise to 3 or 4 with a toxic reference or the study item during foraging activity. However carrying out a field test with more than 2 treatments is very heavy and hazardous in environmental conditions (field plots should be similar size (at least 2 ha) and separated by 4 km at least from one another).

Description and identification of the treatment (i.e. foliar application during the flowering period):

Treatment 1: test item: at the highest expected dose rate/ha while crop flowering and application *not during* foraging activity

Treatment 2: water treated control

Treatment 3: test item: at the highest expected dose rate/ha while crop flowering and application *during* foraging activity

Treatment 4: toxic reference

Treatments 3 and 4 are optional. The list of treatments can be adapted according to the conditions of use of the test item (i.e. seed treatment, soil application, application before flowering).

Number of hives: Seven hives per treatment (= 7 hives per plot). 4 are used for assessing the brood development whereas the 3 others are used for residue analysis.

Definition of test units (plots): a test unit (1 per treatment) consists of one plot containing 7 beehives with colonies of about 20,000 bees each.

2.5 Test Sites, Study Design and Procedures

Crop used for bee foraging

Phacelia tanacetifolia, oil seed rape or mustard can be used as crop support for bee foraging. The study will be performed either during the flowering period or will be initiated before this period according to the type of item product (seed treatment, systemic product, microencapsulation formulation). The exposure is between BBCH 61 to 69.

Study Design

Each test unit is placed in a isolated area and constitutes the elementary basis of the study design. Each plot should be at least 2 ha to supply the necessary food for the forager bees of the 7 hives and ensure an exposure of bees.

Test units of the study are separated from one another by at least 4 km in order to avoid cross-foraging by bees from the different test units. It is recommended to limit the number of attractive crops surrounding the study fields.

Potentially attractive flowering crops or plants in the near surrounding of each plots must be reported in the report.

The hives are placed at the edge of the plot. Four of the seven hives in each plot are used for the brood assessment while the remaining three are equipped with a pollen collection trap and are not used for brood assessments. All colonies will be equipped with a dead bee trap attached to the front of the beehives.

The hives are set up on the plots at the expected crop growth stage BBCH 61-62 at the latest.

Application

The applications in the different plots are performed during crop flowering (BBCH 62-64), 2 days (+/- 1 day) after the Brood Area Fixing Day (BFD 00) or before flowering in case of systemic or microencapsulated foliar product or at the planting date in case of seed treatment or soil application. If requested it is possible to combine application before flowering period followed by an application during the crop flowering for systemic and microencapsulation products.

In case of application during foraging activity, the application can be performed only if the foraging activity in the crop before application reaches at least 3 bees/m^2 on *Phacelia* or 2 bees/m^2 on oilseed rape or mustard.

The application is carried out by using an agricultural broad boom sprayer. Spraying is performed in a way that guarantees a homogenous deposition level over all sprayed areas. The application is performed with a volume of solution approximately 200 L/ha.

Regarding seed treatment and soil product, sowing or application is carried out according to the proposed Good Agricultural Practice.

Weather conditions and climatic parameters at application are recorded. Applications should be carried out in dry conditions with no rainfall predicted for 2 hours, a wind speed below 19 km/h (3 Beaufort = 10 knots) and temperature below 30°C.

The equipment used to apply the products is rinsed after each application of the different treatment by using tap water.

2.6 Assessments

The experimental phase of the study begins when the hives are set up on plot edges. The following assessments are carried out in order to study the effects of the test item:

- strength of the colony, quantitative brood development and food storage
- evolution of the brood development
- amount of harvested pollen (% by weight of *phacelia*/OSR/mustard pollen in each sample)
- residue analysis from different matrix
- bee mortality in dead bee traps
- foraging activity
- possible abnormal behavioural of the bees observed in the field and/or at the hives

For all the above assessments, the data from the test item and control are compared according to the parameters described below.

2.6.1 Strength of the colony, quantitative brood development and food storage

Three apiarist visits are scheduled in order to assess colony development at the following timings:

- the day of their introduction in the field,
- at BFD+28
- at BFD+42.

Parameters assessed are the *adult bee population* and the *quantity of brood* estimated with an adapted Liebefeld method (each side of the comb is separated in 6 equal parts containing about 740 cells each, a full 1/6 comb covered with bees equal to 240 bees), the *quality of the brood* (different development stages observed), and *amount of reserves*. Regarding the adult bee population, only bees on combs are evaluated, flying bees and those on the floor and edge of the hive are not considered. Purpose of theses visits is for comparison between treatments but not for defining the exact population of the colony. Colonies are inspected to confirm the presence of a *healthy queen*. These observations focus on the colony development. Weighting the hives can provide additional information.

