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

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Available published guidances for brood studies

• OECD 237  Honey bee (*Apis mellifera*)
  *Larval toxicity test, single exposure*

• OECD draft guidance document Honey Bee (*Apis mellifera*) *Larval Toxicity Test, Repeated Exposure*

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

• Oomen test (1992)

What is missing?

Methodology on honey brood test under field conditions when product is applied on crop
Referenced documents

This methodology is based on the recommendations from:


• EPPO guideline 170(4), Side effects on honeybees
Test organism

- *Apis Mellifera L.*
- Beehives
  - About 20000 bees
  - Queens from the same maternal origin and same age (<2 years old)
  - Homogeneous on parameters
    - Colony strength
    - Food storage
    - Brood
    - Preparation
  - 10-12 frames
    - 5-6 frames for brood
    - 3-4 frames for storage
List of treatments
(foliar application during the flowering period)

• Mandatory ones
  – Treatment N°. 1: test item, at the highest expected dose rate/ha while crop flowering and application out of foraging activity
  – Treatment N°. 2: water treated control

• Optional ones in addition to the previous ones
  – Treatment N°. 3: test item, at the highest expected dose rate/ha while crop flowering and application during foraging activity
  – Treatment N°. 4: Toxic reference

Local legal rules can prevent the application of treatments 3 and 4.
List of treatments

(foliar application before flowering period or before and during flowering period)

• Mandatory ones
  – Treatment N°1: test item, at the highest expected dose rate/ha applied before flowering period
  – Treatment N°2: water treated control

• Optional ones in addition to the previous ones
  – Treatment N°3: test item, at the highest expected dose rate/ha while crop flowering and application out of foraging activity
  – Treatment N°4: test item, at the highest expected dose rate/ha while crop flowering and application during foraging activity
  – Treatment N°5: test item, at the highest expected dose rate/ha applied before flowering period and during the flowering period however out of foraging activity
  – Treatment N°6: test item, at the highest expected dose rate/ha applied before flowering period and during the flowering period while bees are foraging
  – Treatment N°7: Toxic reference

Local legal rules can prevent the application of treatments 4, 6 and 7
List of treatments (Seed treatment)

• Mandatory ones
  – Treatment N°1: test item, at the highest expected dose rate in kg/ha used at the sowing period
  – Treatment N°2: Untreated control

• Optional ones in addition to the previous ones
  – Treatment N°3: Toxic reference used at the sowing period or toxic reference used at the flowering period
List of treatments
(Soil application before flowering period)

• Mandatory ones
  – Treatment N°1: test item, at the highest expected dose rate /ha used before flowering
  – Treatment N°2: Untreated control

• Optional ones in addition to the previous ones
  – Treatment N°3 : Toxic reference used before application
  or toxic reference used at the flowering period
Crops

• *Phacelia tanacetifolia*

• Oil seed rape

• Bee exposure between BBCH 61-69
Study design

• Test unit: one plot of minimum 2 ha/treatment with 7 beehives
• Test units are separated by minimum 4 km
• It is recommended to limit the number of attractive crops surrounding the study fields
• Hives are placed at the edge of the plot at BBCH 61-62
  – 4 hives are used for brood assessment
  – 3 hives are equipped with pollen collection trap
  – All hives are equipped with dead bee traps

Based on this study design, carrying out field study with more than 3 treatments can be a hard task
Application during the flowering period

- At BBCH 62-64.

- Foraging activity before application should be at least 3 bees/m² on Phacelia or 2 bees/m² on oilseed rape or mustard.

- Application is carried out by using a boom sprayer with a volume of water around 200 L/ha.