2.6.2 Evolution of brood development

The environmental conditions (temperature and humidity) is recorded at each brood assessment.

At the first brood assessment (BFD 00 = Brood Area Fixing Day, expected at crop growth stage BBCH 61-62), a specific identification is assigned on the frame (No. of the hive, position of the frame in the hive and side). A brood comb is taken out from a hive and inspected in order to select areas containing 100 eggs, 100 young larvae and 100 old larvae and photographed. Cells for observation should preferably be selected from the central comb area and cells from closer to the outer frame should only be used in exceptional cases. It is possible to analyse several combs from one hive in order to reach 100 brood cells of each stage in the central part of the comb.

Brood development is assessed on four out of the seven hives per object. The number of combs with brood is recorded at each visit. The evolution of hundred eggs, hundred young larvae and hundred old larvae previously selected is followed in each hive from the Brood Fixing Day (BFD 00) to 22 days after BFD (BFD 22) with a digital imaging tool. An extra assessment is carried out at BFD 28 to confirm the assessment at BFD 22 but is not included in any analysis

At each BFD timing, cell contents are converted into a value presented below for further calculations (Tab. 1).

Table 1 Cell content assessment values

Value	Corresponding contents	Value	Corresponding contents
0	Empty	5	Nectar
1	Egg	6	Pollen
2	Young larvae (L1 - L2)	7	Dead
3	Old larvae (L3 - L5)	8*	Not characterized
4	Pupae (capped cell)		

*if the cell is noted 8, this cell is not included in any calculations

To cover a whole brood cycle (i.e. 21 days for worker honeybees) and the beginning of a new one, pictures are taken 5, 10, 16, 22 and 28 days approximately after BFD 00. The expected brood stage at each assessment date is showed in the tables 2 below. Based on those tables, the cell content assessment values are converted to a brood category for further calculations.

Table 2 Expected brood stage at each BFD and value for index calculation in case of eggs (a), young larvae (b) or old larvae (c) at BFD00

(a)

Assessment day	Expected brood stage in cell	Brood category for index calculation
BFD	Egg	1
5 days ± 1 after BFD	Young larvae or old larvae	2 or 3
10 days ± 1 after BFD	Capped cells	4
16 days ± 2 after BFD	Capped cells shortly before hatch	4
22 days ± 2 after BFD	Empty or reserve cells after hatch or new egg laid	5

(b)

Assessment day	Expected brood stage in cell	Brood category for index calculation
BFD	Young larvae	2
5 days ± 1 after BFD	Old larvae or capped cells	3 or 4
10 days ± 1 after BFD	Capped cells	4
16 days ± 2 after BFD	Capped cells or empty or reserve cells after hatch or new egg laid	4 or 5
22 days ± 2 after BFD	Empty, reserve, egg or larvae after hatch	5

(c)

Assessment day	Expected brood stage in cell	Brood category for index calculation
BFD	Old larvae	3
5 days ± 1 after BFD	Capped cells	4
10 days ± 1 after BFD	Capped cells or empty or reserve cells after hatch or new egg laid	4 or 5
16 days ± 2 after BFD	Empty, reserve, egg or larvae after hatch	5
22 days ± 2 after BFD	Empty, reserve, egg or larvae after hatch	5

Three numeric parameters describe the bee brood development over the time and are explained below; the Brood Termination Rate (BTR), the Brood Index (BI) and the Compensation Index (CI). These values are calculated from the assessment values assigned in raw data and with the use of a specific software (Fiji® - Bee brood Analyzer 2.0). One analysis is performed for each of the three

brood stages selected at BFD 00. Only values from BFD 00 to BFD 22 are analyzed, the assessment carried out at BFD28 is only for confirmation of the values met at BFD 22.

- *Brood Termination Rate BTR*

The *Brood Termination Rate (BTR)* expresses the quantity of cell's failure in percentage for each brood comb at each assessment day.

BTR is calculated by dividing the number of cells that do not reach the expected growth stage (see Tab. II) at a specific assessment day by the total number of cells observed.

$$\text{BTR (\%)} = \frac{\text{Number of cells failed} \times 100}{\text{Number of successful cells} + \text{Number of cells failed}}$$

For example (table II-a, eggs selected at BFD 00), if a cell is empty or contains a new egg after adult bee hatching at BFD 22, development is successful. If the expected bee brood stage was not reached at one of the assessment days or occurred with big delay or if food was stored in the cell before BFD 22, there is a termination of the development and the BTR increases.

If no failure occurred during the brood development, the BTR is equal to 0. Otherwise this rate increases with the number of terminated cells (dead larvae, nymph or significant delay in the development process, or food stored in cells at BFD 05, 10 or 16). Cells noted 0 (empty), 5 (nectar) or 6 (pollen) before hatch (BFD 22) or 7 (dead) or with any unexpected value at a specific BFD are considered to be failures in the brood development ; value of these cells are equal to 0 for the calculation of BTR and the following index BI.

Mean value of BTR for each object is calculated by the average of BTR obtained in each colony belonging to the same modality.

Brood Index BI

The *Brood Index (BI)* is an indicator of bee brood development and is calculated for each brood comb at each assessment day.

If brood cell contents reach the expected brood stage at the specific assessment day (Table II), the cells are classified using the brood category number as defined in Table II. On the opposite, if the expected brood stage is not reached or occurred with big delay or if food is stored in the cells at BFD 05, BFD 10 or BFD 16 in case of eggs at BFD 00 (see table II-a), the cells are valued with 0 at the assessment date and also the following dates, disregarding if cells are again filled with brood.

The Brood Index of a colony is obtained by summing up the value of all cells assessed the same day and divided by the number of observed cells. If all cells present a successful development (expected pattern), BI is equal to 5 which is the maximal and best value for this index.

Mean value of BI is calculated by averaging all BIs of colonies belonging to the same treatment.

Compensation Index CI

The *Compensation Index (CI)* indicates the recovery of a colony and is calculated for each brood comb at each assessment day. Cells containing a brood stage are classified according to categories (from 0 to 8) described in Table 1.Then values are converted to brood categories as reported in the Table 2. If a cell is empty or contains nectar, pollen before hatch (BFD 22) or contains dead larvae or pupae, its value becomes 0, meaning that the cell is empty from any brood stage.

Only values of category at each date of assessment are taken into account, without considering the expected brood stage. Therefore this index does not influence the development value of the brood after termination, suspension or delay.

The Compensation Index of a colony is obtained by summing up the value of all cells assessed the same day and divided by the number of observed cells.

Mean value of CI for an object is calculated from an average of CI's colonies belonging to the same treatment.

2.6.3 Residue analysis in plant and honeybee matrices

Specimens are sampled in each of the 3 dedicated hives and kept frozen according to Standard Operating Procedures at temperature below -15°C. Disposable or washable gloves are worn during sampling, any equipment used is washed between objects and specimens are collected in double (one retained at the test facility and one sent to the analytical laboratory) in a specific container (paper for the pollen, glass or plastic for honey). Then samples are put in identified sealed plastic bags before freezing. Samples from each treatment are stored (or ‚kept' separated from each others.

For each of the 3 hives :

Pollen specimens are collected in clean paper bags 3 and 8 Days After Application (3 DAA and 8 DAA) from the 3 hives set with pollen collection traps. If not enough pollen is collected, stored pollen can be collected directly from frame cells. The collection time can be delayed depending on the weather conditions (e.g. collection before a rainy weather in order to guarantee non fermented pollen). The presence of the characteristic purple colour of pollen of phacelia (yellow for oil seed rape) in the collection traps is monitored and the total amount of pollen in the trap is weighed. The percentage of *phacelia* pollen is expressed as a proportion of the total harvested pollen.

Bee bread specimens are collected 8 DAA as it is the optimal timing for sampling enough quantity of bee bread made of pollen exposed to the test item or water. It is collected from the reserve combs in the brood chamber.

Nectar specimens are collected 8 DAA from newly filled reserve combs in the brood chamber or in the super when available (it is accepted that uncapped cells containing fresh reserves -fluid matter- are filled with nectar).

Honey specimens are preferably collected 20 DAA from honey super. In case of empty super, some fresh honey may be collected from storage frames in the hive.

Bee bread, nectar and honey are manually collected from the 3 dedicated hives per object using clean spoons and jars.

Flowers are collected in the morning after application (1DAA) from 12 different places in plot. Whole inflorescences are sampled in clean paper bags.

Specimen size:

- Pollen = about 10 g of total amount (amount of *phacelia* pollen will depend on the harvest of honeybees and will be reported in the final report)
- Beebread = about 10 g
- Honey = about 50 mL
- Nectar = about 20 mL
- Flowers = about 50 g

2.6.4 Mortality

The number of *dead bees* found in front of the hives is regularly counted and recorded. This procedure is carried out daily from BFD 00 (*i.e.* 2 days before the expected application day) to BFD 22 ± 1 day and then at BFD 26, BFD 36 and BFD 43 ± 2 days.