- Application should be carried out in dry conditions with no rainfall predicted for at least 2 hours, wind speed < 19 km/h and temperature < 30°C.
Application before flowering period

• According to GAP table

• Depending on the GAP table, for foliar application with systemic or microencapsulated formulation, it is possible to combine application before flowering period followed by an application during the crop flowering
Assessments

- 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 matrices
- Bee mortality in dead bee traps
- Foraging activity
- Possible abnormal behavioral 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
Strength of the colony

• 3 Times: the day of the hive settlement, at BFD28 and BFD42

• Parameters:
  – Adult bee population
  – Quantity of brood
  – Quality of brood (different development stages)
  – Amount of reserves
  – Check the presence of healthy queen
Evolution of brood development (1)

- Brood area Fixing Day: BFD00 at BBCH 61-62
  - 2 days before application date
  - 100 eggs, 100 young larvae, 100 old larvae are selected and photographed.
  - Pick exposure is the following days after application.
  - Short term effect on eggs, young larvae and old larvae
  - 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 analyze 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 4 hives out of 7 at BFD05, BFD10, BFD16, BFD22 and BFD28
Previous visual assessment (with acetate sheet)
Picture assessment

Frame identification

Frame marks

Assessed cells
**Bee brood study evaluation**

<table>
<thead>
<tr>
<th>Bee brood analysis of a specific stage at BFD00:</th>
<th>Eggs</th>
<th>Young larvae</th>
<th>Old larvae</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>BFD00</th>
<th>BFD05</th>
<th>BFD10</th>
<th>BFD16</th>
<th>BFD22</th>
<th>BFD28</th>
</tr>
</thead>
<tbody>
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<th>BFD28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eggs</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>Young larvae</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>Old larvae</td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
<td><img src="image16.png" alt="Image" /></td>
<td><img src="image17.png" alt="Image" /></td>
<td><img src="image18.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Bee brood study evaluation

Bee brood analysis of a specific stage at BFD00:

- **Eggs**
- **Young larvae**
- **Old larvae**
### Bee brood study evaluation

#### Bee brood analysis of a specific stage at BFD00:

<table>
<thead>
<tr>
<th>Stage</th>
<th>BFD00</th>
<th>BFD05</th>
<th>BFD10</th>
<th>BFD16</th>
<th>BFD22</th>
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<td>Eggs</td>
<td><img src="image" alt="Eggs" /></td>
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<td>Young larvae</td>
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</tr>
</tbody>
</table>
Evolution of brood development (2)

- At each BFD timing, cell contents are converted into a value for further calculations.

<table>
<thead>
<tr>
<th>Value</th>
<th>Corresponding contents</th>
<th>Value</th>
<th>Corresponding contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Empty</td>
<td>5</td>
<td>Nectar</td>
</tr>
<tr>
<td>1</td>
<td>Egg</td>
<td>6</td>
<td>Pollen</td>
</tr>
<tr>
<td>2</td>
<td>Young larvae (L1 - L2)</td>
<td>7</td>
<td>Dead</td>
</tr>
<tr>
<td>3</td>
<td>Old larvae (L3 - L5)</td>
<td>8*</td>
<td>Not characterized</td>
</tr>
<tr>
<td>4</td>
<td>Pupae (capped cell)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
# Expected brood stage at each BFD and value for index calculation

**Eggs at BFD00**

<table>
<thead>
<tr>
<th>Assessment day</th>
<th>Expected brood stage in cell</th>
<th>Brood category for index calculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFD</td>
<td>Egg</td>
<td>1</td>
</tr>
<tr>
<td>5 days ≈ 1 after BFD</td>
<td>Young larvae or old larvae</td>
<td>2 or 3</td>
</tr>
<tr>
<td>10 days ≈ 1 after BFD</td>
<td>Capped cells</td>
<td>4</td>
</tr>
<tr>
<td>16 days ≈ 2 after BFD</td>
<td>Capped cells shortly before hatch</td>
<td>4</td>
</tr>
<tr>
<td>22 days ≈ 2 after BFD</td>
<td>Empty or reserve cells after hatch or new egg laid</td>
<td>5</td>
</tr>
</tbody>
</table>