Dead pupae found in the dead bee trap (or on the plastic sheet) while counting adult honeybees mortality are also monitored. They are checked for abnormalities, deformations and colour changes and are kept deep frozen with a specific identification.

For seed treatment and soil application, additional mortality assessment can be carried out just after the sowing and during the guttation. For foliar product applied before flowering period, one additional mortality assessment can be performed at the beginning of the flowering period.

2.6.5 Foraging activity

Observations on *foraging activity* are conducted once a day from BFD 00 to BFD 10 (and a complementary count is carried out the day after application) then every two days until BFD 16 ± 1 day. The foraging activity on each field is recorded by counting the number of forager bees on 10 m^2 on two points of the field.

The assessments should be carried out during the bee activity. The assessment timing may be postponed for 1 day depending on the weather conditions.

In case of application during the foraging activity, additional assessments are conducted at least once just before the application and two times after the application (about 1 h and 3 h after the application).

2.6.6 Observation on behaviour of the bees

At the time of observation of foraging activity, the behaviour (and possible behavioural anomalies) of the bees is observed, both on the crop and at the entrance of the hives, and recorded.

2.7 Monitoring site

After wilting of flowers from field site, all hives from the test are transported to an unique monitoring site until 42 days after application, close to forest or crops apart from expected chemical sprays. At the monitoring site, bees should have access to sufficient naturally available pollen and nectar sources. Details on the location of the monitoring site as well as potentially available bee attractive plants are reported in the final report.

2.8 Statistical Analysis

A statistical analysis is performed on the *brood development results* (BTR, BI and CI). Currently in 2014 the software Fidji is used for the assessment of numeric pictures along the different timings and runs statistical analysis on the brood evolution. Any other dedicated software could be developed for this purpose.

2.9 Validity Criteria

Each object is represented by one plot. The validity criteria of an individual trial are as follows:

Before the application:

- The adult bee daily mortality between the treatments should be similar. The difference of the average mortality among treatments the day before application should not exceed 60%.

- The foraging activity should be significant (at least 3 bees/m^2 on *phacelia* or 2 bees/m^2 on oilseed rape or mustard) in each field and comparable between treatments.

After the application

- The daily mortality in the control must be similar before and after the application. The difference in the control between the average adult bee mortality the day after the application should not exceed by 50% the mortality average found the day before the application.

After the Brood area Fixing Day:

- Assuming that eggs are recorded at BFD, and assuming a normal brood development, mean brood indexes should increase at further assessments: from eggs (1) to larvae (2-3), then pupae stage (4) and finally empty cells after hatch or new eggs (5).

- The termination rate in the control should be lower than 30%.

- Any other phenomena that have been considered as abnormal in the course of the study will be reported.

In case of soil treatments or seed treatments validity criteria should be adapted before and after exposure. When there is no application during crop growing, the validity criteria should be assessed during flowering on the control plot only, with a foraging activity of at least 3 bees/m^2 on *phacelia* or 2 bees/m^2 on oilseed rape or mustard.

3. Summary Table

Target organism	**Honeybees**	
Status	GLP multi-site study	
Study type	Short term effects on brood development, foraging, mortality and behaviour of adult honeybees in field conditions. Specimen sampling for the purpose of residue analysis	
Crop	*Phacelia*	
Number of objects	2	
Number of hives	7 per object (4 hives for brood assessments and 3 to collect samples)	
Target settlement of hives	Beginning of crop flowering	
Target application timing	During flowering, about 2 days (± 1 day) after BFD*, out of bee foraging (in the evening)	
Assessment times (± 1 day)	2 days before the expected application day	Brood Fixing Day (BFD 00) Colony strength and development
	Daily from 2 DBA* to 20 DAA*, then once at 26 DAA, 34 DAA and 41 DAA	Bee mortality
	On the application day (just before the application performed in the evening) and the day after	Additional bee mortality records
	Daily from 2 DBA to 14 DAA	Bee foraging activity
	Once at 0 DBA and 1 DAA	Additional bee foraging activity
	1 DAA (in the morning)	Flower sampling
	5 days after BFD (BFD 05)	Brood development
	10 days after BFD (BFD 10)	Brood development
	16 days after BFD (BFD 16)	Brood development
	22 days after BFD (BFD 22)	Brood development
	28 days after BFD (BFD 28)	Brood development (for information only)
	26 DAA and 40 DAA	Colony strength and development
	3 and 8 DAA	Pollen sampling
	8 DAA	Bee bread sampling
	8 DAA	Nectar sampling
	20 DAA	Honey sampling
Crop destruction	yes	