**Young larvae at BFD00**

<table>
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<th>Assessment day</th>
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<tbody>
<tr>
<td>BFD</td>
<td>Young larvae</td>
<td>2</td>
</tr>
<tr>
<td>5 days ≈ 1 after BFD</td>
<td>Old larvae or capped cells</td>
<td>3 or 4</td>
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</tr>
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<td>16 days ≈ 2 after BFD</td>
<td>Capped cells or empty or reserve cells after hatch or new egg laid</td>
<td>4 or 5</td>
</tr>
<tr>
<td>22 days ≈ 2 after BFD</td>
<td>Empty, reserve, egg or larvae after hatch</td>
<td>5</td>
</tr>
</tbody>
</table>
## Expected brood stage at each BFD and value for index calculation (2)

**Old larvae at BFD00**

<table>
<thead>
<tr>
<th>Assessment day</th>
<th>Expected brood stage in cell</th>
<th>Brood category for index calculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFD</td>
<td>Old larvae</td>
<td>3</td>
</tr>
<tr>
<td>5 days ≡ 1 after BFD</td>
<td>Capped cells</td>
<td>4</td>
</tr>
<tr>
<td>10 days ≡ 1 after BFD</td>
<td>Capped cells or empty or reserve cells after hatch or new egg laid</td>
<td>4 or 5</td>
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<tr>
<td>16 days ≡ 2 after BFD</td>
<td>Empty, reserve, egg or larvae after hatch</td>
<td>5</td>
</tr>
<tr>
<td>22 days ≡ 2 after BFD</td>
<td>Empty, reserve, egg or larvae after hatch</td>
<td>5</td>
</tr>
</tbody>
</table>
Numeric parameters

• Brood Termination Rate (BTR)

• Brood Index (BI)

• Compensation Index (CI)
Residue analysis in plant and honeybee matrices

• Sampling collection from 3 hives
• Pollen : 3 and 8 DAA from pollen trap
• Bee bread : 8 DAA from the brood chamber
• Nectar : 8 DAA from newly filled reserve combs in the brood chamber or in the super when available
• Honey : 20 DAA from honey super
• Flowers : 1 DAA from 12 different places in plots, whole inflorescences are sampled
Mortality assessments
Adult and Pupae

• Count dead bees in front of the hives daily from BFD00 (i.e. 2 days before the expected application day) to BFD22 ± 1 day and then at BFD26, BFD36 and BFD43 ± 2 days.
• Timing on the day of application: just before the application and the day after
• Dead pupae are also checked of abnormalities, deformations and colour changes and are kept deep frozen with a specific identification.
Foraging activity

• Once a day from BFD00 to BFD10 then every two days until BFD16 ± 1 day.
• By counting the number of forager bees on 10 m² on two points of the field.
• In case of application during the foraging activity, additional assessments are conducted at least once just before the application and twice after the application (about 1 h and 3 h after the application).
Observations on Behavior of the bees

The behaviour (and possible behavioural anomalies) of the bees is observed and recorded as well on the crop and at the entrance of the hives, at the same time as the observation of foraging activity.
Monitoring site

After flowers from field site wilt, hives 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 have access to sufficient naturally available pollen and nectar sources.
Benefit from field studies

- Normal colonies (> 20,000 bees)
- Large surfaces to forage (min 2 ha)
- Empty comb for eggs (2/3 days before BFD)
- Possibility to chose eggs, young larvae and older larvae
- Sufficient matrices for residue analysis (pollen, nectar, honey, flowers...)
- BTR usually < 30%
- And...
## Data 2010 - 2014

<table>
<thead>
<tr>
<th>4 semi-field studies (3 hives / modality)</th>
<th>4 field studies (3 to 4 hives / modality)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean control BTR at BFD05 Min 13 % ; max 96 %</td>
<td>Mean control BTR at BFD05 Min 6 % ; max 19 %</td>
</tr>
<tr>
<td>Mean control BTR at BFD22 Min 50 % ; max 97 %</td>
<td>Mean control BTR at BFD22 Min 12 % ; max 27 %</td>
</tr>
</tbody>
</table>
Data (2010-2014)
The population size, related to the foraging activity, is an influential parameter on BTR.

- **Semi-field studies:**
  - risk of stop for the queen to lay eggs
  - It seems small colonies (in nuclei) have better indices, but less exposure

- **Field studies:**
  - normal queen behaviour
Thank you for your attention